

PACE4 regulates proliferation, migration and invasion in human breast cancer MDA-MB-231 cells

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Abstract. PACE4 is one of the proprotein convertases (PC) that participate in the post-translational activation of inactive proteins, leading to mature, biologically active proteins. The processing reactions occur in pairs of basic amino acids. PACE4 is an extracellular PC that binds to growth factors and several components of the extracellular matrix contributing to tumor progression. In the present study, the *PACE4* gene was silenced by small interfering RNA (siRNA), and the knockdown human breast cancer MDA-MB-231 cells showed significantly reduced proliferation, migration and invasion rates. Flow cytometry analysis indicated that downregulation of *PACE4* increases the percentage of cells arrested at the G0/G1 phase. Moreover, the expression of genes involved in cell growth, invasion and adhesion, i.e., *IGF-2*, *MMP9* and *MPZL2* was significantly decreased following siRNA-mediated silencing of *PACE4*. Taken together, these results indicate that *PACE4* plays an important role in human breast cancer, and that it might represent a novel target for breast cancer therapy.

Introduction

PACE4 is one of the neuroendocrine-specific mammalian subtilisin-related endoproteases and a calcium-dependent serine proteinase. PACE4 can cleave precursor proteins at basic residues within the motif (K/R)-(X)_n-(K/R)_↓, where n=0, 2, 4, 6 and X is any amino acid except Cys (1). Eight isoforms of PACE4 have been reported: PACE4A-I, PACE4A-II, PACE4B, PACE4C, PACE4CS, PACE4D, PACE4 E-I and PACE4E-II (2). These isoforms are distributed in different

tissues in humans, and have been shown to function in the secretory pathway (3).

Breast cancer has become a common tumor, threatening women's physical and mental health. It is a complex tumor, and one of its most common causes is genetic mutations. A number of reports have provided evidence for an association between genes such as *BRCA-1*, *BRCA-2*, *p53*, *PTEN* etc., with breast cancer (4,5). The common features of cancer cells, namely, increased proliferation, migration and tissue invasion, lead to uncontrolled division and allow cells to invade surrounding tissues as well as basement membranes, thereby accelerating malignant growth and cancer progression. The protein PACE4 is strongly involved in malignancy, due to its activity that allows to generate biologically active proteins. There is evidence that aberrant expression of PACE4 is involved in different types of cancer. For example, overexpression of PACE4 in murine cells leads to increased invasiveness both *in vitro* and *in vivo* (6), and enhances tumor malignant phenotypes in breast cancer (7). Another report indicated that PACE4 plays an important role in enhancing the progression of prostate cancer (8). The critical role of PACE4 in promoting proceedings of tumors renders it an attractive target for cancer treatment.

PACE4 was shown to play a pivotal role in the extracellular matrix (ECM) (9). Numerous proteins such as growth factors, cell adhesion molecules, and oncogene products are active in the ECM. All these proteins are important in the occurrence and progression of cancer. Thus, PACE4 may also be involved in cancer. A previous study indicated that α 1-PDX and ppPACE4 (PACE4 prosegment) can increase cell motility, migration and invasion (7). In this context, we aimed to explore the direct effect of PACE4 in breast cancer cells and further investigate the roles of PACE4 in this type of cancer.

In the present study, an RNA interference approach was adopted to silence the expression of the *PACE4* gene in the MDA-MB-231 cell line and thereby, explore the role of PACE4 in breast cancer. We studied the proliferation, migration and invasion of MDA-MB-231 cells following siRNA transfection, and also detected cell-cycle changes following the reduction in the expression of *PACE4*. Breast cancer is a tumor related to hormones, and thus, the relationship between PACE4 and 17 β -estradiol was also investigated. In order to elucidate the

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mechanism underlying the PACE4 effects on MDA-MB-231 cells, the expression of genes involved in cell growth, invasion and adhesion, such as matrix metalloproteinase-9 (*MMP-9*), insulin like growth factor 2 (*IGF-2*) and myelin protein zero-like 2 (*MPZL2*), was also studied following siRNA-mediated inhibition of *PACE4* expression.

Materials and methods

Cell culture. Human breast cancer MDA-MB-231 and MCF-7 cells were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China) and cultured in HyClone™ Dulbecco's modified Eagle's medium (DMEM) containing 10% HyClone™ fetal bovine serum (HyClone), as well as Gibco® penicillin (100 IU/ml) and streptomycin (100 µg/ml) in a 37°C, 5% CO₂ incubator (all from Thermo Fisher Scientific, Rockford, IL, USA).

