Overexpression of neogenin inhibits cell proliferation and induces apoptosis in human MDA-MB-231 breast carcinoma cells

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Abstract. Neogenin has been documented as playing an important role in cancer development. Although an elevated expression of neogenin has been detected in human breast cancer, the role of neogenin in breast cancer cells is not clearly understood. In the present study, we investigated neogenin in breast cancer cell proliferation, migration and apoptosis. We found that neogenin overexpression markedly reduced the proliferation and migration of breast cancer cells ($P<0.05$). Neogenin overexpression resulted in a reduction in the apoptosis rate. Inhibition of neogenin expression by neogenin siRNA dramatically promoted the proliferation and migration of breast cancer cells, whereas it inhibited cell apoptosis. Furthermore, we found that BMP-2-induced phosphorylation of Smad1/5/8 which was inhibited by neogenin overexpression. The present study demonstrates that neogenin may be a tumor suppressor in breast cancer. Neogenin may serve as a potential diagnostic marker and therapeutic target for breast cancer.

Introduction

Breast cancer is the most common malignant tumor in women, and the leading cause of cancer mortality in females that causes approximately half a million deaths each year worldwide (1,2). Although recent substantial progress has been achieved in treatments involving chemotherapy, surgery and radiation therapy, breast cancer is still difficult to cure because of the propensity of these tumors to form distant metastases (3,4) and the distinct subtypes that exist (5-7). Therefore, to cure breast cancer, we should understand the relevant molecular mechanisms involved in breast cancer metastasis. Evidence suggests that many characteristics and markers, such as the progesterone receptor, histological grade, HER2/ERBB2 status, the estrogen receptor, p53 mutational status and neogenin are able to classify heterogeneous breast cancers (1,8). More recently, previous studies have indicated that neogenin expression may be inversely correlated to the tumorigenicity of human breast cancer (8); however, the specific function of neogenin in the progression of breast cancer is unclear.

Neogenin, a homologue of the DCC (deleted in colorectal cancer) receptor group, encodes a 1461 amino acid identity. Neogenin is widely distributed in the CNS and is a dependent receptor of the repulsive guidance molecule a (RGMa) (9-11). Previous studies have suggested that neogenin plays an important role in cell to cell recognition, tissue growth regulation, cellular differentiation, cell migration, cell apoptosis, angiogenesis, epithelial cell renewal and histogenesis (12-16). It has been reported that neogenin is expressed in many adult tissues, and abnormal expression of neogenin has been found in a variety of human cancers, such as pancreatic (17), colon cancer (18), esophageal squamous cell carcinoma (ESCC) (19), gliomas (20) and breast cancer (8). Subsequent studies revealed that altered expression of neogenin may lead to loss of pro-apoptotic activity and may even cause tumorigenesis (21). There is some evidence to suggest that downregulation of neogenin accelerates glioma progression through promoter methylation and its overexpression in SHG-44 induced apoptosis (20). Moreover, Lee and colleagues (8) reported that neogenin expression is downregulated in human breast cancer relative to the normal breast tissue.

A protein which can regulate cancer-relevant cellular functions such as cellular proliferation and apoptosis may be the potential source of molecular signaling pathways commonly disrupted in cancer cells (22,23). Evidence suggests that bone morphogenetic proteins (BMPs) regulate many mammalian physiological and pathophysiological processes (24). BMPs bind to kinase receptors, thereby activating Smad transcription factors. Moreover, it has been reported that neogenin is a receptor for BMPs (24). Thus, we speculated that neogenin could modulate Smad signal transduction through binding with BMPs. In the present study, we demonstrated that neogenin overexpression can inhibit cell proliferation and

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migration; moreover, promoting cell apoptosis. The present study provides the first direct evidence in breast cancer cells that neogenin overexpression can result in cell growth inhibition and apoptosis.

Materials and methods

Antibodies. A rabbit monoclonal phospho-specific antibody to Smad1/5/8 was obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-Smad1 monoclonal antibody, rabbit anti-neogenin monoclonal antibody, mouse anti-β-actin monoclonal antibody, HRP-conjugated rabbit anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were obtained from Abcam (Cambridge, MA, USA).

