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Stable SET knockdown in breast cell carcinoma inhibits cell migration and invasion

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ABSTRACT

Breast cancer is the most malignant tumor for women, however, the mechanisms underlying this devastating disease remain unclear. SET is an endogenous inhibitor of protein phosphatase 2A (PP2A) and involved in many physiological and pathological processes. SET could promote the occurrence of tumor through inhibiting PP2A. In this study, we explore the role of SET in the migration and invasion of breast cancer cells MDA-MB-231 and ZR-75-30. The stable suppression of SET expression through lentivirus-mediated RNA interference (RNAi) was shown to inhibit the growth, migration and invasion of breast cancer cells. Knockdown of SET increases the activity and expression of PP2Ac and decrease the expression of matrix metalloproteinase 9 (MMP-9). These data demonstrate that SET may be involved in the pathogenic processes of breast cancer, indicating that SET can serve as a potential therapeutic target for the treatment of breast cancer.

1. Introduction

Breast cancer is the most common malignant tumor for women around the world, and its incidence rate is increasing in recent years. Like most cancers, breast cancer is a systemic disease, and many factors and biological active substances such as growth factors, cytokines and hormones are involved in the complex pathogenetic processes. The abnormality of those active substances-mediated signal transduction pathways can lead to excessive amplification of certain genes that cause normal cells to accept the signal of abnormal proliferation, differentiation and growth, and eventually result in cancerogenesis [1].

SET, also termed I2PP2A, PHAPII and TAF-1b, was first identified in 1992 in a study of leukemia [2]. SET is known as inhibitor-2 of protein phosphatase 2A (I2PP2A) and exists at elevated levels in the brains of those with Alzheimer's disease relative to normal age-matched controls [3] and in leukemia cancer cells [4]. SET is overexpressed in many cancer cells including Wilm tumors [5], pancreatic tumors [6], prostate cancer [7], lung tumors [8], ovarian cancer [9] and testicular cancers [10]. It has also been demonstrated that SET is involved in neuronal apoptosis [11]. As an Endogenous inhibitor of PP2A, SET-mediated PP2A inhibition occurs via dephosphorylation of proteins, such as the protein kinase B (Akt) [12] and extracellular signal-regulated kinase (ERK) [13]. SET also regulate apoptosis, the cell cycle as well as cell motility through its functions of controlling histone acetylation, beta-adrenergic receptor phosphorylation and granzyme activity [14–17].

Because SET has such a diversity of functions, we hypothesized that SET is involved in breast tumor. In this study, we described the construction and characterization of a stable SET siRNA breast cancer cells MDA-MB-231 and ZR-75-30 and explored the effects of SET knockdown on the breast tumor cells.

2. Materials and methods

2.1. Chemicals and reagents

The human breast cancer cells MDA-MB-231 and ZR-75-30 were obtained from the American Type Culture Collection. 293T

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cells were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences (Chinese Academy of Science, China). RPMI 1640 and culture medium, fetal bovine serum (FBS), penicillin–streptomycin and trypsin were purchased by Gibco/Invitrogen (Carlsbad, CA, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit for cell viability was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The matrigel was obtained from BD Company (BD Biosciences, USA). The mouse monoclonal antibodies against SET, MMP-9 and GAPDH were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The PP2A immunoprecipitation phosphatase assay kit and PP2Ac antibody were purchased from Millipore (Billerica, MA, USA). The pLVX-shRNA1 expression vector and lentivirus transfection packaging kit (Lenti-X Packaging System) were purchased from Clontech (Mountain View, CA, USA).

2.2. Cell culture

The human breast cancer cell lines was cultured in RPMI 1640 medium containing 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2.

Constructs, production of recombinant lentivirus vectors, and generation of stable SET siRNA cell lines.

One pair of siRNA oligonucleotides targeting human SET was designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).The annealed oligonucleotides were cloned into the BamHI and EcoRI sites of pLVX-shRNA1 using compatible restriction sites flanking the oligo siRNA target sequence. The blank plasmid was used as a control.