Small interfering RNA transfection. Three pairs of siRNAs were used in order to achieve the most effective inhibition of *PACE4* expression (si-*PACE4*-1 to -3). The siRNAs were synthesized by GenePharma (Shanghai, China) and target the human *PACE4* transcript (GenBank accession no., NM_002570.3), at positions 1,011, 1,196, and 2,118 of the sequence. The cultured MDA-MB-231 cells were transfected with the siRNAs using the HiPerFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and three siRNA concentrations (50, 100 and 150 nM) were tested. Cells transfected with an unrelated siRNA pair (5'-CUCCGAACGUGUCACGUTT-3') and cells cultured with the transfection reagent alone were used as the negative control (NC) and the mock, respectively.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the cells using the E.Z.N.A.® Total RNA Kit II (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. The extracted RNA was reverse transcribed using the ReverTra Ace® qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The relative expression of *PACE4* following siRNA transfection was analyzed by qPCR. The PCR was performed three times using the SYBR Green kit (Roche Diagnostics, Mannheim, Germany) and the following primers: *PACE4* forward (F), 5'-AAG CAA GGG AAG TTG AAA GA-3', and reverse (R), 5'-CAC TGA AGG TGT GGT ACG-3'; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*); used to standardize the level of expression of *PACE4*) F, 5'-CAC CAT CTT CCA GGA GC-3', and R, 5'-AGT GGA CTC CAC GAC-3'; *MMP9* F, 5'-CCC TGC CAG TTT CCA TT-3', and R, 5'-CCA TCA CCG TCG AGT CA-3'; *IGF-2* F, 5'-GCT TCT ACT TCA GCA GGC-3', and R, 5'-GTC CCT CTC GGA CTT GG-3'; *MPZL2* F, 5'-CTC TAA CAG TGA CCT GGA ATT T-3', and R, 5'-GAA GGA TGG AGG CAT CG-3'. The qPCR reactions were performed on a LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany), with the following cycling conditions: an initial denaturation step at 95°C for 15 sec, 45 cycles at 95°C for 10 sec and 60°C for 30 sec, followed by 10 sec at 72°C for final extension. The comparative threshold cycle (Ct) method was used to analyze the relative expression of mRNA. PCR products were subjected to 1%

agarose gel electrophoresis to confirm the specificity of the amplification.

Western blotting. Total protein was extracted by incubation on ice for 30 min with RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) containing protease inhibitors. The preparation was centrifuged at 14,000 x g for 30 min at 4°C. The protein concentration was measured with the Bio-Rad protein assay system (Hercules, CA, USA). Following incubation at 100°C for 5 min with loading buffer, 30 µg of the total protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Amersham, Piscataway, NJ, USA) by wet transfer, and blocked in 10 nM Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) skim milk. Next, the membranes were incubated overnight at 4°C with specific anti-human primary antibodies (anti-*PACE4* at 2,000-fold dilution, or anti-*GAPDH* at 1,000-fold dilution). Following a wash with phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween-20 (PBST), the membranes were incubated with the corresponding secondary antibody (goat anti-rabbit immunoglobulin G-HRP; 1:5,000 dilution) for 1 h at 37°C. All antibodies were purchased from Abcam (Cambridge, UK). Detection was performed using an enhanced chemiluminescence (ECL) kit (KeyGen Biotech, Nanjing, China). The film was scanned and analyzed with Quantity One Analysis software (Bio-Rad).

Cell proliferation assay. The human breast cancer MDA-MB-231 cells were seeded onto 96-well culture plates and incubated until they reached 80% confluence. The cultured cells were transfected with si-*PACE4* (150 nM). After growing for 24, 48 and 72 h, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml) were added to each well, and the incubation was continued for an additional 4 h at 37°C. Then, the MTT solution was removed, and 150 µl of dimethyl sulfoxide (Solarbio, Beijing, China) were added and incubated at room temperature for 10 min to allow extraction of the MTT-formazan products. The absorbance was measured at 490 nm with a UV/VIS spectrophotometer (Yuanxi-UV5500, Shanghai, China) and the measurements were repeated three times.