Cell culture and transfection. The human breast cancer cell lines MDA-MB-231, MCF-7 and T47D cells (all cell types from the American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA), 1% penicillin-streptomycin and 1% glutamine. All the cells were grown and maintained at 37˚C under a humidified atmosphere of 5% CO_2. MDA-MB-231 cells were transiently transfected in a 24-well plate with either human neogenin cDNA (pcDNA3.1-neogenin) or the control vector pcDNA3.1 using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. These cells were assayed 24, 48, 72 and 96 h after transfection.

MTT proliferation assay. Cell proliferation in MDA-MB-231, MCF-7 and T47D cells was detected using the MTT assay according to a method previously described (4). Briefly, transfected cells and control cells were plated in 96-well plates at $5x10^3$ cells/well and cultured in DMEM for 48 h. Next, the culture medium was replaced with 100 μl of fresh DMEM, then 20 μl MTT (5 mg/ml) was added to the cells for another 4 h at 37˚C. Formazan crystals were dissolved in 200 μl of dimethyl sulfoxide (DMSO) and the absorbance was measured at λ 595 nm with a spectrophotometer (Multiskan MK3; Thermo Fisher Scientific, Waltham, MA, USA).

Transwell migration assays. MDA-MB-231 cell migration was detected according to the method described in a previous study (16). Briefly, MDA-MB-231 cells were transfected with neogenin, and then the cells ($5x10^3$ cells/well) were added to the upper Transwell (Corning Costar, Corning, NY, USA) chambers with 0.5 mg/ml collagen type I (BD Biosciences, Seoul, Korea) coated filters 24 h after transfection. DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin and 1% glutamine was added to the lower chamber and incubation was continued for 24 h. Wide-field microscopy was used to quantify the cells that migrated to the lower chamber. Cells were counted at five randomly selected areas in each well.

Detection of apoptotic cells by flow cytometry. At 48 h after transfection, apoptosis of MDA-MB-231 cells was detected by flow cytometry. Subsequently, the cells were stained with Annexin V-FITC and propidium iodide (PI) for 20 min at room temperature. The apoptotic cells were then analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA) according to the instruction of the Annexin V-FITC Apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China).

Total RNA extraction and quantitative reverse transcription-PCR. Neogenin mRNA level was detected by the RT-PCR method (25). Total RNA was extracted using standard methods (26,27). Approximately 2 μg of total RNA was reverse transcribed into first strand cDNA using random primers for qRT-PCR analysis. The primer pairs used for PCR are as follows: neogenin (24): forward, 5'-GGAAGGAGGGGAATGAGACC-3' and reverse, 5'-AATCACGGGTAGGGTAGGTA-3'; β-actin forward, 5'-TCCCTGGAGAAGAGCTACGA-3' and reverse, 5'-AGGAAGGAAGGCTGGAAGAG-3'. All the primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Quantitative RT-PCR was done using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Interpretation of the relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (28). β-actin mRNA was used as an internal control.

Western blot analysis. We performed western blot analysis as previously described (29). The cells were homogenized and lysed with RIPA lysis buffer (Beyotime, Nantong, China). The protein concentration was measured using a BCA protein assay kit (Beyotime). Equal amounts of protein lysate (40 μg/lane) were separated on 12% SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. Then, the cells were incubated with primary antibodies specific for neogenin, Smad1/5/8 and β-actin. The blots were rinsed in TBST, and further incubated in HRP-conjugated rabbit anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG. Bound proteins were visualized using enhanced chemiluminescence (ECL) reagent (Boehringer Mannheim, Mannheim, Germany).

SiRNA transfection. Breast cancer MDA-MB-231 cells with the neogenin protein were transfected with neogenin siRNA or the control siRNA (siMock) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The coding strand of human neogenin siRNA (16) was 5'-AGAUCUGGAGGUUUCACAUCUUUGG-3'. The siRNA oligonucleotides were obtained from Shanghai Sangon. Neogenin siRNA and siMock-transfected cells were used for further experiments. Neogenin mRNA and protein levels were determined by RT-PCR and western blotting 24 h after transduction.

Statistics analysis. All data were obtained from at least three independent experiments and are expressed as mean ± SD. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data were analyzed using analysis of variance (ANOVA) and Student's t-test. $P<0.05$ was considered to indicate a statistically significant result.

