

BCSC-1 suppresses human breast cancer metastasis by inhibiting NF- κ B signaling

DALIN DI¹, LEI CHEN², YINGYING GUO¹, LINA WANG¹, CHUNLING ZHAO³ and JIYU JU¹

¹Department of Immunology, Weifang Medical University, Weifang, Shandong 261053; ²Department of Hematology, Affiliated Hospital of Weifang Medical University, Weifang, Shandong 261031; ³School of Biological Science, Weifang Medical University, Weifang, Shandong 261053, P.R. China

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Abstract. Breast cancer suppressor candidate-1 (BCSC-1; also termed von Willebrand factor A domain containing 5A and LOH11CR2A) is a newly identified candidate tumor suppressor gene that has been implicated in several types of cancer in previous studies. However, there have been few reports about the association between BCSC-1 and human breast cancer in recent years. In the present study, the expression of BCSC-1 in breast cancer was determined by immunohistochemistry (IHC) staining of tissue microarrays and clinical tissue specimens. Subsequently, BCSC-1 gene expression was evaluated in different breast cancer cell lines by quantitative polymerase chain reaction and the MDA-MB-231 cell line was selected for further use in subsequent experiments, due to its low BCSC-1 expression. An MDA-MB-231 cell line with stable overexpression of BCSC-1 was established through transfection with plasmid containing the BCSC-1 gene, and then screening for G418 resistance. Wound-healing, migration and invasion assays were conducted to detect the effect of BCSC-1 on MDA-MB-231 cells. Furthermore, changes in matrix metalloproteinases (MMPs), osteopontin (OPN) and the nuclear factor- κ B (NF- κ B) pathway were detected in the current study. Additionally, stable silencing of BCSC-1 expression in MCF-7 cells was performed using a lentivirus. The results of IHC indicated that BCSC-1 is expressed at low levels in breast cancer tissues compared with in normal breast tissue. Results of the wound healing, migration and invasion assays demonstrated that BCSC-1 overexpression reduced

the metastasis ability of MDA-MB-231 cells *in vitro*. Further research confirmed that the BCSC-1 overexpression reduced the expression levels of MMP7, MMP9 and OPN, and the phosphorylation of NF- κ B p65. Furthermore, inhibition of BCSC-1 via lentivirus-mediated RNA interference revealed that the downregulation of BCSC-1 increased the invasive ability of MCF-7 cells. In summary, the results demonstrated that BCSC-1 is expressed at low levels in breast cancer tissues, and that it can suppress human breast cancer cell migration and invasion, potentially altering the expression of MMP7, MMP9, OPN, and the activity of the NF- κ B pathway. Therefore, BCSC-1 may be useful as a biomarker for the treatment of breast cancer in the future.

Introduction

Breast cancer is one of the most common heterogeneous malignant tumors in women. It affects developed and developing countries, and its incidence continues to increase by ~3% each year (1). Despite substantial progress in improving diagnostic and therapeutic methods in recent years, breast cancer remains the second leading cause of cancer-associated mortality in women; and there were >500,000 mortalities in 2011 according to the World Health Organization statistics (2). Previous research suggests that recurrent metastasis in patients with breast cancer remains incurable and only 3% of cases can achieve complete remission for >5 years (3). The malignant biological behavior of breast cancer is regulated by multiple genes. The occurrence and increased tumorigenesis of breast cancer are associated with the loss of tumor suppressor genes (4).

Previous studies have confirmed that the transfection of tumor suppressor genes can significantly reduce the growth and metastatic ability of breast cancer cells (5). Breast cancer suppressor candidate-1 (BCSC-1; also termed von Willebrand factor A domain containing 5A and LOH11CR2A) is a newly identified tumor suppressor gene localized at chromosome 11q23-q24. It encodes a protein with 786 amino acids and a molecular mass of 86 kDa. BCSC-1 contains two conserved domains: An inter- α -trypsin inhibitor domain at the N-terminus and a von Willebrand factor type A domain at the C-terminus. Northern blot analysis of BCSC-1 gene expression indicated a lack of expression in 33/41 (80%) tumor cell

Correspondence to: Dr Jiyu Ju, Department of Immunology, Weifang Medical University, 7166 Baotongxi Street, Weifang, Shandong 261053, P.R. China
E-mail: jujiyu@163.com

Dr Chunling Zhao, School of Biological Science, Weifang Medical University, 7166 Baotongxi Street, Weifang, Shandong 261053, P.R. China
E-mail: zhaochunlingbj@163.com

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lines, and ectopic expression of BCSC-1 led to the suppression of tumorigenicity *in vitro* and *in vivo* (6). Other studies have also demonstrated that BCSC-1 is lost or expressed at low levels in different carcinomas, including lung adenocarcinoma, nasopharyngeal carcinoma, esophageal squamous carcinoma and melanoma (7-9). Our previous study confirmed that BCSC-1 can suppress the malignant biological behavior of various types of tumor cells including human CNE-2L2 nasopharyngeal carcinoma cells and human NCI-H446 small cell lung cancer cells. The metastatic ability of these tumor cells was significantly reduced following transfection with the BCSC-1 gene (8). These data suggest that BCSC-1 may exert tumor suppressor activity, and that low expression of BCSC-1 may lead to increased tumorigenesis. In the present study, the expression level of BCSC-1 was detected in breast cancer using tissue microarray with immunohistochemical methods. Tissue microarrays, also termed tissue chips, are an important branch of biochip technology. Many specimens of different individuals are arranged in a regular array of the same carrier in a tissue microarray. Tissue microarrays are used to study the expression of the same gene or protein molecule in different cells or tissues. Additionally, a stably transfected MDA-MB-231 cell line with high BCSC-1 expression was established, and the effects of BCSC-1 on the biological behavior of breast cancer were investigated.