The pseudotyped lentiviruses were produced in 293T cells by cotransfection with the recombinant siRNA plasmid, the blank plasmid and the three Clontech Lenti-X HT packaging vectors. After 48 h of transfection, the cell supernatants were collected and centrifuged at 500×*g* for 10 min, and the soluble supernatant fractions containing the lentiviruses were collected and stored for later use.

The transduction of lentiviruses was performed using 4.0 μg/ml polybrene. About 24 h post-infection, media was replaced with media containing 1.0 μg/ml puromycin for days. siRNA knockdown efficiency was determined by Q-PCR and Western blot analysis. The stable SET siRNA cell line was passaged for follow up assays.

2.3. Quantitative real-time polymerase chain reaction (Q-PCR)

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Germany). cDNAs were generated according to the manufacturer's protocol (TaKaRa, Dalian, China). GAPDH was used as the internal control. The sequences of the PCR primers and expected sizes of the amplification products for SET and GAPDH were as follows: 5′-GTCAAACGCAGAATAAAGCC-3′ (sense), 5′-ATCATCCATATCGGGAACCAA-3′ (antisense), and 230 bp for SET; and 5′-TCTGACTTCAACAGCGACACC-3′ (sense), 5′-CTGTTGCTGTAGCCAAATTCGT-3′ (antisense), and 309 bp for GAPDH. The RT-PCR reactions contained 12.5 μl SYBR Premix Ex TaqTM, 0.5 μl of 50× ROX Reference DyeII, 1 μl of sense primer, 1 μl of antisense primer, 2 μl of cDNA, and 8 μl of ddH_2O. The samples were amplified using a ABI 7900HT Fast Real-Time PCR System (ABI, USA) and the following amplification conditions: one cycle at 95 °C for 30 s, 1 cycle at 60 °C for 30 s, followed by 40 cycles at 72 °C for 30 s. The data were analyzed using the software provided with the Stratagene 7900HT detector.

2.4. Western-blot analysis

Cells were grown in six-well plates and collected after the indicated treatment and lysed as previously. Protein concentration was determined by BCA protein assay kit according to the manufacturer's recommendations (Beyotime Biotech, China). The resulting whole lysates (20 μg of total protein) were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and electrically transferred to polyvinylidene difluoride membrane (PVDF; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were blocked in 5% milk dissolved in Tris-buffered saline (TBS), 0.04% Tween-20 and then probed with antibodies against SET (1:1000), PP2Ac (1:1000), MMP-9 (1:1000) and GAPDH (1:2000). The blots were developed with horseradish peroxidase-conjugated secondary antibodies and incubated in Supersignal® West Dura Extended Duration Substrate (Thermo scientific) and detected with ECL™ Western blotting detection system (ImageQuant™ RT, GE Healthcare).

2.5. Cell proliferation assay

Cell proliferation was determined using a MTT assay as described previously [18]. Briefly, The control cells, psiRNA-SET cells and pLVX (the blank plasmid) cells were seeded (2.0×10^3 cells/well) in 96-well flat-bottom plates (Corning, USA), Twenty microliters of a 5 mg/ml MTT solution was added to each well at the time points indicated (24, 48, 72, 96, 120, 144 and 168 h), and the reactions were left to incubate for 4 h. Sample absorbance at 490 nm was measured and the absorbance value was used to calculate cell proliferation.

2.6. In vitro scratch assay

A total of 2×10^5 cells were seeded into a 24-well plate. Reference points near the "scratch" were marked to ensure the use of the same area for the image acquisition. After a 24-h incubation period, confluent monolayers of cells were scratched with 10 μl pipette tip to form a gap space of approximate 0.6 mm. PBS was used to wash out the cell debris three times, and serum-free medium was added to each well. The photomicrographs of scratch were taken at four predefined locations at 6, 12 and 24 h after the scratch in control cells, psiRNA-SET cells and pLVX cells. The distances between the 2 edges of the scratch were measured using Image-Pro Plus 6.0 software at the reference points and analyzed statistically.