Results

Increased neogenin levels in breast cancer cell lines MDA-MB-231, MCF-7 and T47D cells transduced with pcDNA3.1-neogenin. As a result of the RT-PCR and western

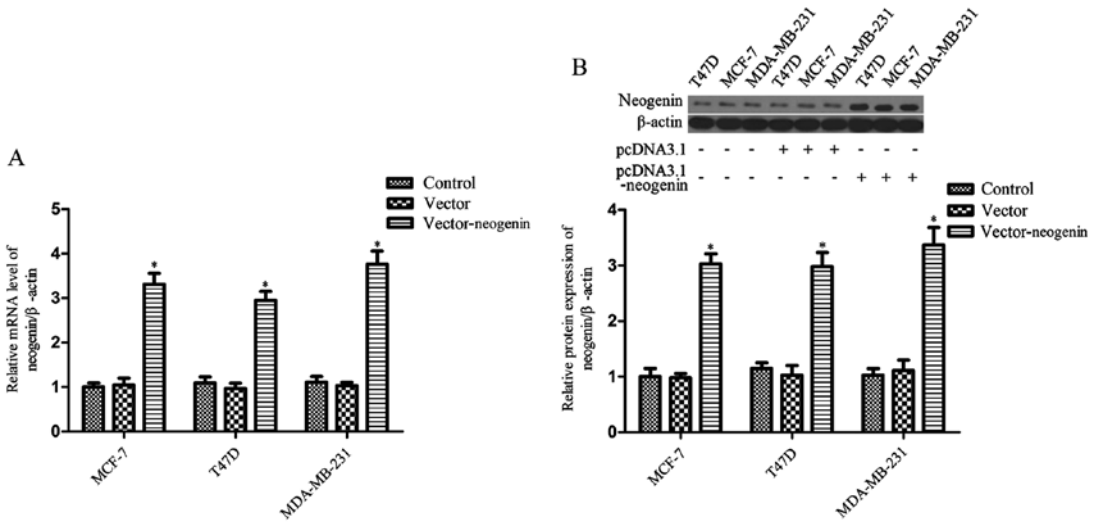

Figure 1. Analysis of neogenin mRNA and protein expression in human breast cancer cells after transduction. (A) Neogenin mRNA levels were measured by RT-PCR and normalized to β-actin. (B) The expression of neogenin protein was determined by western blot analysis. The relative levels of the neogenin normalized by β-actin values are shown in the graph. Values are presented as mean ± SD. *P<0.05 vs. control.

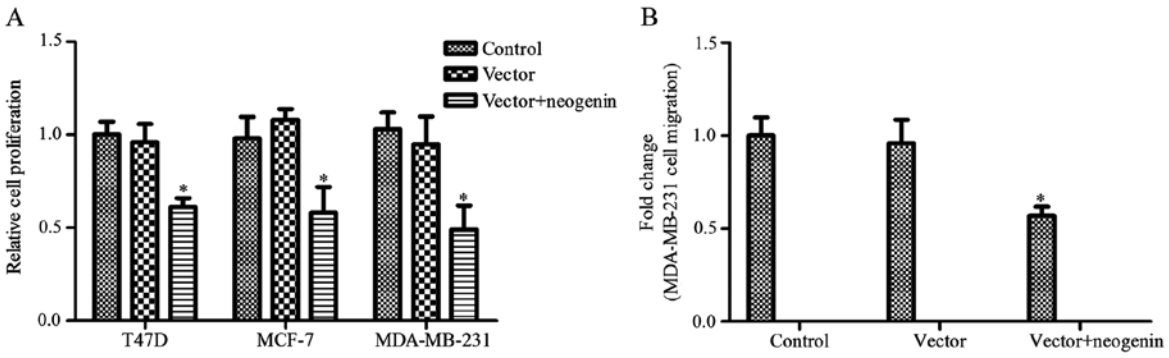

Figure 2. The effect of neogenin overexpression on breast cancer cell proliferation and migration. (A) Cell proliferation was assessed in three breast cancer cell lines (T47D, MCF-7 and MDA-MB-231) 48 h after transfection with pcDNA3.1-neogenin. (B) Cell migration of MDA-MB-231 breast cancer cells 48 h after transfection. Data are presented as mean ±SD. *P<0.05 vs. control.

