Abstract. Mesenchymal stem cells (MSCs) are known to migrate to tumor tissues and to play an important role in cancer progression. However, the effects of MSCs on tumor progression remain controversial. The purpose of the present study was to detect the effects of human umbilical cord-derived MSCs (hUC-MSCs) on the human breast cancer cell lines MDA-MB-231 and MCF-7 in vitro and the underlying mechanisms. MSCs were isolated and identified from umbilical cord tissues. MDA-MB-231 and MCF-7 cells were treated with conditioned medium (CM) from 10 and 20% umbilical cord MSCs (UC-MSCs), and the resulting changes in proliferation and migration were investigated. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and plate clone formation assays were used to assess the effect on proliferation, and the effects of CM on MDA-MB-231 and MCF-7 migration were assessed through scratch wound and Transwell migration assays. The expression of cell proliferation- and metastasis-related genes and proteins and activation of the ERK signaling pathway were analyzed by RT-PCR and western blot assays. UC-MSCs are characteristically similar to bone marrow MSCs (BM-MSCs) and exhibit multipotential differentiation capability (i.e., osteoblasts and adipocytes). The MTT, plate clone formation, scratch wound and Transwell migration assay results revealed that 10 and 20% CM promoted the proliferation and migration to higher levels than those observed in the control group. Our findings showed that UC-MSC-CM inhibited E-cadherin expression, increased the expression of N-cadherin and proliferating cell nuclear antigen (PCNA) and enhanced the expression of ZEB1, a transcription factor involved in epithelial-to-mesenchymal transition (EMT), through activation of the ERK pathway. U0126, an inhibitor of ERK, reversed the effects of UC-MSC-CM on breast cancer cell proliferation and migration. We conclude that UC-MSCs promote the proliferation and migration of breast cancer cell lines via activation of the ERK pathway.

Introduction

Breast cancer is the most common malignancy and is the leading cause of cancer-related death in females worldwide (1,2). At present, the main clinical therapy strategies include chemotherapy, surgery and radiotherapy, but all of these have side effects (3,4). Mesenchymal stem cells (MSCs) are known to migrate to tumors, and it is possible to exploit the behavior of MSCs as a tumor-targeting method for cell-based cancer therapy. The effects of MSCs on tumor progression remain controversial, and in particular, it is not clear whether the clinical application of MSCs leads to unforeseen and unwanted side effects.

Tumor development has been recognized as the result of the interaction between tumor cells and their surrounding supporting tissues (5). The mutual interactions of tumor cells and stromal cells through direct contact to various cytokines and chemokines in a paracrine manner are thought to modulate tumor progression (6-8). Several studies indicate that MSCs promote tumor proliferation and metastasis (9,10), whereas other studies suggest that MSCs display intrinsic antitumor activities (11-13). This discrepancy requires further investigation.

The bone marrow is the main source of MSCs, but their collection from the bone marrow is extremely difficult. The proliferative and multilineage differentiation capacities of bone marrow-derived MSCs (BM-MSCs) decreases with aging (14). However, umbilical cord collection is convenient...
and is not associated with any ethical or legal issue (15). Many studies have confirmed that the proliferative and differentiation abilities of umbilical cord MSCs (UC-MSCs) are greater than those of BM-MSCs (16). Therefore, UC-MSCs are considered a promising source of stem cells for cancer therapy.

UC-MSCs have been found to target many primary solid tumors and their metastases (17,18). UC-MSCs secrete interferon-β (IFN-β), which was found to reduce the growth of human MDA-MB-231 breast carcinoma cells by inducing apoptosis (19). It was recently shown that the intratumoral injection of rat umbilical cord matrix stem cells (rUC-MSCs) caused regression of rat mammary carcinomas (20). Human umbilical cord Wharton's jelly stem cells (hWJSCs) have been shown to have anti-inflammatory potential by reducing the expression of inflammatory mediators (21). Taken together, the results indicate that UC-MSCs exhibit an anticancer effect. However, it remains unclear whether UC-MSCs are safe in cancer clinical therapy.