2.7. Transwell matrigel invasion assay

Cell invasion assay was performed in a 24-well Transwell (Corning, USA) on a polycarbonate filter (8 μm pore size) pre-coated with 30 μg of matrigel (BD Biosciences, USA). A total of 2×10^4 control cells, psiRNA-SET and pLVX cells each suspended in 0.2 ml of fresh medium without FBS were added to the upper well of the chamber, and 600 μl of medium supplemented with 10% FBS was added to the lower well. After incubation at 37 °C in 5% CO_2 for 24 h, the cells on the upper chamber were removed by a cotton swab. Invaded cells on the bottom of the membranes were fixed with methanol and stained with 0.1% crystal violet solution, and then photographed under the microscope. The cell numbers were counted by Image-Pro Plus 6.0 software in five randomly selected fields at 100-fold magnification.

2.8. PP2A activity assay

PP2A activity was evaluated using the Millipore PP2A immunoprecipitation phosphatase assay kit as described previously [19]. Briefly, tumor cells were washed as described above, and lysed with phosphatase extraction buffer containing 20 mM imidazole–HCl, 2 mM EDTA, 2 mM EGTA (pH 7.0) and protease inhibitors. The lysates were sonicated, centrifuged, diluted and incubated as described above. Then washed the PP2A immune complexes and

assayed the phosphatase activity. PP2A activity was determined by colorimetric analysis of the free phosphate that the enzyme released from the phosphopeptide substrate. The relative activities were estimated from standard phosphate curves.

3. Results

3.1. Construction of stable SET siRNA breast cancer cells

In order to silence SET expression in MDA-MB-231 and ZR-75-30 cells, we designed one pair of siRNA oligonucleotides targeting the SET gene. The oligonucleotides encoding the siRNAs were cloned into a self-inactivating lentivirus vector containing the human U6 promoter upstream of the siRNA multiple cloning sites, and the resulting constructs were verified by DNA sequencing.

To determine the efficiency of SET siRNAs of stably transfected siRNA MDA-MB-231 and ZR-75-30 cells, SET mRNA and protein levels were measured by Q-PCR and Western-blot analysis, respectively. After 15 days of puromycin selection, transfection with the empty lentivirus vector (pLVX) did not significantly affect SET levels, but the transfection of psiRNA-SET construct inhibited SET expression efficiently (Fig. 1). These data demonstrated successful construction of stable SET knock-down breast cancer cells.

3.2. Knockdown of SET significantly inhibits cell proliferation and migration of breast cancer cells

To assess the potential effect of SET knockdown on the proliferation of breast cancer cells, we measured cell viability using the MTT assay. As shown in Fig. 2A and B, we found that the growth of SET knockdown MDA-MB-231 and ZR-75-30 cells were significantly inhibited after the culture of 7 days when compared to the control cells. The growth of breast cancer cells were not obviously different between the empty vector and the control cells. These data demonstrated that knockdown of SET significantly attenuates the proliferation of breast cancer cells.

Since cell migration is an initial step in the metastatic process of breast cancer, we further explored the role of SET in the migration of MDA-MB-231 and ZR-75-30 cells by scratch assay. As shown in Fig. 2, the migration (corresponding to wound healing capacity) was significantly inhibited in SET knockdown MDA-MB-231 (Fig. 2C and E) and ZR-75-30 (Fig. 2D and F) cells compared with the control cells after 12 and 24 h of culture, whereas it were similar in the empty vector cells and the control cells.

3.3. Knockdown of SET expression suppresses the invasion of breast cancer cells

For metastasis, tumor cells must transverse basement membrane to reach connective tissues. Invasion of tumor cells has been used as a model system to evaluate migratory abilities of tumor cells through the basement membrane [20]. To determine whether SET could also enhance the invasive potential of breast cancer cells, we performed transwell matrigel invasion assay. With this assay, MDA-MB-231 and ZR-75-30 cells that invaded through the matrigel were counted. As shown in Fig. 3, compared with the parent breast cancer cells, SET knockdown breast cancer cells displayed decreased cell invasion apparently and no difference were observed between the control cells and the empty vector cells.