blot analysis of the three cell lines (MDA-MB-231, MCF-7 and T47D), the data show that neogenin mRNA and protein expression was weak (Fig. 1). Then, in order to further understand the role of neogenin in breast cancer, neogenin was overexpressed in the MDA-MB-231, MCF-7 and T47D cell lines by transfection. Cells were harvested after 48 h and neogenin expression was analyzed by RT-PCR and western blot analysis. The results show that neogenin mRNA and protein levels in the cells that were transduced with pcDNA3.1-neogenin for 48 h were much higher than in the control group (Fig. 1). The expression of neogenin was also upregulated in MDA-MB-231, MCF-7 and T47D cells transduced with pcDNA3.1-neogenin for 24, 72 and 96 h (data not shown).

Effect of the overexpression of neogenin on breast cancer cell proliferation and migration. The neogenin overexpression vector was transfected into MDA-MB-231, MCF-7 and T47D cells, and then cell proliferation was measured by the MTT assay. As shown in Fig. 2A, the proliferation of MDA-MB-231, MCF-7 and T47D cells was greatly decreased with neogenin overexpression. Neogenin overexpression resulted in a 39, 42 and 51% decrease in the T47D, MCF-7 and MDA-MB-231 cell numbers, respectively. These results indicate that neogenin overexpression can inhibit the proliferation of all three breast cancer cell lines. Thus, we selected the breast cancer cell line MDA-MB-231 for further study. We also assessed the effects of neogenin overexpression on MDA-MB-231 cell migration. The results show that the migration of MDA-MB-231 cells was significantly decreased after neogenin overexpression (Fig. 2B).

Induction of apoptosis after neogenin overexpression in the breast cancer cell line MDA-MB-231. Reports have shown that neogenin overexpression can induce apoptosis in the human glioma cell line SHG-44 (20). Moreover, the present study indicates that neogenin overexpression inhibits breast cancer cell proliferation. Thus, we tested whether the change in the MDA-MB-231 cell numbers in our studies was also mediated by neogenin-induced apoptosis. Apoptosis was measured by flow cytometric analysis; the results are shown in Fig. 3. Flow cytometry showed that 33.9% of the cells that were transfected with neogenin underwent apoptosis compared to 5.6% in the

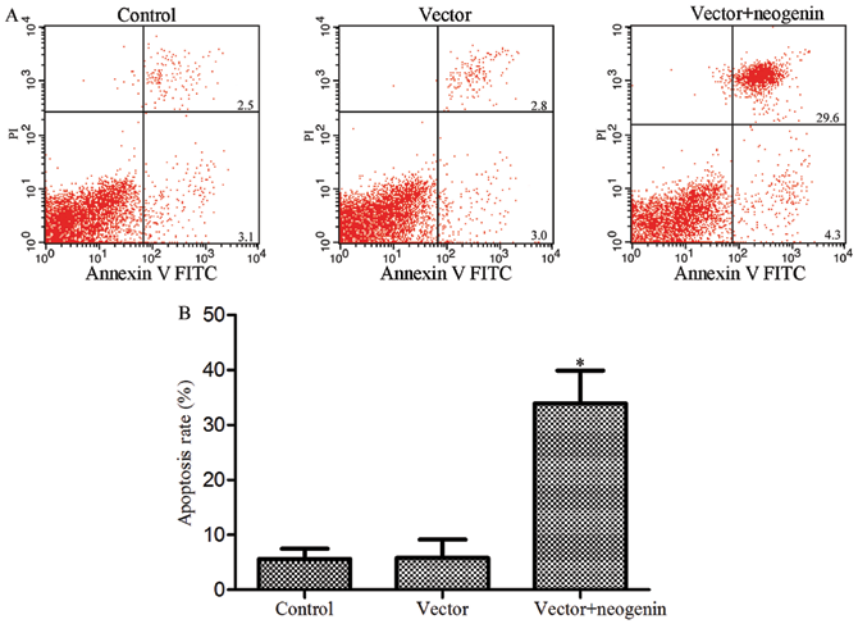

Figure 3. The effect of neogenin overexpression on breast cancer MDA-MB-231 cell apoptosis. (A) Flow cytometric analyses of MDA-MB-231 cell apoptosis. Cells were treated with pcDNA3.1-neogenin vector or blank vector for 48 h. (B) Quantitative analysis of the apoptotic rate after transfection. Data are presented as mean ± SD. *P<0.05 vs. control.