Previous studies have suggested that matrix metalloproteinases (MMPs) exert specific proteolytic activity on components of the ECM, and the overexpression of MMPs has been linked to the invasiveness of breast cancer cells, a process that may be regulated by the nuclear factor- κ B (NF- κ B) pathway (10,11). Osteopontin (OPN) is a multifunctional, secretory, phosphorylated glycoprotein that can promote cell adhesion and migration. In recent years, OPN has been identified as a critical protein participating in the malignant transformation of tumor cells (12,13). Thus, changes in the expression of MMPs (particularly, MMP-2, MMP-7 and MMP-9), OPN and the NF- κ B signaling pathway were evaluated in the current study. Lentivirus-mediated RNA interference (shRNA) methods were used to knockdown BCSC-1 gene expression in MCF-7 breast cancer cells. Stably silenced BCSC-1 in MCF-7 cells resulted in a higher capacity for metastasis. These results suggest that BCSC-1 may be a potential anticancer target in breast cancer.

Materials and methods

Tissue microarrays and clinical specimens for immunohistochemistry. A tissue microarray containing 69 cases of breast cancer tissue and 3 cases of normal breast tissue was purchased from Alenabio (Xian, China; cat. no. BC08013a). Additionally, 40 pathologically and clinically confirmed patients with breast cancer were recruited from the Breast Surgery Centre of Weifang People's Hospital (Weifang, China) from February to May 2016 in order to obtain cancer and adjacent non-tumor tissue samples for further investigation of BCSC-1. All patients were required to provide written informed consent and the experiments were approved by the Institutional Ethics Committee of Weifang Medical University. Thus, a total of 109 cases of breast cancer tissues and 43 cases of adjacent non-tumor tissues were included in the present study. All of the patients were women,

and ranged from 28-76 years old. All patients were pathologically and clinically confirmed with breast cancer, patients with incomplete information were excluded. The fresh tissue samples were stored at -80°C until use for immunohistochemistry. Prior to immunohistochemistry, samples were fixed in 10% formalin solution and embedded in paraffin. The paraffin sections were cut at $5\ \mu\text{m}$ thickness and were deparaffinized in xylene for 20 min, rehydrated in 100, 95, 80 and 70% ethanol for 10 min each step at room temperature and incubated in 3% H_2O_2 for 10 min at room temperature to block endogenous peroxidase. All slides were placed into 10% citrate buffer and boiled at 90°C for 30 min for antigen retrieval, then were incubated in blocking solution containing 10% goat serum (Bioss, Beijing, China) at 37°C for 15 min. Then incubated with a mouse anti-human BCSC-1 antibody (cat. no. ab64977; 1:1,000; Abcam, Cambridge, MA, USA) at 4°C overnight. The corresponding biotin-labeled goat anti-mouse IgG (ready-to-use reagents) were added at 37°C for 30 min and reacted with 100 μl horseradish peroxidase (HRP)-labeled avidin (ready-to-use reagents) at 37°C for 30 min, and 100 μl diaminobenzidine chromogenic substrate (ready-to-use reagents) were added at 37°C for 10 min in a dark box, according to the manufacturer's instructions of Streptavidin-Peroxidase Immunohistochemical staining kit (cat. no. SP-0022; Bioss). Cytoplasmic and membranous staining intensity were categorized as follows: Absent, 0; weak, 1; moderate, 2; and strong, 3. The percentage of stained cells were categorized as follows: No staining, 0; 1-10%, 1; 11-50%, 2; 51-80%, 3; and 81-100%, 4. The final score for each tissue was calculated by multiplying the staining and the percentage score. The final scores were categorized as: <2, negative (-); 3-4, weak (+); 6-8, moderate (++) and 9-12, strong (+++).

Cells and cell culture. Human MDA-MB-231 and MCF-7 breast cancer cell lines, and the 293T cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and incubated at 37°C and 5% CO_2 .

Generation of an MDA-MB-231 cell line stably overexpressing BCSC-1. MDA-MB-231 cells were routinely cultured in DMEM; 1×10^5 cells/well were seeded into a 6-well plate for transfection with BCSC-1 plasmid. Transfection was conducted when ~80% cell confluence was reached. The full length of the human BCSC-1 gene (NM_014622) was amplified from plasmid pcDNA4/myc-His A-BCSC-1 (8) and cloned into the eukaryotic expression vector pcDNA3.1, and the plasmid containing BCSC-1 gene was stored routinely in our laboratory. The following primer pairs were used in this study: BCSC-1, forward, 5'-GTGGAATTCTATGGTGCCTTCTGTGGCCTAC-3' and reverse, 5'-ATTCTCGAGCGAAAGGCAAAGATAGCAGGA-3'. pcDNA3.1/BCSC-1 (4 μg) and the empty pcDNA3.1 vector (negative control) were transfected into MDA-MB-231 cells with Lipofectamine™ 2000. The cells were cultured in DMEM containing 600 $\mu\text{g}/\text{ml}$ G418 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for selection, and a stable BCSC-1 expression cell line was obtained 4 weeks later. Quantitative

Table I. Primer sequences used in the present study.