3.4. Knockdown of SET expression increases the activity and expression of PP2A and decreases MMP-9 expression in breast cancer cells

Given the inhibitory effect of SET on PP2A activity, we explored the effect of SET knockdown on PP2A activity in MDA-MB-231 and ZR-75-30 cells. As shown in Fig. 4D, knockdown of SET resulted in a significant increase in the activity of PP2A in breast cancer cells compared with the control cells. The PP2A activity of breast cancer cells was not obviously different between the empty cells and the control cells.

In addition, we measured the protein levels of PP2Ac by Western-blot analysis. As shown in Fig. 4, we found that the expression of PP2Ac protein was significantly elevated in SET knockdown MDA-MB-231 and ZR-75-30 cells (Fig. 4C) compared with the control cells, whereas the expression levels was similar between the empty vector and the control cells.

MMP-9 has been reported to be associated with invasion and metastasis of cancer, we further examined the effects of SET knockdown on the expression of MMP-9 by Western-blot analysis. As shown in Fig. 4, knockdown of SET by siRNA decreased MMP-9 expression by 47.75% and 43.6%, respectively in MDA-MB-231 and ZR-75-30 cells (Fig. 4E) compared with the control cells. There was no difference between the control cells and the empty vector cells.

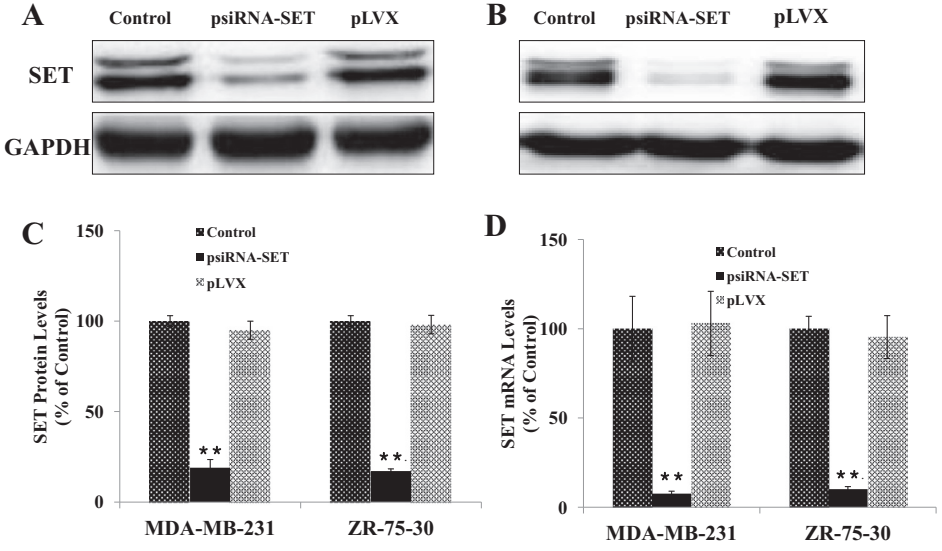

Fig. 1. The expression of SET is downregulated by siRNA in breast cancer cells. The representative Western blot is shown as (A) MDA-MB-231 and (B) ZR-75-30. (C) The analysis of SET protein expression. The data represent mean ± SD of at least three independent experiments. $^{**}P < 0.01$ compared with the control cells. (D) The levels of SET mRNA were measured by Q-PCR in breast cancer cells. The data represent mean ± SD of at least three independent experiments. $^{**}P < 0.01$ compared with the control cells.