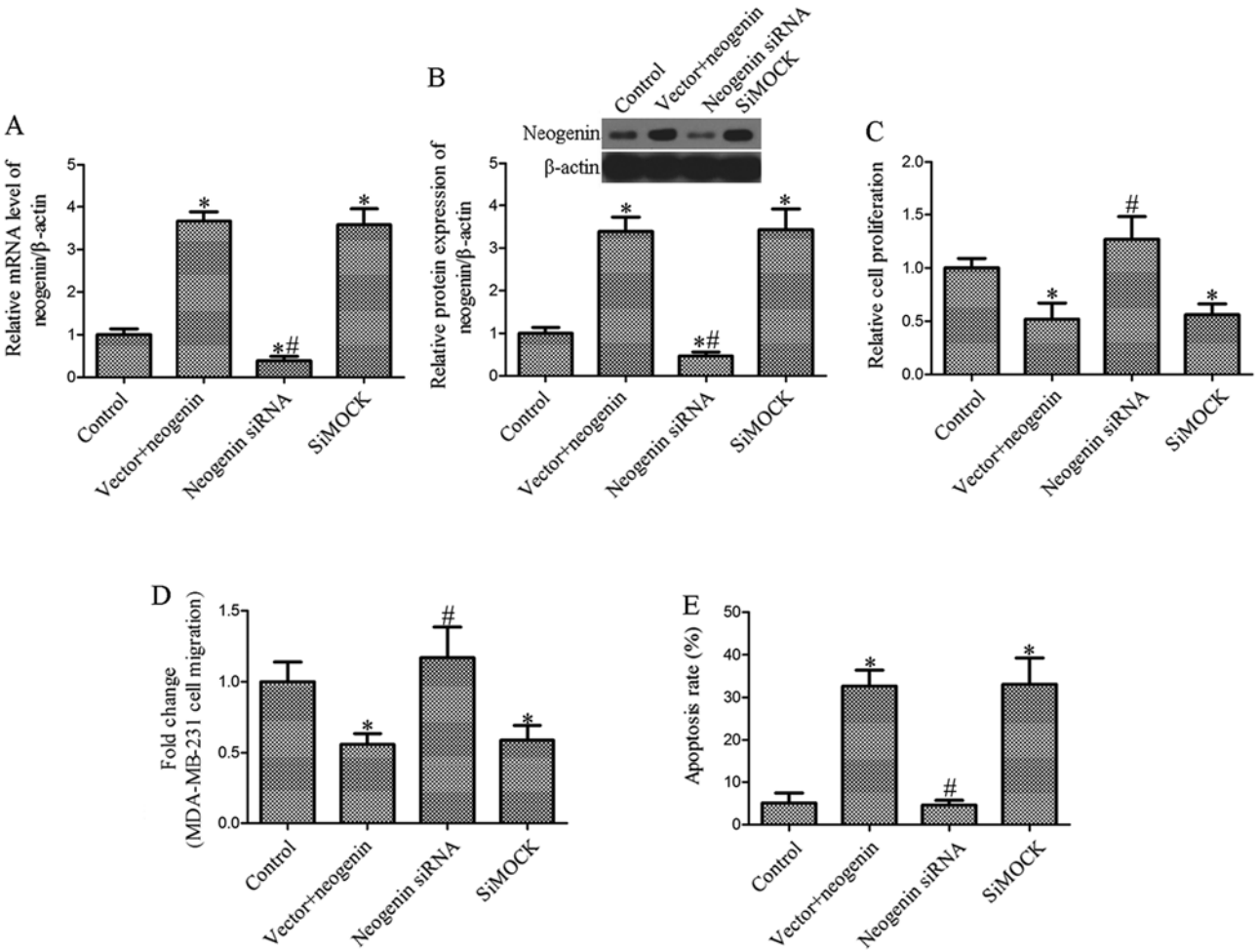

Figure 4. The effect of neogenin siRNA on the proliferation, migration and apoptosis of MDA-MB-231 transfected cells. (A) RT-PCR analyses of neogenin mRNA expression in neogenin siRNA and siMock treated cells. (B) Neogenin expression in neogenin siRNA and siMock treated cells by western blot analysis. The relative levels of neogenin normalized by β-actin values are shown in the graph. (C) The proliferation of MDA-MB-231 cells after neogenin siRNA and siMock treatment. (D) Cell migration of MDA-MB-231 breast cancer cells transfected by siRNA or siMock. (E) Cell apoptosis was detected by flow cytometry. Cells were treated with neogenin siRNA and siMock. Data are presented as mean ± SD. *P<0.05 vs. control, #P<0.05 vs. neogenin overexpression group.