The MDA-MB-231 cell line is a triple-negative breast cancer cell line (22) that exhibits stronger drug resistance and a tendency to manifest recurrence and metastasis. The MCF-7 cell line is an estrogen receptor-positive, hormone-dependent breast cancer cell line. In the present study, we sought to ascertain whether UC-MSCs have the capability to affect the migratory potential of MCF-7 cells, which have very low metastatic potential (23), and whether UC-MSCs exert differential effects in MDA-MB-231 and MCF-7 cells. The molecular mechanism of UC-MSCs on cancer cells remains unclear. Thus, a better understanding of the molecules or mechanism that regulates the proliferative and migratory behaviors of breast cancer cells is essential to the development of novel effective therapies. Thus, the present study focused on the molecular mechanism underlying these effects.

**Materials and methods**

**Cell culture.** The cells were cultured in Dulbecco's modified Eagle's medium with low glucose (L-DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and streptomycin under mycoplasma-free conditions at 37°C in 5% CO₂. The human breast cancer cell lines MCF-7 and MDA-MB-231 were a gift from Dr W. Zhu (Department of Medicine, Jiangsu University, China).

**Isolation and culture of human umbilical cord MSCs (hUC-MSCs).** Fresh umbilical cords were collected from informed, consenting mothers at the First People's Hospital of Zhenjiang (China) and rapidly processed. Moreover, hUC-MSCs were isolated within the optimal processing period of 6 h. The cords were rinsed twice with phosphate-buffered saline (PBS) supplemented with penicillin and streptomycin to remove any blood and cord vessels. The washed cords were subsequently cut into 1 mm³ pieces, floated in L-DMEM containing 10% FBS, penicillin and streptomycin, and incubated at 37°C with 5% CO₂. The medium was replaced every 3 days after the initial culture. When well-developed colonies of fibroblast-like cells appeared after 10 days, the cultures were trypsinized and passaged into a new flask for further expansion, and the medium was changed every 3 days. The experimental protocol was approved by the Jiangsu University Ethics Committee.

**Flow cytometry.** After the third passage, the cells were trypsinized (0.25% trypsin EDTA), washed twice with PBS and stained on ice with monoclonal antibodies against FITC-CD34, HLA-DR, PE-CD29, CD44 and CD90 (Becton-Dickinson, San Jose, CA, USA). PE-IgG1 and FITC-IgG1 were used as isotype controls. The stained cells were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson).

**Osteogenic and adipogenic differentiation in vitro.** The differentiation of UC-MSCs was assessed in the third passage. The cells were cultured in medium that contained either osteogenic reagents [0.1 μM dexamethasone, 10 mM β-glycerophosphate, 50 mg/l ascorbic acid and 4 μg/ml basic fibroblast growth factor (bFGF)] (all from Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks or adipogenic reagents (1 μM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine, 5 ng/ml insulin, 60 μM indomethacin and 100 μM hydrocortisone) (all from Cyagen, Guangzhou, China) for 3 weeks. Two or three weeks later, the degree of osteogenic differentiation was assessed by Alizarin Red staining, and the intracellular lipid accumulation was visualized by Oil Red O staining.

**Generation of conditioned media.** UC-MSCs were plated to 70% confluency in 35-mm plates with 10% FBS L-DMEM and allowed to adhere overnight at 37°C in 5% CO₂. The following day, the media was removed, and the cells were washed twice with PBS. The cells were then re-incubated with non-serum culture media. After 12 h, the conditioned medium (CM) was collected and passed through a 0.45-μm filter (Sigma-Aldrich). CM aliquots were frozen at -20°C until required (not exceeding 2 weeks). To prepare different concentrations of UC-MSC-CM (10 and 20%), 100% UC-MSC-CM was diluted accordingly in freshly prepared L-DMEM with 10% FBS.