Cell migration and invasion assays. Cell migration was measured by a wound healing assay. The cells were plated onto 24-well plates and scratched linearly in multiple areas with a cell scraper. Then, the cells were transfected with 150 nM siRNA targeting *PACE4*, or with the negative and the transfection reagent control. The same area of each wound was examined at 0 and 24 h to quantify the cells that had migrated into the wound.

The cell invasion assay was performed in a Costar® Transwell apparatus coated with a Matrigel filter (Corning, NY, USA). Cells were plated at a density of 3x10⁴ cells/well, and incubated with 150 nM of siRNA in the upper chamber of the apparatus for 8 h, while the lower compartment was filled with DMEM with 10% FBS. The upper and the lower chamber were filled with DMEM without FBS and incubated for 12 h. The non-invading cells at the upper surface of the filter were scraped with cotton swabs, and the invading cells were stained Giemsa

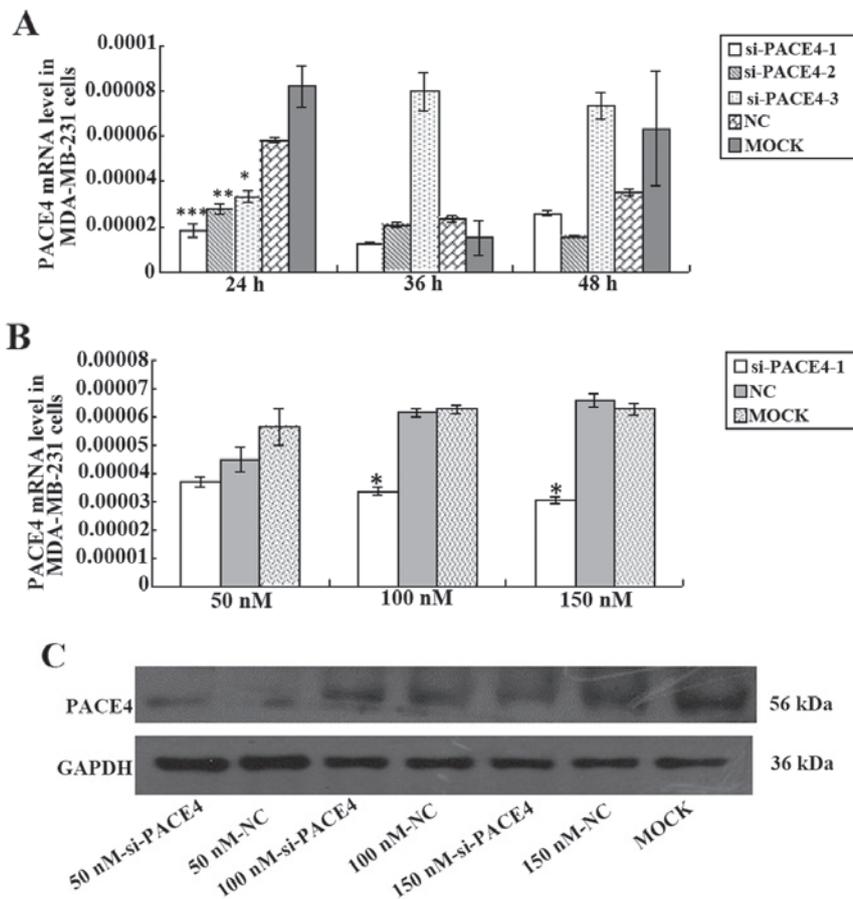


Figure 1. PACE4 expression in MDA-MB-231 cells following transfection with three different pairs of small interfering RNA (siRNA) (A) at different time-points, and (B) at different concentrations of the most effective siRNA, si-PACE4-1. *P<0.05, **P<0.01, and ***P<0.001 compared to the NC and MOCK controls. (C) Western blot analysis was used to measure the PACE4 protein expression level. NC, negative control; MOCK, transfection reagent control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(Solabio, China). The number of cells that had invaded through the membrane was quantified in 5 random fields of a fluorescence microscope (ECLIPSE50i, Nikon, Tokyo, Japan) at x100 magnification.