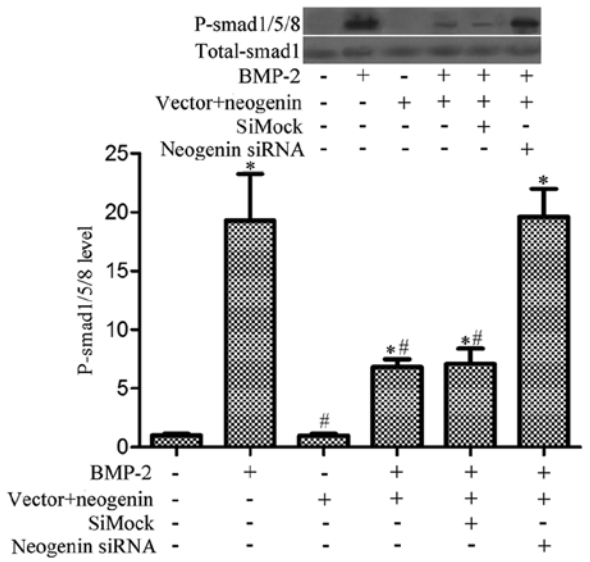

Figure 5. Effects of neogenin overexpression on the phosphorylation of Smad1/5/8. MDA-MB-231 cells were transfected with pcDNA3.1-neogenin, neogenin siRNA or control siRNA (siMock) and were treated with rhBMP-2 for 30 min. The relative levels of P-Smad1/5/8 normalized by Smad1 and were expressed as fold changes vs. Smad1. Data are presented as mean ± SD. *P<0.05 vs. control, #P<0.05 vs. BMP-2 treated group.

control group which were not transfected (P<0.05) and 5.8% in the vector group which were transfected with the empty vector (P<0.05). Similar to those reported in literature (8), these results further suggest that neogenin may be a breast cancer suppressor by inducing apoptosis in breast cancer cells.

Effect of the ablation of neogenin on breast cancer cell proliferation, migration and apoptosis. To determine whether siRNAs inhibit the expression of neogenin, we first investigated the effects of siRNA on neogenin mRNA and protein expression in MDA-MB-231 cells transfected with neogenin. The level of neogenin was measured by RT-PCR and western blot analysis after transfection of siRNAs into the breast cancer cells. The results showed that the level of neogenin in breast cancer MDA-MB-231 cells transfected with neogenin siRNA was significantly decreased (P<0.05; Fig. 4A and B). Then, we determined the effect of neogenin silencing on cell proliferation, migration and apoptosis. As shown in Fig. 4C, MDA-MB-231 cell growth was significantly increased in the neogenin siRNA-transfected group compared with the siMock-transfected group. Furthermore, we found that cell migration following the ablation of neogenin considerably increased the migration of MDA-MB-231 cells (Fig. 4D). Moreover, MDA-MB-231 cell apoptosis was also markedly decreased in the siRNA-transfected group (Fig. 4E). Our results showed that the effect of neogenin on proliferation, migration and apoptosis is associated with the overexpression of neogenin.

Overexpression of neogenin suppresses BMP-2-induced phosphorylation of Smad1/5/8 in breast cancer cells. Reports have suggested that BMP2 may act as a tumor suppressor by promoting apoptosis in many cell types, such as mature colonic epithelial and human colorectal cancer cells (30,31). Moreover, some evidence indicates that BMP-2 can induce the phosphorylation of Smad1/5/8, which is prevented by neogenin (24). Furthermore, an important role for the Smad1/5/8 signaling pathway in migration was described in the bone marrow stromal cells (32). Therefore, we investigated if neogenin-induced breast cancer cell migration and growth inhibition is related to the BMP-2-induced Smad1/5/8 signaling pathway. We treated MDA-MB-231 cells with rhBMP-2 (0.1 mg/ml) for 30 min, and then analyzed the phosphorylation state of the receptor proteins Smad1/5/8 using an antibody that specifically recognizes phosphorylated Smad1/5/8. The results showed that phosphorylation of Smad1/5/8 was significantly decreased in neogenin overexpressing cells (Fig. 5). These data indicate that the Smad1/5/8 pathway is inhibited in neogenin-transfected cells.