Gene	Accession	Forward	Reverse
BCSC-1	NM_014622	5'-TGCTTCTGCCCCATTGAAGA-3'	5'-CTGTGCTGGTCCTTGTGAC-3'
β-actin	NM_001101.4	5'-CCTAGAAGCATTTCGCGGTGG-3'	5'-GAGCTACGAGCTGCCTGACG-3'
MMP-2	NM_001302510.1	5'-GATACCCCTTTGACGGTAAGGA-3'	5'-CCTTCTCCCAAGGTCCATAGC-3'
MMP-7	NM_002423.4	5'-AGATGTGGAGTGCCAGATGT-3'	5'-TAGACTGCTACCATCCGTC-3'
MMP-9	NM_004994.2	5'-TCTATGGTCCTCGCCCTGAA-3'	5'-CATCGTCCACCGGACTCAAA-3'
OPN	NM_001251830.1	5'-CTCCATTGACTCGAACGACTC-3'	5'-CAGGTCTGCGAAACTTCTTAGAT-3'

BCSC-1, breast cancer suppressor candidate-1; MMP, matrix metalloproteinase; OPN, osteopontin.

polymerase chain reaction (qPCR), western blot analysis and immunocytochemistry methods were performed in order to detect changes in BCSC-1 expression in the MDA-MB-231 cells following transfection.

Immunocytochemistry. Cells were seeded into 6-well plates (150,000/well) at 37°C for 24 h and fixed in 4% polyformaldehyde at room temperature for 15 min, then incubated with 0.5% Triton X-100/PBS solution at room temperature for 20 min and followed with 3% H₂O₂ at room temperature for 15 min, then incubated in blocking solution containing 10% goat serum at 37°C for 15 min. Cells were incubated with mouse anti-human BCSC-1 antibody (1:1,000; cat. no. ab64977; Abcam) at 4°C overnight, then incubated with 100 μl biotin-labeled goat anti-mouse IgG (ready-to-use reagents) and reacted with 100 μl HRP-labeled avidin (ready-to-use reagents) and stained with 100 μl diaminobenzidine (ready-to-use reagents), according to the manufacturer's instructions of Streptavidin-Peroxidase Immunohistochemical staining kit (cat. no. SP-0022; Bioss). The slides stained with hematoxylin at room temperature for 30 sec, then dehydrated in 70, 80, 95 and 100% ethanol, and xylene at room temperature for 5 min each step. The results were observed and imaged by using an electron microscope (Olympus BX43; Olympus Corporation, Tokyo, Japan).

RNA extraction and reverse transcription (RT)-qPCR. Total cellular RNA from MDA-MB-231 cells was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequently, 0.5 μg of total RNA was reverse transcribed into cDNA using an RT reaction kit (cat. no. DRR036s; Takara Biotechnology Co., Ltd., Dalian, China) with incubation at 37°C for 15 min, 85°C for 5 sec and 4°C for 10 min. qPCR was performed using SYBR™ PremixEX Taq (cat. no. DRR820s; Takara Biotechnology Co., Ltd.) in a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland). The reaction conditions were as follows: Predegeneration at 95°C for 1 min, then 35 cycles of denaturation at 94°C for 30 sec, renaturation at 55°C for 30 sec and extension at 72°C for 45 sec. The primer sequences are listed in Table I. The relative mRNA expression levels were determined using the 2^{-ΔΔCq} formula. Cq was the cycle quantitation; ΔCq = Cq (target gene) - Cq (β-actin) (14).

Western blot analysis. Cells (10⁶ cells/ml) were harvested by scraping from the wells and washed twice with PBS.

Proteins were extracted using a protein extraction solution (cat. no. P0013; Beyotime Institute of Biotechnology, Haimen, China) and the concentrations were measured using a bicinchoninic acid assay protein assay kit (cat. no. P0010; Beyotime Institute of Biotechnology), then 40 μg of protein per lane was separated by 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes (cat. no. FFN05; Beyotime Institute of Biotechnology). The membranes were blocked with 5% skim milk at 37°C for 1 h and incubated with primary antibodies against BCSC-1 (cat. no. sc-137568; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-human β-actin (cat. no. AA128; 1:500; Beyotime Institute of Biotechnology) at 4°C overnight, then incubated with the corresponding secondary antibody conjugated to horseradish peroxidase (cat. nos. A0181 and A0216; 1:1,000; Beyotime Institute of Biotechnology) for 2 h. The detection of specific proteins was performed with an enhanced chemiluminescence kit (Sangon Biotech Co., Ltd., Shanghai, China), according to the recommended procedure. The visualized bands were imaged and analyzed using ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA), which measured the density of each band using β-actin as the loading control.

Cell invasion and migration assays in vitro. For invasion and migration assays, MDA-MB-231 cells were serum-starved overnight. For the invasion assay, 5x10⁵ cells were seeded into Matrigel (BD Biosciences, San Jose, CA, USA) pre-coated chambers with 8.0-μm pores (Corning, Inc., Corning, NY, USA) and cultured for 48 h. For the migration assay, 1x10⁵ cells were seeded onto uncoated membranes and cultured for 12 h. Cells remaining on the upper filter surfaces were removed with sterile swabs, and the invaded or migrated cells were fixed in 70% precooled methanol for 1 h, then stained with 1% crystal violet at 37°C for 10 min. The numbers of invaded or migrated cells were counted in five randomly selected fields under an inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Scratch wound-healing assay. MDA-MB-231 cells were cultured in DMEM with 10% FBS. Cells were seeded into 6-well plates at a density of 1x10⁵ cells/ml to form a confluent monolayer. The monolayer was scratched with a sterile 200 μl pipette tip across the center of the well at the indicated time points (0 and 24 h). The extent of cell migration was imaged and measured using an NIS electron microscope (Nikon Corporation).