Cell cycle changes detected by flow cytometry. Cells were plated in 6-well culture plates at a density of 1×10^5 cells/ml in 2 ml of DMEM, and were transfected with siRNA as described above. The cells were then harvested at 24 h by addition of trypsin (0.25%; Solarbio), incubation for 5 min and centrifugation at 500 g for 10 min. The cells were washed twice with PBS, fixed overnight with 70% cold ethanol, and stained for 30 min at 37°C with 1 ml of propidium iodide solution (50 µg/ml; Beyotime Institute of Biotechnology), containing 50 µg/ml RNase A (MacGene Biotechnology, Beijing, China). The cell number at different phases of the cell cycle was determined on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and the DNA content was analyzed with the ModFitLT V3.0 software (BD Biosciences).

PACE4 expression induced by 17β-estradiol. MDA-MB-231 and MCF-7 cells were cultured for 3 days in phenol red-free DMEM medium (MacGene Biotechnology) containing 10% certified charcoal-stripped-FBS (Biological Industries,

Kibbutz Beit-Haemek, Israel), and were next treated with 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} mol/l of 17β-estradiol (Sigma-Aldrich, St. Louis, MO, USA) for 24 or 36 h. Cells cultured in phenol red-free DMEM medium were used as the control. The mRNA level of PACE4 was quantified by RT-qPCR, as described above.

Statistical analysis. Data were expressed as the mean ± standard deviation (SD). All results were confirmed in at least three independent experiments. Significant differences among three or more datasets were analyzed by a one-way analysis of variance (ANOVA) using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered to indicate statistically significant differences.

Results

PACE4 is successfully silenced by siRNA. To select the most effective PACE4-specific siRNA, RT-qPCR (data not shown) and western blot analyses were performed to detect the expression of PACE4 at the mRNA and protein level. We used three pairs of siRNA at concentrations 50, 100, 150 nM, and three periods of transfection, 24, 36 and 48 h. The most efficient silencing of PACE4 was achieved with 150 nM (Fig. 1B and C) of siRNA-1, incubated with the cells for