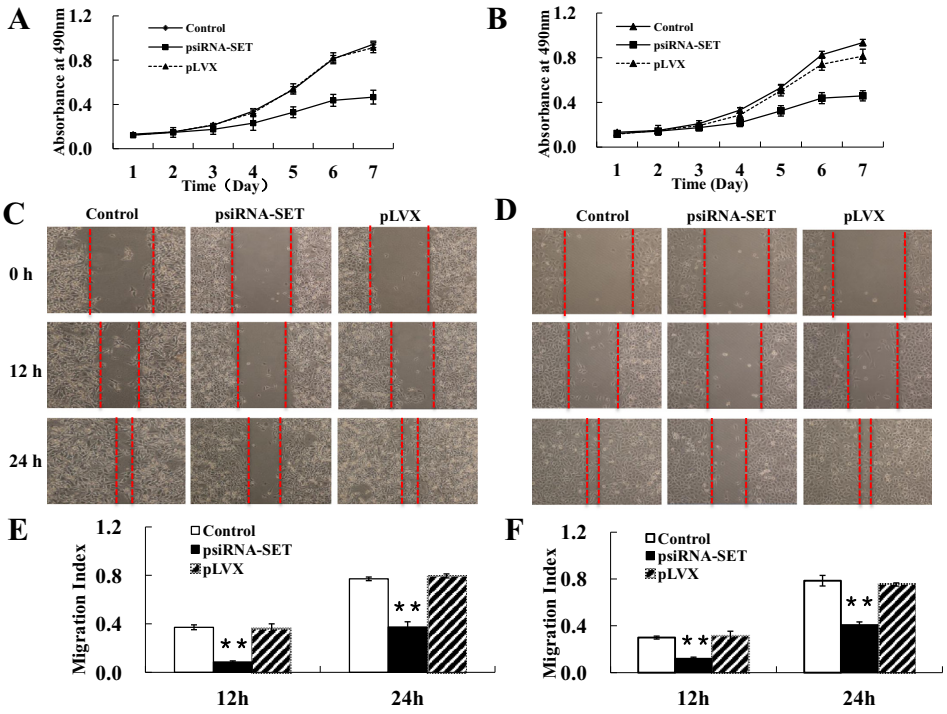

Fig. 2. SET knockdown inhibits the proliferation and migration of breast cancer cells. Growth of curves of MDA-MB-231 (A) and ZR-75-30 (B) cells were measured with MTT assay. The experiment was performed in triplicate, and the data represent the mean ± SD of three independent experiment. Straight wound area of MDA-MB-231 (C) and ZR-75-30 (D) were generated in each culture well at 0, 12, 24 h. The bars represent the migration index for MDA-MB-231 (E) and ZR-75-30 (F), expressed as a value relative to the distance moved by the cell monolayer. The data are presented as mean ± SD. The experiments were repeated at least three times. $^{**}P < 0.01$ compared with the control cells.