Changes in MMPs, OPN and NF- κ B in MDA-MB-231 cells affected by BCSC-1 overexpression. MDA-MB-231 cells were cultured in DMEM and harvested, following which changes in MMPs and OPN in MDA-MB-231 cells were detected by RT-qPCR and western blot analysis. The primer sequences used for RT-qPCR are described in Table I, the antibodies used for western blot analysis were as follows: MMP-2 (cat. no. ab92536; 1:1,000); MMP-7 (cat. no. ab205525; 1:1,000) (both from Abcam); MMP-9 (cat. no. 13667; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA); OPN (cat. no. ab69498; 5 μ g/ml; Abcam); NF- κ B/p65 (cat. no. 8242; 1:1,000); phospho-NF- κ B p65 (Ser536; cat. no. 3033; 1:1,000) (both from Cell Signaling Technology, Inc.); mouse anti-human β -actin (cat. no. AA128; 1:500; Beyotime Institute of Biotechnology); the corresponding secondary antibody conjugated to horseradish peroxidase (cat. nos. A0208 and A0216; 1:1,000; Beyotime Institute of Biotechnology). The detailed operating procedures of RT-qPCR and western blot were performed according to the aforementioned protocols.

Lentivirus-mediated short hairpin RNA (shRNA) knockdown of BCSC-1 gene expression and the effect on MCF-7 cell metastasis capacity. Lentiviral production and transductions were performed. The plasmids psPAX2 and pMD2.G were purchased from Addgene, Inc. (Cambridge, MA, USA). Briefly, an interference sequences specific to the human BCSC gene (LV-BCSC-1) and a negative control sequence (LV-NC) were designed and cloned into a PLKO.1-SP6-PGK-GFP vector (Animal Core Facility of Nanjing Medical University, Nanjing, China). A three-plasmid lentiviral packaging cell system (vector plasmid + psPAX2 + pMD2.G) was used for the co-transfection of 293T cells using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The supernatants containing the lentivirus were collected after 48 h and purified with a 0.4 μ m filter, then the lentiviral titer was determined. MCF-7 cells were infected with lentiviral particles at a multiplicity of infection of 5, followed by puromycin selection for 10 days. The efficacy of transfection was evaluated by calculating the number of fluorescent-positive cells with flow cytometry (BD FACSVerse™; BD Bioscience) and FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR, USA). The changes in BCSC-1 expression were detected by qPCR and western blotting methods. Changes to the invasive ability of MCF-7 cells were measured by an invasion assay, according to the aforementioned procedures. The target sequences are described in Table II.

Statistical analysis. Data are presented as the mean \pm standard deviation and were analyzed using SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA). A χ^2 test was used to analyze the immunohistochemical expression of BCSC-1. Differences between two groups were analyzed using the Student's t-test. Differences between more than two groups were analyzed using one-way analysis of variance. The least significant difference method was used to conduct multiple comparisons in cases of homogeneity of variance, whereas the Games-Howell method was applied in cases of heterogeneity of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BCSC-1 is expressed at low levels in breast cancer tissues. To investigate the exact effect of BCSC-1 in breast cancer, the expression of BCSC-1 in breast cancer tissue was investigated using microarrays and clinical tissue specimens using immunohistochemistry. Of the 109 breast cancer samples, 45 (41.28%) were negative, 30 (27.53%) were weakly positive, 23 (21.1%) were moderately positive and 11 (10.09%) were strongly positive. In normal breast tissue samples or adjacent non-tumor tissues, 6 (13.95%) were negative, 7 (16.28%) were weakly positive, 14 (32.56%) were moderately positive and 16 (37.21%) were strongly positive. BCSC-1 expression in breast cancer tissue was significantly lower than in normal breast or adjacent non-tumor tissues. These results suggest that BCSC-1 is expressed at low levels in breast cancer and may be involved in the pathogenesis of breast cancer (Fig. 1 and Table III).

Establishment of an MDA-MB-231 stable cell line overexpressing BCSC-1. An MDA-MB-231 cell line with stable high BCSC-1 expression, and an empty plasmid cell line, was successfully established by transfection with the BCSC-1 plasmid and subsequent screening with G418. The results of RT-qPCR and western blotting demonstrated that the mRNA and protein expression levels of BCSC-1 in MDA-MB-231 cells were significantly increased in the BCSC-1 group compared with in the control group (Fig. 2A and B), and these results were further confirmed by immunocytochemistry methods (Fig. 2C).

Overexpression of BCSC-1 can inhibit the metastasis capacity of MDA-MB-231 cells in vitro. To clarify the association between BCSC-1 and the metastatic capability of MDA-MB-231 cells, an MDA-MB-231 cell line with stable high BCSC-1 expression and an MDA-MB-231 cell line with empty plasmid were established as the control. It was subsequently identified that overexpression of BCSC-1 inhibited the metastasis capacity of MDA-MB-231 cells *in vitro*. In the invasion assay, 27.5 \pm 2.08 cells/field in the BCSC-1 group invaded to the lower chamber, while 47.25 \pm 3.59 cells/field invaded in the control group (Fig. 3A). In the migration assay, 56.25 \pm 3.5 cells/field migrated in the BCSC-1 group and 95.75 \pm 4.35 cells/field migrated in the control group (Fig. 3B). In the migration and invasion assays, the number of BCSC-1-overexpressing cells that passed through the membrane was significantly lower than the cells of the control group. In the wound-healing assay, the average migration distance recorded in the BCSC-1 group was 112.2 \pm 13.57 μ m, which was significantly shorter, compared with that of the control group (238.2 \pm 22.16 μ m) (Fig. 3C).

Expression of MMPs and OPN, and NF- κ B transcriptional activity in MDA-MB-231 cells is affected by BCSC-1 gene overexpression. The expression levels of MMPs (MMP-2, MMP-7 and MMP-9) and OPN were detected by RT-qPCR and western blot. The results demonstrated that the levels of MMP-7, MMP-9 and OPN were reduced in cells overexpressing BCSC-1 compared with the vector control cells (Fig. 4A and B). NF- κ B/p65 is a critical factor involved in tumor metastasis. The effect of BCSC-1 gene overexpression

Table II. Target sequences used in LV-mediated short hairpin RNA interference.