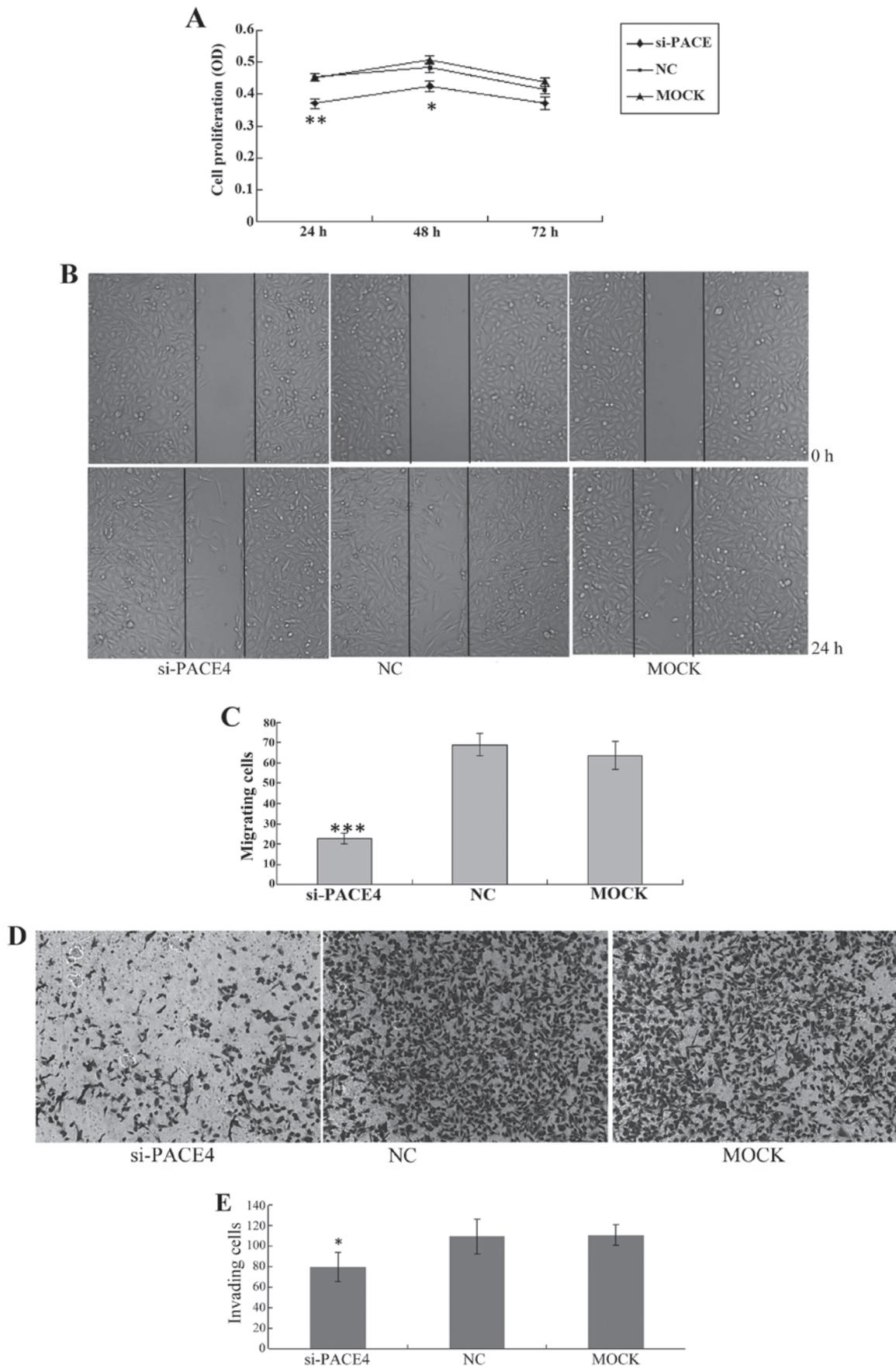


Figure 2. Cell proliferation, migration and invasion in MDA-MB-231 cells transfected with the PACE4 small interfering RNA (siRNA). (A) The MTT assay was used to determine cell proliferation. OD, optical density. (B) Cell migration was detected by a wound healing assay. (D) A Transwell assay was performed to study the cell invasive ability. The average number of cells (C) migrating into the wound and (E) invading through the Transwell membrane was calculated. Three independent experiments were performed. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared to the NC and MOCK controls. NC, negative control; MOCK, transfection reagent control.