**MTT and plate colony formation assays.** The cells were plated at a density of 2.5x10³ cells/well in a 96-well plate in 180 μl of L-DMEM and allowed to attach overnight. The cells were then treated with 0, 10 and 20% CM for 48 h. MTT (20 μl) was added to each well for the last 4 h. Once the reaction was terminated, the solution was discarded, and 150 μl of dimethyl sulfoxide was added to each well. The 96-well plate was shaken to ensure complete solubilization of the purple formazan crystals. The absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay reader. For the colony formation assay, the cells were plated at a density of 200 cells/plate in a 6-well plate. After culturing for 10 days, the colony units were fixed with methanol and stained with crystal violet for 30 min before washing with water and air-drying. The clones with >150 cells were counted with an optical microscope, and the average values are reported.

**Scratch wound assay.** The cells were grown to confluence and then scratched with a 0.2-ml pipette tip. The resulting debris was removed by gentle washing with medium. The cells were subsequently placed in an incubator. The cells were maintained in the presence of 0, 10 and 20% CM for 24 h, respectively. Images of the closing wound were acquired with a tendency to manifest recurrence and metastasis. The MCF-7 cancer cell line (22) that exhibits stronger drug resistance and...
an inverted microscope and analyzed using Image software (National Institutes of Health, Bethesda, MD, USA).

Transwell migration assay. First, 0, 10 or 20% CM was added to the bottom chambers. Then, 5x10^4 MCF-7 cells and 2.5x10^5 MDA-MB-231 cells were plated in 100 µl of non-serum L-DMEM and added to the top of the chambers (Corning, Lowell, MA, USA), and the plates were then incubated for 12 h at 37°C. The cells on the top part of the filter were removed by scrubbing twice with a cotton swab. The migrating cells were fixed in formaldehyde and stained with crystal violet. Four low-power fields (magnification, x200) were randomly selected from each chamber to observe the cells and count the stained migrated cells. Each experimental group was assessed in triplicate.

Reverse transcriptase-polymerase chain reaction (RT-PCR). MCF-7 and MDA-MB-231 cells were treated with CM (0, 10 or 20%) for 48 h. The total RNA was isolated with TRIzol reagent (Invitrogen), and 1 µg of RNA was processed for cDNA synthesis with Superscript II reverse transcriptase (Cinnagen, Iran), 200 µM dNTPs, 10 pM of each primer, reaction buffer, and MgCl2 (Takara, Japan). PCR was performed using 1 µg of cDNA sample with 0.3 U of Taq polymerase (Cinnagen, Iran), 200 µM dNTPs, 10 pM of each primer, reaction buffer, and MgCl2 (Takara, Japan) in a 25-µl volume. PCR amplification was performed for 35 cycles using an ABI 2720 Thermal Cycler (Applied Biosystems). The cycling conditions were 94°C for 30 sec, and a final extension at 72°C for 10 min was performed. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The specific primers for PCR were designed as follows: β-actin sense, 5'­-CACGAAACTACCTTCAA­CTC-3' and antisense, 5'-CATACTCTC­TGGTGT­GGCTTA­CT-3'; E-cadherin sense, 5'-CGATT­GCGCA­CTAC­TCT-3' and antisense, 5'-TGT­GCT­G­AGG­AT­GT­G­TA­AG-3'; and N-cadherin sense, 5'-AGT­C­ATA­C­C­CT­G­CT­GA­TCT-3' and antisense, 5'-AGG­CTT­GCC­T-3'.

Western blot assay. MCF-7 and MDA-MB-231 cells were treated with CM (0, 10 and 20%) for 48 h. The total cellular protein was extracted using RIPA lysis buffer. Samples containing 100 µg of protein were separated on 10% SDS-PAGE gels (Beyotime, Shanghai, China) and transferred electrophoretically to a PVDF membrane (Millipore Corp., Billerica, MA, USA), and the membrane was then blocked with 5% (w/v) skim milk in TBST (20 mM Tris-HCl, 0.15 M NaCl, and 0.05% Tween-20) for 1 h at room temperature. The membranes were then incubated with primary antibodies at 4°C overnight, washed in TBST and incubated for 1 h with a goat anti-rabbit secondary antibody. The reactions were visualized using an ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). The western blot data are representatives from 3 independent experiments. The intensities of the bands obtained from the western blot assays were quantified using the Gel Image analysis software (Lane 1D, Beijing, China). The following primary antibodies were used: p-ERK and T-ERK (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PCNA (1:1,000; Bioworld, Minneapolis, MN, USA), E-cadherin, N-cadherin and ZEB1 (1:1,000; Cell Signaling Technology, Beverly, MA, USA) and GAPDH and the secondary antibody (1:2,000; Kangcheng Bio-Engineering, Shanghai, China).