Target name	Target sequences	Target site
LV-BCSC-1	5'-GAGTTTACCTATAGGCTGTTA-3'	1,048-1,068
LV-NC	5'-CCTAAGGTTAAGTCGCCCTC-3'	n/a

LV, lentivirus; BCSC-1, breast cancer suppressor candidate-1; NC, negative control.

Table III. Expression of BCSC-1 in breast cancer as evaluated by immunohistochemistry.

Samples	n	BCSC-1 immunostaining [n (%)]				χ^2	P-value
		Negative (-)	Weak (+)	Moderate (++)	Strong (+++)		
Breast cancer tissue	109	45 (41.28)	30 (27.53)	23 (21.1)	11 (10.09)	22.89	<0.001
Adjacent non-tumor tissue	43	6 (13.95)	7 (16.28)	14 (32.56)	16 (37.21)		

BCSC-1, breast cancer suppressor candidate-1.

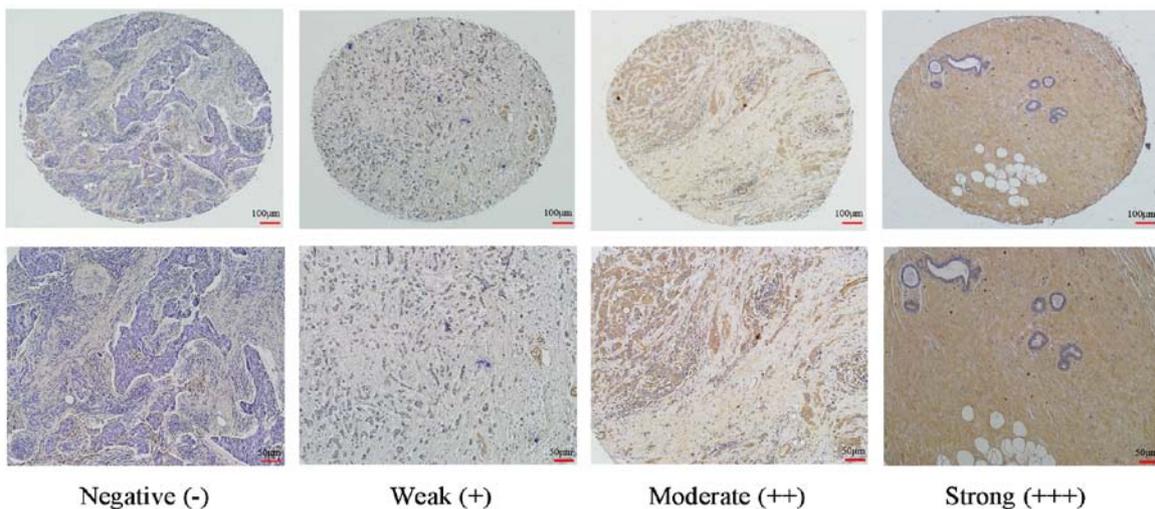


Figure 1. BCSC-1 expression in breast cancer and normal breast tissues. A tissue microarray containing both breast cancer samples and normal breast tissue samples was immunohistochemically stained using an anti-BCSC-1 antibody, then imaged under a light microscope at low (x40) and high (x200) magnification. Normal breast tissue exhibited strong expression (+++), while breast cancer tissues exhibited moderate expression (++) , weak expression (+) and negative expression (-). Representative images are shown. BCSC-1, breast cancer suppressor candidate-1.

on NF- κ B/p65 activity was determined via western blot analysis. The results revealed that BCSC-1 expression decreased NF- κ B/p65 activity, as compared with the control group, indicating that BCSC-1 may mediate breast cancer cell metastasis through the NF- κ B pathway (Fig. 4C and D).

Efficient knockdown of BCSC-1 expression by lentiviral-mediated RNA interference (RNAi) and its effect on the invasion ability of MCF-7 cells. To investigate the function of BCSC-1 in breast cancer cells, lentivirus-mediated RNAi technology was used to knockdown BCSC-1 gene expression in MCF-7 cells that exhibited high BCSC-1 expression. At 4 days after lentiviral infection, the cells emitted bright fluorescence visible under a fluorescent microscope (Fig. 5A). The number of positive cells was determined using flow cytometry, and the results

from FlowJo software analysis indicated that the infection efficiency was >80% (Fig. 5B). Results from RT-qPCR and western blotting indicated that BCSC-1 expression was downregulated in the LV-BCSC-1 group compared with in the LV-NC and blank group (Fig. 5C and D). Results from the invasion assay indicated that 23.5 ± 2.39 and 24.75 ± 1.71 cells/field invaded in the blank and LV-NC groups, respectively, which was significantly higher than the LV-BCSC-1 group (34 ± 3.16 cells/field; $P < 0.05$). This indicated that the downregulation of BCSC-1 increased MCF-7 cell invasion capacity *in vitro* (Fig. 5E).

Discussion

Breast cancer is one of the most common type of malignancy diagnosed among women worldwide (2). Metastasis is the main