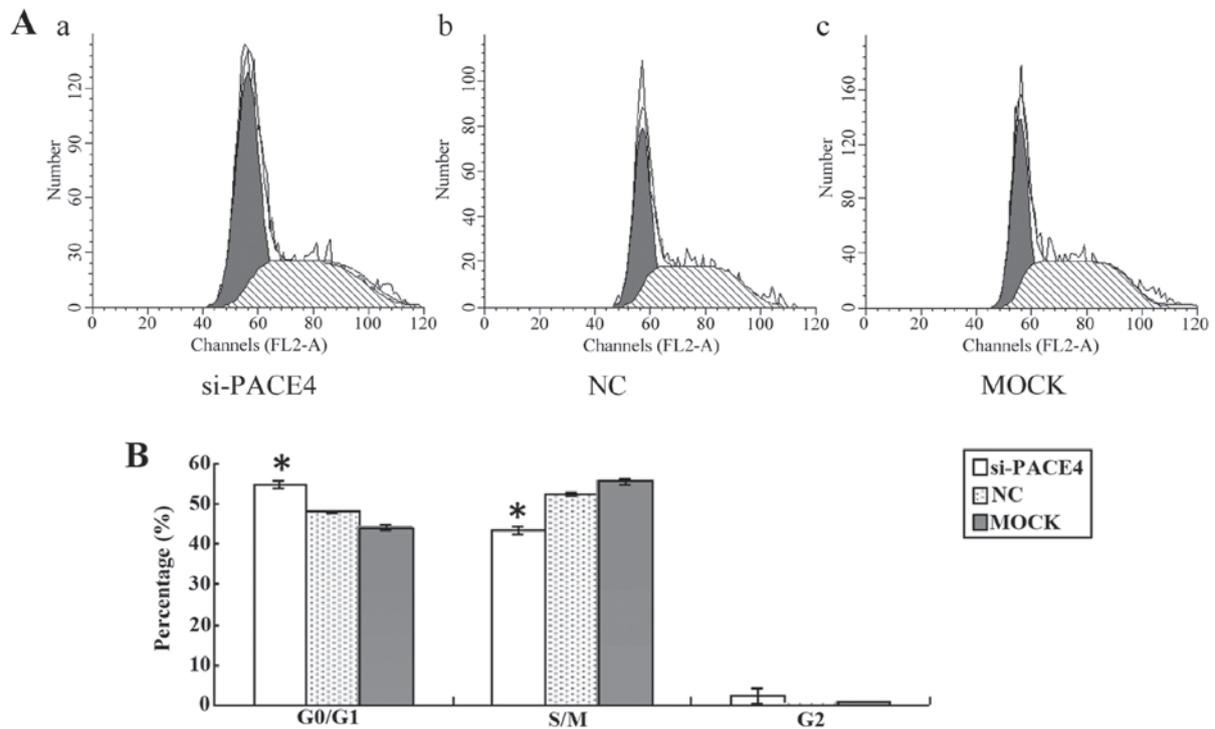


Figure 3. Silencing of *PACE4* induces cell-cycle arrest in MDA-MB-231 cells. (A) Representative pictures of flow cytometry and (B) percentage of cells at the different cell-cycle phases. Three independent experiments were performed. Data are expressed as means \pm SD. * $P < 0.05$ compared to the NC and MOCK controls. NC, negative control; MOCK, transfection reagent control; si-PACE4, small interfering RNA targeting *PACE4*.

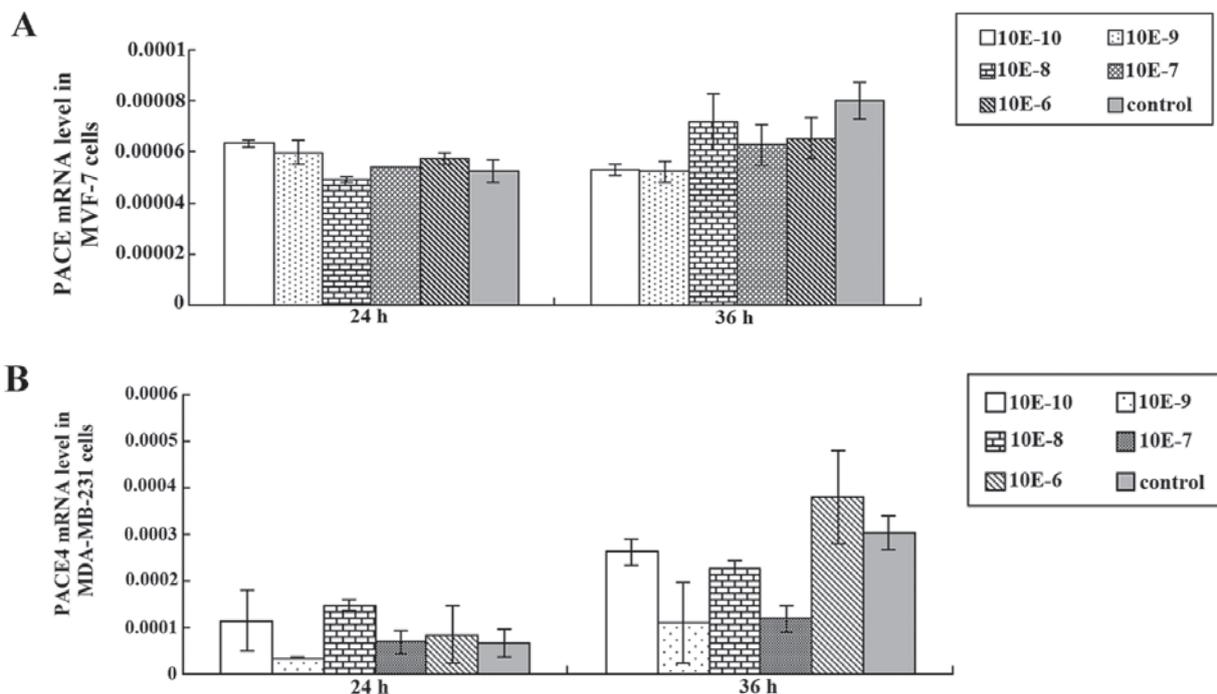


Figure 4. mRNA levels of *PACE4* in response to 17β -estradiol treatment. Five estrogen concentrations were tested in (A) MCF-7 cells and (B) MDA-MB-231 cells at 24 and 36 h. Three independent experiments were performed. Data are expressed as means \pm SD. * $P < 0.05$, compared to the control (untreated cells).

24 h (Fig. 1A). These conditions were used in the following experiments.

PACE4 silencing decreases the MDA-MB-231 proliferation rate. To analyze cell proliferation following siRNA-mediated silencing of *PACE4*, the MTT assay was performed. The

results showed that the growth rate of the cells transfected with the si-PACE4 is slower than that of the NC and mock control groups (Fig. 2A). This finding indicated that the human breast cancer cell line MDA-MB-231 is sensitive to reduced levels of *PACE4*, since silencing of the gene had an inhibitory effect on the growth of these cells.