Statistical analysis. Differences between more than two groups were analyzed by one-way ANOVA with the Newman-Keuls multiple comparison test using the GraphPad Prism V5.0 software program (GraphPad, San Diego, CA, USA). The results are expressed as the mean ± SD of 3 different replicates from individual assays. p<0.05, p<0.01 and p<0.001 were considered to indicate statistically significant differences.

Results

Morphology and differentiation potential and surface antigens of UC-MSCs. After 7 to 10 days of initial culture, the long spindle-shaped fibroblastic cells began to form colonies and became confluent (Fig. 1A). A multilineage differentiation potential is the functional standard for verifying the identity of MSCs. The differentiation of UC-MSCs was apparent after 2 or 3 weeks of induction under specific media. At the end of the second or third week, the UC-MSCs were capable of differentiating into osteocytes and adipocytes, as shown by positive staining of Oil Red O (Fig. 1B) and Alizarin Red (Fig. 1C). The surface antigens of MSCs were positive for CD29, CD44, and CD90 but negative for CD34 and HLA-DR (Fig. 1D).

UC-MSCs enhance the proliferation of MCF-7 and MDA-MB-231 cells. We hypothesized that various soluble factors secreted by stem cells are capable of affecting cancer cell growth; thus, we further investigated the effects of CM derived from UC-MSCs on breast cancer cells. The MT Assay of MCF-7 and MDA-MB-231 cells cultured in UC-MSC-CM (0, 10 and 20%) revealed proliferation rates of 0.342±0.015, 0.557±0.066 and 0.534±0.047 for MCF-7 cells and 0.143±0.017, 0.275±0.046 and 0.299±0.060 for MDA-MB-231 cells, respectively (Fig. 2A). The increases in the proliferation rates of MCF-7 and MDA-MB-231 cells with 10 and 20% CM were statistically significant compared with the rates observed in the control groups (p<0.05). After 10 days of culture, the plate clone formation rates obtained for the control group and the 10 and 20% CM groups were 0.040±0.013, 0.088±0.013 and 0.173±0.025 for MCF-7 cells and 0.143±0.017, 0.275±0.046 and 0.299±0.060 for MDA-MB-231 cells, respectively (Fig. 2A). The increases in the proliferation rates of MCF-7 and MDA-MB-231 cells with 10 and 20% CM were statistically significant compared with the rates observed in the control groups (p<0.05). After 10 days of culture, the plate clone formation rates obtained for the control group and the 10 and 20% CM groups were 0.040±0.013, 0.088±0.013 and 0.173±0.025 for MCF-7 cells and 0.143±0.017, 0.275±0.046 and 0.299±0.060 for MDA-MB-231 cells, respectively (Fig. 2A). These differences were statistically significant (p<0.05, Fig. 2B and C). Our western blot results showed that treatment with CM increased the PCNA protein levels in breast cancer cells (Fig. 2D). The data also revealed that the PCNA expression level in MCF-7 and MDA-MB-231 cells treated with 10 and 20% CM presented significant differences compared with the control groups (Fig. 2E, p<0.05).