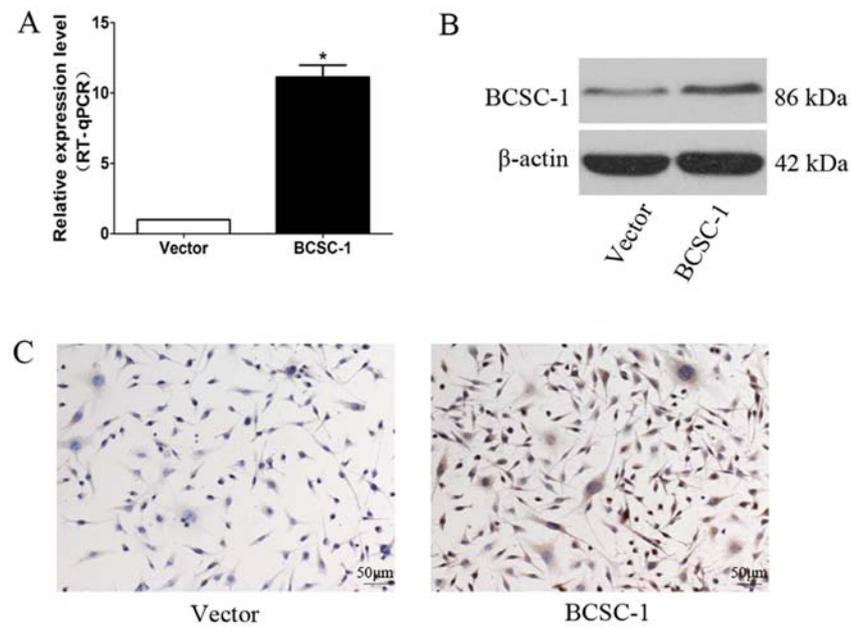


Figure 2. Establishment of an MDA-MB-231 cell line stably overexpressing BCSC-1. Changes in BCSC-1 expression levels in MDA-MB-231 cells transfected with the BCSC-1 gene were detected by (A) reverse transcription-quantitative polymerase chain reaction, (B) western blotting and (C) immunocytochemistry. * P <0.05 vs. vector control group. BCSC-1, breast cancer suppressor candidate-1.

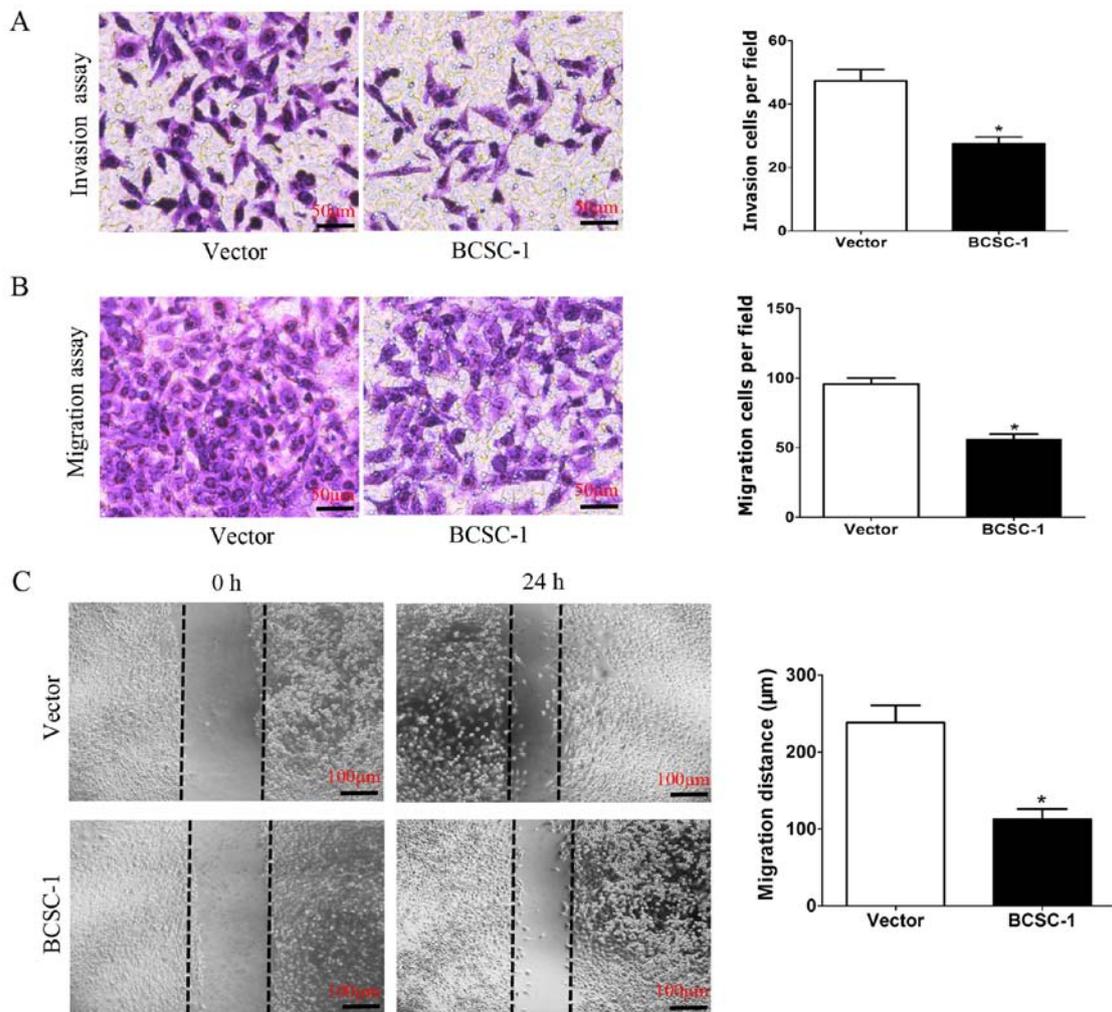


Figure 3. Metastatic ability of MDA-MB-231 cells following the overexpression of BCSC-1. The number of migratory MDA-MB-231 cells in the BCSC-1 group were detected using a (A) migration assay or (B) an invasion assay; cells were stained with crystal violet. (C) The migration distance of the MDA-MB-231 cells was detected using a wound-healing assay. * P <0.05 vs. vector control group. BCSC-1, breast cancer suppressor candidate-1.