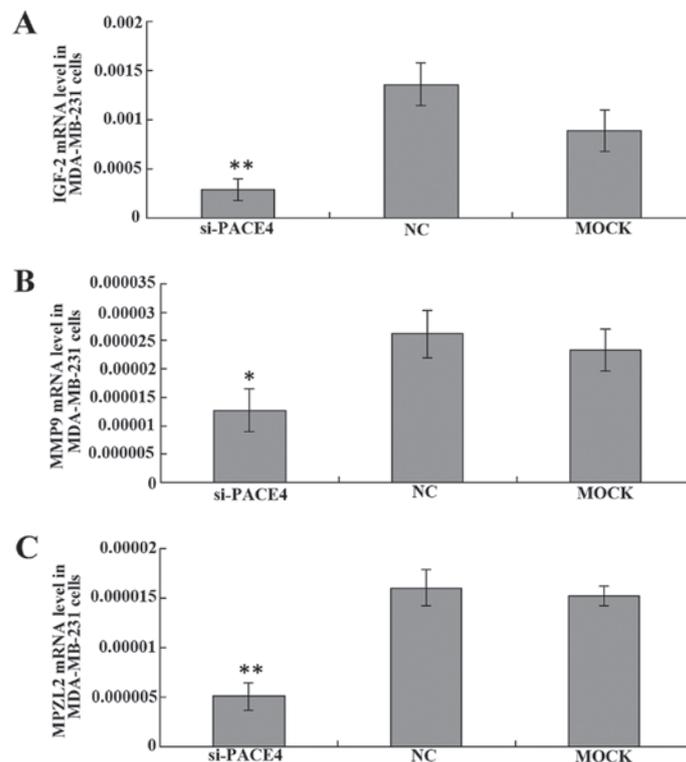


Figure 5. mRNA levels of key genes following *PACE4* silencing in MDA-MB-231 cells. The expression of (A) insulin like growth factor 2 (*IGF-2*), (B) matrix metalloproteinase-9 (*MMP9*) and (C) myelin protein zero-like 2 (*MPZL2*) was determined by reverse transcription-quantitative PCR. Three independent experiments were performed. Data are expressed as means \pm SD. * $P < 0.05$, and ** $P < 0.01$, compared to the NC and MOCK controls. NC, negative control; MOCK, transfection reagent control; si-*PACE4*, small interfering RNA targeting *PACE4*.

Silencing of PACE4 reduces MDA-MB-231 cell migration and invasion. Cell migration (Fig. 2B) and invasion (Fig. 2D) assays were performed to investigate whether *PACE4* affects cell migration and invasion. The results showed that, following *PACE4* silencing, cell migration and invasion were both reduced compared to the NC and mock controls. The number of cells that migrated into the wound (Fig. 2C) and invaded through the Matrigel filter (Fig. 2E) were quantified, and were both found to be significantly decreased compared to the controls.

PACE4 may induce G0/G1 phase arrest in breast cancer cells. Flow cytometry was used to investigate the effects of the *PACE4* knockdown on the cell cycle. Two types of results are shown in Fig. 3: typical images of flow cytometry showing the cell-cycle phase distribution of the cells (Fig. 3A), and the results of quantification and statistical analyses of cell-cycle phase distribution data at 24 h (Fig. 3B). These results showed that, following transfection with si-*PACE4* for 24 h, the populations of cells at the G0/G1 phase remained increased, which indicates that *PACE4* may induce G0/G1 arrest in breast cancer cells.

The expression of PACE4 in response to 17 β -estradiol treatment in MDA-MB-231 and MCF-7 cells. An earlier study showed that the *PACE4* gene expression level significantly correlates to the concentration of the cells in estrogen receptors (10). Based on this finding, we investigated whether the expression of *PACE4* is affected by 17 β -estradiol using RT-qPCR in the human MDA-MB-231 and MCF-7 cell lines. The results showed that the mRNA level of *PACE4* is not significantly

different between treatments with different concentrations of 17 β -estradiol (Fig. 4).