UC-MSCs promote the migration of MCF-7 and MDA-MB-231 cells. In this study, we sought to determine whether UC-MSCs affect the migratory potential of the normally non-metastatic MCF-7 cell line and the high-metastatic MDA-MB-231 cell line. In the Transwell migration assay, the mean numbers of migrated MCF-7 cells in the lower fields after 12 h were 46.50±12.40, 77.75±6.02 and 91.00±8.52, whereas the
mean numbers of MDA-MB-231 cells were 76.50±5.97, 112.50±10.28, and 140.50±5.79, respectively. There were significant differences between the control group and the CM groups (p<0.05, Fig. 3A and B). Scratch wounds were
inflicted in cells pretreated with or without UC-MSC-CM for 24 h, whereas differences were observed between the groups after 24 h of treatment (Fig. 3C). The wound closure ratios for MCF-7 cells were 23.18±3.73, 28.65±2.61 and 35.83±2.88% in the control, 10 and 20% CM groups, respectively. The difference obtained with 10% CM was not significant, but that obtained with 20% CM was statistically significant (p<0.05, Fig. 3D). These results indicate that MCF-7 cells have very low metastatic potential. The wound closure ratios for the MDA-MB-231 cells were 68.00±4.16, 86.00±9.89 and 114.80±9.22 in the control and 10 and 20% CM groups, respectively, and the values obtained for the CM groups were significantly higher compared with that obtained for the control group (p<0.05, Fig. 3D).
**E-cadherin and N-cadherin expression.** The RT-PCR and western blot results showed that treatment with CM downregulated E-cadherin and increased N-cadherin (Fig. 4A and B, p<0.05). The results revealed that the mRNA and protein expression levels of N-cadherin in breast cancer cells were significantly higher than those in the control group. In addition, there was a significant difference in the expression of E-cadherin in the MCF-7 and MDA-MB-231 cells between the CM and control groups. The CM-induced migration of UC-MSCs may be achieved by the suppression of E-cadherin and the stimulation of N-cadherin expression.

**Protein expression of UC-MSCs and the effect of ERK inhibitor U0126 on breast cancer cells.** To investigate whether CM downregulates E-cadherin expression by modulating the transcription factor ZEB1, we examined ZEB1 protein levels. Treatment with CM significantly increased ZEB1 expression by ~4-fold in the MCF-7 cells and 3-fold in the MDA-MB-231 cells compared with the control groups (p<0.05, Fig. 5Ab). These findings indicate that the effect of UC-MSCs on breast cancer cell migration may be achieved by EMT. We then analyzed the activation of the ERK pathway. Treatment with 10 and 20% CM enhanced the p-ERK levels in the MCF-7
and MDA-MB-231 cells (p<0.05, Fig. 5Aa). To determine whether the MAPK/ERK signaling pathway is involved in the CM-induced increase in ZEB1 protein levels, the cells were treated with the ERK inhibitor U0126 (Promega, Madison, WI, USA) in the presence or absence of CM. Notably, U0126 significantly decreased the ZEB1 and p-ERK protein levels (Fig. 5Bc and d). These results are consistent with those of previous studies that demonstrated that MAPK/ERK is an upstream factor of ZEB1 activation in ovarian cancer cells in vitro (24) and that MAPK/ERK signaling is required in IGF-1-induced ZEB1 expression in prostate cancer cells (25).

The ERK inhibitors significantly decreased N-cadherin expression and increased basal E-cadherin expression (Fig. 5Cf and g) as well as markedly diminished but not completely abolished the CM-induced suppression of E-cadherin expression. This finding suggests that MAPK/ERK signaling is required for MSC-derived CM-induced E-cadherin downregulation. Furthermore, U0126 significantly decreased the PCNA protein levels (Fig. 5Ce). Taken together, these results indicate that the MAPK/ERK pathway is involved in CM-induced breast cancer cell proliferation and migration.