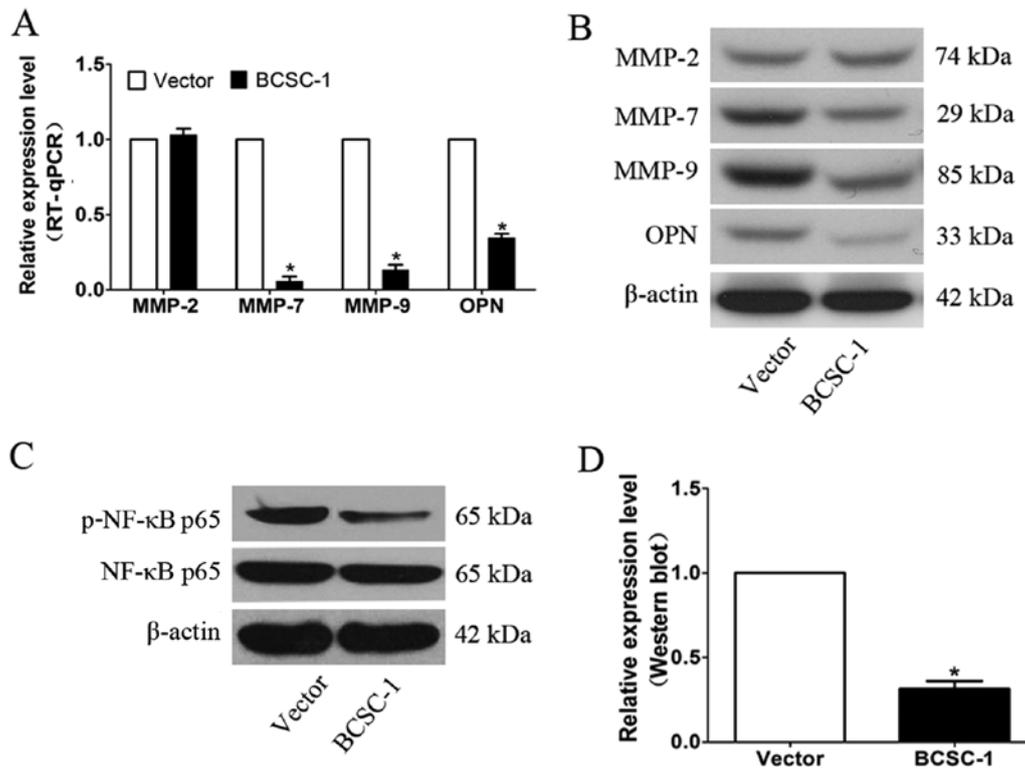


Figure 4. Expression of MMPs, OPN and the NF-κB proteins in MDA-MB-231 cells following the overexpression of BCSC-1. The expression of MMPs (MMP-2, MMP-7 and MMP-9) and OPN was detected by (A) RT-qPCR and (B) western blotting. (C) Expression of the NF-κB signaling pathway in MDA-MB-231 cells was detected by western blotting and (D) relative expression level of pNF-κB normalized to β-actin. *P<0.05 vs. vector control group. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; BCSC-1, breast cancer suppressor candidate-1; MMP, matrix metalloproteinase; OPN, osteopontin; pNF-κB, phospho-NF-κB; NF-κB, nuclear factor-κB.

cause of mortality in patients with breast cancer. The function and mechanism of tumor suppressor genes in the invasion and metastasis processes of tumor cells have increasingly received the attention of researchers in recent years (15).

BCSC-1 is a novel tumor suppressor gene, recently identified by Martin *et al* (6). The BCSC-1 gene is located at chromosome 11q23 and encodes a predicted 786 amino acid protein containing two conserved domains: A vault protein inter-α-trypsin inhibitor domain at the N-terminus and a von Willebrand factor type A domain at the C-terminus (6). Previous study has demonstrated that BCSC-1 is either not expressed or is expressed at low levels in a variety of tumors, and that the ectopic expression of BCSC-1 can reduce the tumorigenicity of carcinoma cells. BCSC-1 can inhibit melanoma tumor formation *in vivo* and tumor cell proliferation *in vitro* through downregulating melanogenesis associated transcription factor expression, resulting in a switch of melanoma from a proliferative phenotype to a migratory phenotype (9). Our prior study indicated that BCSC-1 has a key role in the tumorigenesis of nasopharyngeal carcinoma by increasing E-cadherin and α-catenin expression through the Wnt signaling pathway (8). We also confirmed that BCSC-1 was expressed at low levels in human esophageal squamous cell carcinoma, and that the downregulation of BCSC-1 was associated with the grade of tumor differentiation (7). However, the association between BCSC-1 and breast cancer is remains poorly understood.

In the present study, the expression levels of BCSC-1 in breast cancer tissues were determined via immunohistochemistry

analysis of a tissue microarray; the results indicated that BCSC-1 was expressed at low levels in breast cancer, compared with in normal breast tissues (P<0.05). Subsequently, cancer and adjacent non-tumor tissue samples were obtained from 40 patients with breast cancer for further confirmation, and the results were concordant. This indicated that BCSC may be involved in the pathogenesis of breast cancer. Following this, the human MDA-MB-231 breast cancer line, with low BCSC-1 expression, was selected for use in the current study. It was observed that the ectopic expression of BCSC-1 inhibited the metastatic ability of cells *in vitro* compared with control cells.

MMPs are zinc-dependent endopeptidases that have an important role in the invasion and metastasis of breast cancer by degrading various extracellular matrix proteins; this may be regulated by the NF-κB signaling pathway (10,11). OPN is a multifunctional secretory phosphorylated glycoprotein that can promote cell adhesion and migration. OPN is considered to be a critical protein involved in the malignant transformation of tumor cells. OPN has a close association with MMPs, and stimulates MMP expression via the NF-κB signaling pathway (16). Thus, the expression of MMPs, OPN and the NF-κB p65 were detected in the present study. The results demonstrated that overexpression of BCSC-1 inhibited the expression of MMP-7, MMP-9 and OPN, and that NF-κB/p65 activity was decreased in MDA-MB-231 cells, indicating that the anti-metastatic effects of BCSC-1 in human breast cancer may be mediated through inhibition of the NF-κB signaling pathway.