The MMP9, IGF-2 and MPZL2 gene levels are decreased following transfection with si-PACE4. To investigate the mechanism underlying the *PACE4* silencing-mediated effects on the breast cancer cell line MDA-MB-231, the expression of the *MMP9*, *IGF-2* and *MPZL2* genes was studied following siRNA transfection. qRT-PCR experiments were performed, and the results showed that all three genes show significantly reduced expression following transfection with si-*PACE4* (Fig. 5).

Discussion

In recent years, the relationship between *PACE4* and cancer has been extensively studied. Overexpression of *PACE4* was demonstrated in skin, lung and prostate cancer (8,11,12). However, other studies reported markedly reduced *PACE4* expression levels in human ovarian endometrial cancer cells (13,14). As to breast cancer, *PACE4* was found overexpressed, and the study of Lapierre *et al* (7) demonstrated that overexpression of the prosegment pp*PACE4* in the breast cancer cell line MDA-MB-231 significantly enhances cell motility, migration and invasion of collagen *in vitro*, although the role of *PACE4* in these processes was not fully clarified. The abnormal expression of *PACE4* has overall become an emerging research focus in numerous cancer studies.

In the present study, we transfected different pairs of siRNA targeting the *PACE4* gene (si-*PACE4*-1 to -4) in the breast cancer cell line MDA-MB-231, and examined the

degree of *PACE4* silencing by RT-qPCR and western blotting. The results showed that the expression of *PACE4* is efficiently silenced by si-*PACE4*. The highest transfection efficiency was achieved at 24 h of transfection with 150 nM of si-*PACE4*-1. The MTT assay also indicated that cell growth is prominently decreased at 24 h of siRNA transfection compared to the control groups. *PACE4* has been shown to play a vital role in the activation of numerous growth factors, such as IGF-2. IGF-2 is involved in tumor progression by stimulating cell proliferation, and can be activated by *PACE4* at Arg104 (14). We presumed that *PACE4* may promote cell proliferation through activation of growth factors such as IGF-2. To confirm this, we studied the expression of the *IGF-2* gene by RT-qPCR following transfection of MDA-MB-231 cells with si-*PACE4*, and the result showed that *IGF-2* expression is significantly decreased in the si-*PACE4*-transfected cells. We also found that silencing of *PACE4* blocks the cell cycle transition from the G0/G1 to the S phase. Taken together, these results indicated that si-*PACE4* may inhibit cell proliferation.

MMPs are enzymes involved in a number of pathological processes, and play a vital role in tumor invasion and metastasis, by damaging almost all the proteins of the ECM. MMP9 was identified as an important target of *PACE4*, degrading the ECM and eventually resulting in enhanced invasiveness (15). To investigate the role of *PACE4* in the process(es) enhancing the invasive ability of breast cancer MDA-MB-231 cells, we studied the expression of the *MMP9* gene by RT-qPCR. This analysis showed that, following *PACE4* silencing, the *MMP9* level is significantly decreased. This result is consistent with the findings of Lapiere *et al* (7), who reported that overexpression of pp*PACE4* can increase the *MMP9* activity.

The MPZL2 protein, which contains a putative serine phosphorylation site and is specifically regulated during T cell maturation, is believed to mediate cell adhesion through a homophilic interaction (16). The results of another study by our lab on rheumatoid arthritis (unpublished data) also indicated that MPZL2 may be an important target of *PACE4*. In this study, RT-qPCR detection of the *MPZL2* mRNA level showed that its expression is significantly decreased upon si-*PACE4* transfection of MDA-MB-231 cells. All the above suggest that MPZL2 may represent an important protein in cell differentiation.

It is well known that the occurrence and development of breast cancer is closely related to estrogens (17). In this study, we chose MCF-7 cells (estrogen receptor-positive cells) and MDA-MB-231 cells (estrogen receptor-negative cells) to explore the relationship between *PACE4* gene expression and estrogen content. However, in contrast to a previous study showing that *PACE4* expression significantly correlates to the estrogen receptor content (10), our results did not identify any association between these two parameters.

In conclusion, our study showed that silencing of *PACE4* can decrease proliferation, migration and invasion in breast cancer cells. An earlier report proposed that *PACE4* inhibition may disrupt the function of numerous proteins critical for tumorigenesis, and that attenuating *PACE4* overexpression in cancer may constitute a therapeutic approach (18). Therefore, the *PACE4* gene may be a promising inhibition target in the context of breast cancer treatment.

Acknowledgements

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