**Effects of the ERK inhibitor U0126 on CM-stimulated cell proliferation and migration.** To determine whether the ERK pathway is involved in the effects of CM on breast cancer cells, we used the ERK inhibitor U0126 to specifically block the MAPK/ERK pathway in MCF-7 and MDA-MB-231 cells. As shown in Fig. 6A and B, the MTT assay results showed that U0126 treatment significantly decreased breast cancer cell proliferation (p<0.05). The proliferation rates obtained were 0.477±0.065, 0.2820±0.036, 0.7267±0.068 and 0.4220±0.058 for MCF-7 cells and 0.267±0.048, 0.170±0.040, 0.415±0.045, and 0.264±0.044 for MDA-MB-231 cells. Our results also showed that CM-induced cell migration was markedly diminished but not totally abolished by treatment with U0126 (p<0.05). The numbers of migrated MCF-7 cells were 46.50±12.40, 9.200±2.168, 91.00±8.524 and 18.25±1.708, whereas the numbers of migrated MDA-MB-231 cells were 78.33±4.509, 28.67±2.082, 138.7±7.572 and 54.25±5.058 (Fig. 6C and D). This finding also suggests that other pathways may be involved in the response of breast cancer cells to hUC-MSCs.

**Discussion**

In the present study, UC-MSCs from human umbilical cord tissues showed a homogenous immunophenotype and multilineage differentiation potential (osteoblast and adipocyte lineages). We demonstrated that these were homogeneously positive for the mesenchymal cell markers CD29, CD90 and CD44 but negative for CD34 and HLA-DR. These results showed that UC-MSCs were Alizarin Red-positive and Oil Red O-positive after induction. Taken together, the findings suggest that the isolated adherent cells from the umbilical cord were in fact MSCs.

Furthermore, we observed the effects of UC-MSCs on the proliferation and migration of the human breast cancer cell lines MCF-7 and MDA-MB-231 in vitro. The MTT cell proliferation results showed that UC-MSC-CM significantly stimulates breast cancer cell proliferation. Therefore, it was
suggested that UC-MSCs may exert certain increasing effects on the growth of breast cancer cells in vitro. The statistical analysis of the scratch wound and Transwell migration assay results revealed that CM significantly promoted MCF-7 and MDA-MB-231 cell migration. Our results are consistent with those of previous studies (28-31).

E-cadherin functions as a cell-cell adhesion protein and tumor-suppressor that is silenced in many malignancies (32). E-cadherin is known to suppress tumor invasion, and the re-expression of E-cadherin in E-cadherin-deficient tumors reverts cells to a less invasive phenotype (33,34). Some findings indicate that hMSCs decrease cell-to-cell contact and decrease epithelial cell adhesion markers (i.e., E-cadherin) in breast cancer cells (35,36). Several transcription factors have been identified to suppress E-cadherin, including Twist, Snail, Slug and ZEB1, via their interaction with the E-box binding site in the E-cadherin promoter (37,38). In the present study, we demonstrated that CM reduced the E-cadherin protein and mRNA levels and increased N-cadherin and ZEB1 expression via activation of the MAPK/ERK signaling pathways. Finally, our results found that the downregulation of E-cadherin mediated by CM enhanced the migration of breast cancer cells.

PCNA is a well-defined regulator of DNA replication and cell cycle control (39). Treatment with CM significantly increased the PCNA protein levels. An inhibitor of ERK was able to downregulate the expression of PCNA. Furthermore, this effect was regulated through ERK nuclear translocation, resulting in enhanced PCNA expression. These findings suggest that UC-MSC-CM induced the proliferation of MCF-7 and MDA-MB-231 cells via the MAPK/ERK pathways.

However, our results are contrary to those of previous studies, which suggest that hUC-MSCs inhibit the growth of breast cancer cells (40,41). We speculate that this discrepancy may be related to the sources and numbers of the MSCs, differences in the culture and experimental methods, the type and site of the carcinoma, or a combination of these factors. We believe that UC-MSCs provide potential for cancer therapy, and further study of UC-MSCs will offer a better understanding of the relationship between MSCs and tumor progression and the mechanism governing this relationship.

Acknowledgements

The present study was supported by the Foundation of Jiangsu University for Seniors (grant no. 11JDG0089) and the Innovation Project of Cultivating Graduates of Jiangsu Province (grant no. CXLX13_689) and the Science Foundation of Kunshan (grant no. KS1331).

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