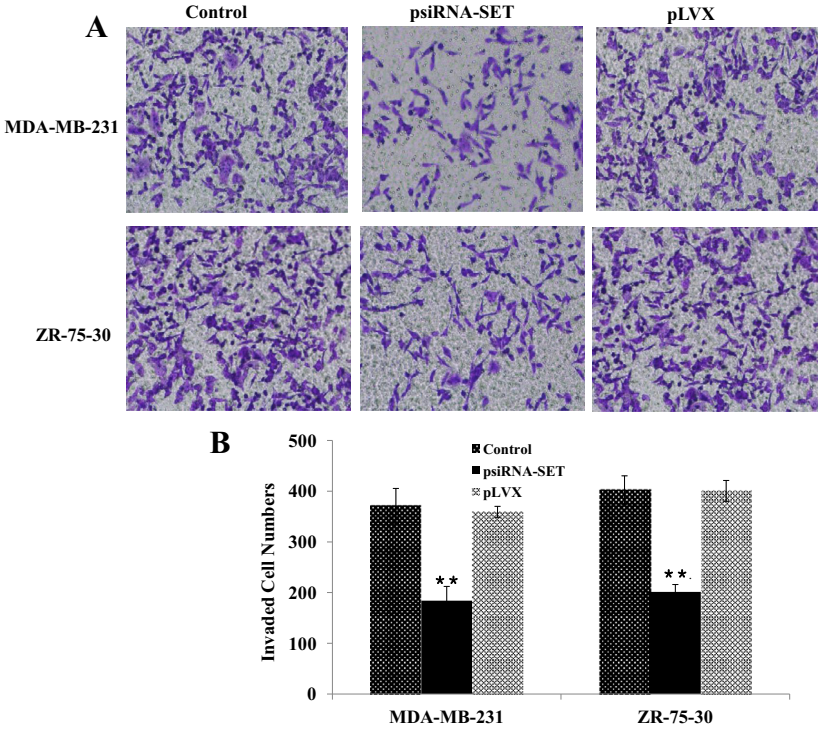

Fig. 3. SET knockdown inhibits the invasion of breast cancer cells. (A) The invasion of MDA-MB-231 and ZR-75-30 were examined using the Transwell system. Representative images are presented. (B) After 24 h treatment with matrigel, the number of successfully invading control, psiRNA-SET, and pLVX cells was counted. The data are presented as mean ± SD. The experiments were repeated three times. $^{**}P < 0.01$ compared with the control cells.

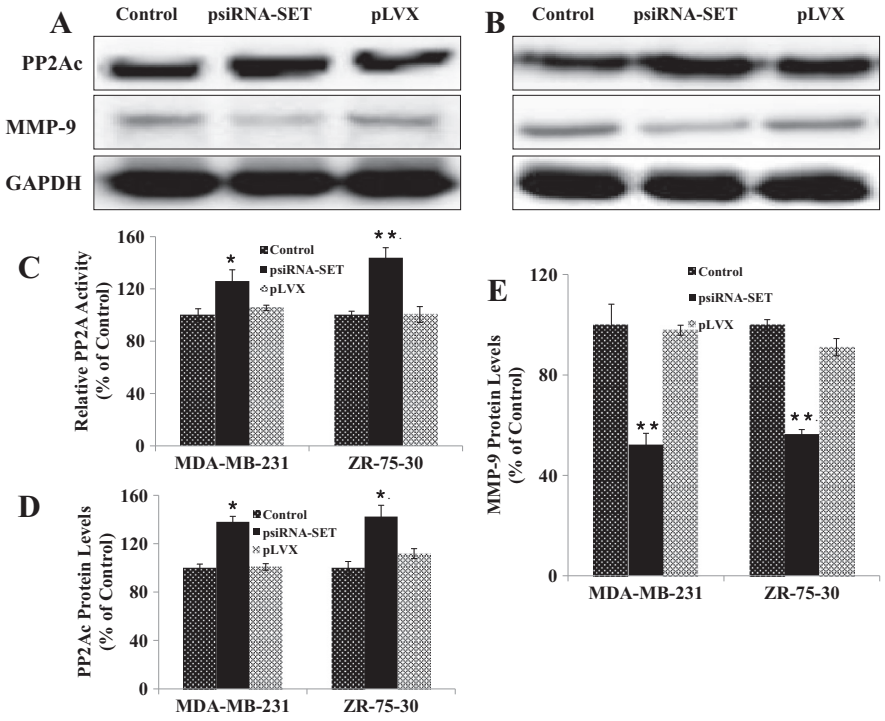

Fig. 4. SET knockdown increases expression of PP2Ac and the activity of PP2A and decreases MMP-9 expression in breast cancer cells. The expression of PP2Ac and MMP-9 protein in MDA-MB-231 (A) and ZR-75-30 (B) cells were examined by Western-blot analysis. The blots are representative of three independent experiments. GAPDH serves as a loading control. (C) PP2A activity was assayed using the Millipore PP2A immunoprecipitation phosphatase assay kit. The data represent mean ± SD of at least three independent experiments. $^{*}P < 0.05$ compared with the control cells. $^{**}P < 0.01$ compared with the control cells. The analysis of PP2Ac (D) and MMP-9 (E) protein expression. The data represent mean ± SD of at least three independent experiments. $^{*}P < 0.05$ compared with the control cells. $^{**}P < 0.01$ compared with the control cells.