Discussion

The main findings of the present study are as follows: i) neogenin is weakly expressed in breast cancer cells, and neogenin overexpression can inhibit breast cancer cell growth and migration; ii) neogenin overexpression can promote breast cancer MDA-MB-231 cell apoptosis; iii) neogenin silencing has no apparent effect on MDA-MB-231 cell growth, migration or apoptosis; and iv) neogenin overexpression is able to inhibit BMP-2-induced Smad1/5/8 phosphorylation. The results of the present study indicate that neogenin inhibits the progression of breast cancer *in vitro*, which can be explained by the growth and migration inhibition and pro-apoptosis effects of neogenin in breast cancer cells.

Breast cancer, a serious threat to the health of females, is a malignant tumor associated with the fastest growing female mortality rate, far surpassing lung cancer (33,34). The incidents of breast cancer are increasing at an annual rate of 3% in China (34). Treatment for breast cancer is far from satisfactory, and some evidence suggests that breast cancer is a genetic disease (35). The balance of oncogenes and tumor suppressor genes plays an important role in the regulation of cellular physiological processes and an abnormal balance may affect cell proliferation, differentiation, apoptosis and drug resistance (36,37). Thus, it is possible that identifying novel targets may prevent or enhance the treatment of breast cancer (38). Evidence has suggested that neogenin is abnormally expressed in various cancers, including bladder cancer (8).

Although Meyerhardt and co-workers (39) suggested that neogenin is expressed in breast cancer cell lines and indicated that neogenin expression is unchanged in cancer, including bladder cancer, some studies have shown that neogenin expression is lower, in prostate (40), colon (41) and breast cancer (8). Our results are consistent with the existing data (8) which suggest that the expression of neogenin in breast cancer cells is inversely associated with the tumorigenicity of breast cancer. Considering the results of RT-PCR and western blot analysis on the three cell lines (T47D, MCF-7 and MDA-MB-231), the data show that neogenin was weakly expressed in these cells (Fig. 1). In order to study the effects of neogenin on the progression of breast cancer, we transfected the recombinant expression vector pcDNA3.1-neogenin into the breast cancer cell lines T47D, MCF-7 and

MDA-MB-231. The RT-PCR and western blot analysis results show that the expression of neogenin in the three cell lines was significantly upregulated (Fig. 1). Then, we demonstrated that a high level of neogenin was correlated with a decrease in cell proliferation and migration and an increase in cell apoptosis (Figs. 2 and 3). Neogenin siRNA was used to silence the expression of neogenin in neogenin-transfected cells. The results suggest that neogenin siRNA increased the cell number and migration and decreased apoptosis (Fig. 4). The data from the present study indicate that neogenin was able to inhibit the progression of breast cancer.

The functions of BMPs in cancer are situational and complex (42); our observations show that neogenin expression inhibits BMP-2-induced phosphorylation of Smad1/5/8 in breast cancer cells. Treatment of these cells with rh-BMP-2 led to an increase in the level of phosphorylation of Smad1/5/8; however, neogenin overexpression induced a marked decrease in the phosphorylation of Smad1/5/8. Moreover, the extent of the Smad1/5/8 phosphorylation in the siMock group was less than that in the neogenin siRNA group.

In summary, neogenin may play an important role in the progression of breast cancer. Upregulation of neogenin reduced breast cancer cell proliferation, inhibited migration and induced apoptosis. Collectively, neogenin can be considered a tumor suppressor in breast cancer. We demonstrated that neogenin expression may be inversely correlated to breast cancer. However, the specific mechanism of action of neogenin in breast cancer cells remains to be determined. Future studies on the role of neogenin in breast cancer will address these issues and enhance our knowledge of breast cancer.

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