4. Discussion

As a lethal disease, 90% of human cancer deaths are caused by metastasis. Breast cancer has the highest incidence rate for cancer in women in industrialized countries and poor prognosis due to its strong metastatic ability [21]. SET-mediated PP2A inhibition is an important regulatory mechanism for a number of physiological and pathological processes, including proliferation, differentiation, apoptosis and carcinogenesis [22]. In vitro studies showed that up-regulation of SET was associated with the cytotoxicity effect of TCE in human hepatic L-02 cells, and these effects could be attenuated by knockdown of SET [19]. It was reported that the SET complex protein was associated with the degree of tumor differentiation through the immunohistochemical test of ovarian cancer patients [9]. SET was over-expressed in the womb, stomach, colon and rectum cancer tissues based on the SET mRNA analysis [23]. In the present study, we used RNAi to knockdown SET in breast cancer cells MDA-MB-231 and ZR-75-30, and the result indicated that lentivirus-delivered shRNA could downregulate SET overexpression with great efficiency and specificity.

Invasion and migration are the two important characteristic of breast cancer cells, and cell proliferation is the basement of migration. In order to determine the potential mechanisms of breast cancers, we tested the effect of SET knockdown on growth, migration and invasion. The MTT assay data indicated that lentivirus vector-mediated silencing of SET in breast cancer cells could efficiently inhibit tumor growth in vitro. Similar to the observed changes in cell proliferation, the invasion and migration assay showed that the invasive capability of breast cancer cells was significantly decreased after the transfection of siRNA. The result of our study is consistent with the decreasing effects of COG112 on cellular migration and invasion through interacted with SET and inhibited its association with Ras-related C3 botulinum toxin substrate 1 (Rac1) in human U87 glioblastoma and MDA-MB-231 breast adenocarcinoma cancer cell lines [10]. These results demonstrate that SET play an important role in the invasion and migration in breast cancer cells.

It is known that degradation of extracellular matrix and basement membrane is an essential step for tumor invasion and metastasis, and these process involves the action of matrix metalloproteinases [24]. Increasing evidence suggest that MMP-9 is a kind of protease hydrolysate and type IV collagen is the specific function domain [25,26]. Stromal MMP-9 is reported to be associated with tumor invasion and metastasis [27]. The high expression of MMP-9 will affect the prognosis of tumor patients. The activation of Aryl hydrocarbon receptor (AhR) pathway in gastric cancer AGS cells induced MMP-9 expression accompanied enhanced cell migration and invasion [25]. Our data provided further evidence of positive correlation between MMP-9 and cells migration and invasion, which showed that SET silencing decreased the expression of MMP-9 and inhibited the migration and invasion in breast cancer cells MDA-MB-231 and ZR-75-30.

However, our results were contrary to the results from the studies of head and neck squamous cell carcinoma, which data showed that stable SET knockdown reduced cell proliferation but promoted cell migration and invasion, MMP-9 and MMP-2 activities [28]. These results demonstrated that the regulation of SET is complex and suggested that SET play a different role in various tumors and various tumors may respond differently to SET knockdown.

As a tumor suppressor enzyme involved in the regulation of oncoproteins in various cancers [21,22], several studies have demonstrated that targeting PP2A to increase its activity has shown promise [29]. As an inhibitor of PP2A, SET might promote the occurrence of tumor through inhibiting PP2A. Our data showed

that the knockdown of SET upregulated the expression of PP2Ac and increased the activity of PP2A. These results indicated that the inhibitory effects of knockdown of SET on the growth, migration and invasion of MDA-MB-231 and ZR-75-30 cells might involved in the regulatory function of SET on PP2A. The result of our study that SET knockdown induced MMP-9 down-regulation and PP2A up-regulation is similar with the negative correlation observed in human leukemia cells between MMP-9 and PP2A [30].

In summary, our study demonstrated that lentivirus vector-mediated silencing of SET inhibited the growth, migration and invasion of breast cancer cells MDA-MB-231 and ZR-75-30 with a possible involvement of MMP-9 and PP2Ac. These data suggest that SET play an promoting role in human breast cancer and may serve as a potential therapeutic target in the treatment of breast cancer.

Acknowledgments

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