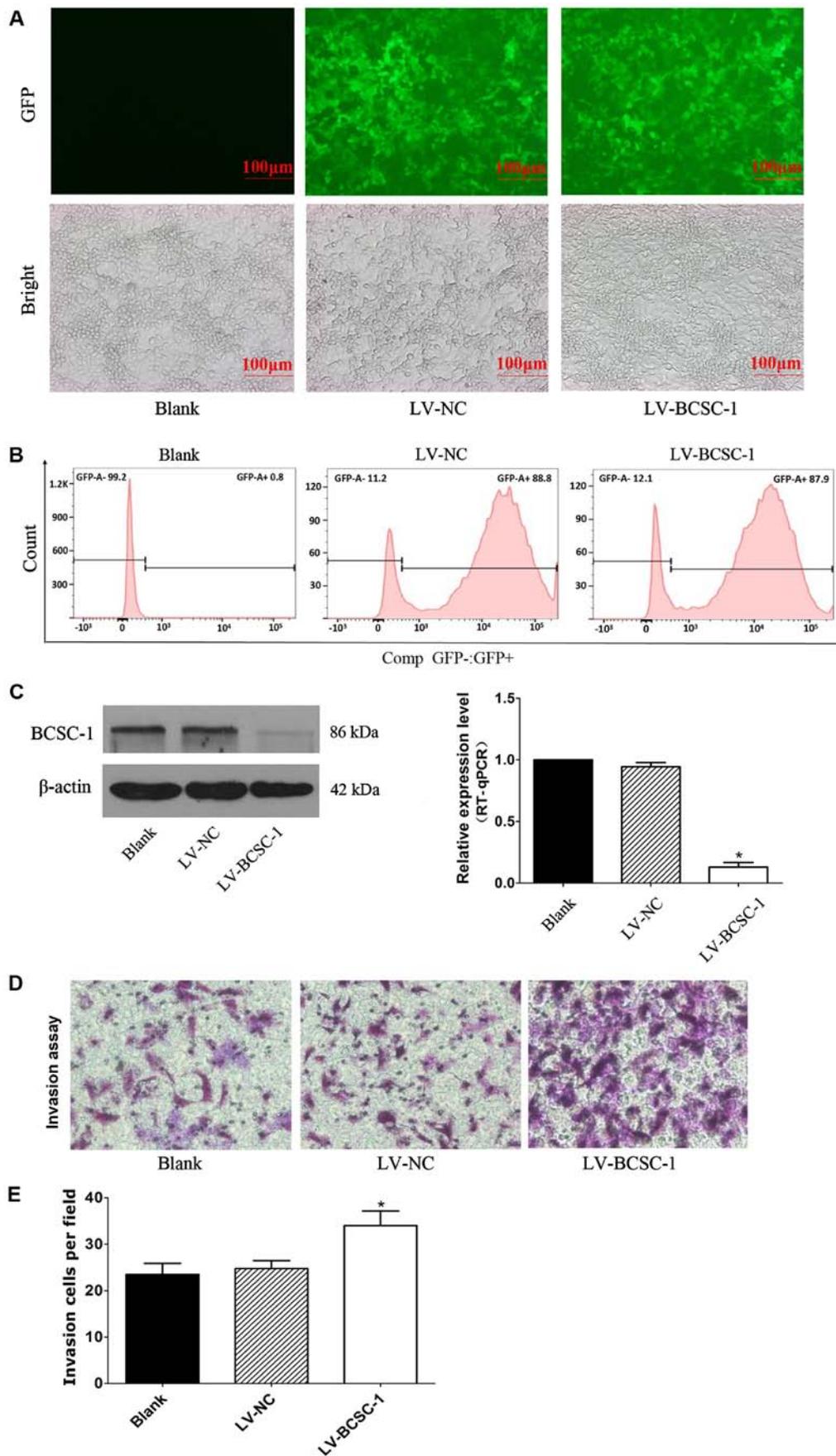


Figure 5. Efficient knockdown of BCSC-1 expression by lentiviral-mediated RNA interference in MCF-7 cells. (A) Green fluorescence emitted by MCF-7 cells following infection with the BCSC-1-containing lentivirus was visualized under a fluorescent microscope. (B) The infection efficiency was determined by flow cytometry. The levels of BCSC-1 were detected by (C) western blotting and (D) RT-qPCR. (E) The metastatic ability of MCF-7 cells was detected by an invasion assay and cells stained with crystal violet. * $P < 0.05$ vs. vector control group. GFP, green fluorescent protein; LV, lentivirus; NC, negative control; BCSC-1, breast cancer suppressor candidate-1.

As a transcription factor, NF- κ B is present in the cytoplasm of most cells and translocates to the nucleus when activated. Activation of NF- κ B can be induced by stress, cigarette smoke, viruses, bacteria, inflammatory cytokines and tumors, among other factors (17). Previous studies have demonstrated that NF- κ B can affect a wide range of biological behaviors, including inflammation, cell cycle and apoptosis (18). NF- κ B also has important roles in the occurrence and development of malignancy. Aberrant constitutive activation of NF- κ B has been observed in large variety of cancer cells, including liver cancer, lung cancer and stomach cancer, and the inhibition of NF- κ B activity can reduce chemoresistance (19-22). The angiogenesis of breast cancer depends on inflammatory factors, such as chemokines (interleukin-8 and C-X-C motif chemokine ligand 8) or growth factors (vascular endothelial growth factor) produced by neutrophils or macrophages following activation of the NF- κ B signaling pathway. In a previous report, high levels of NF- κ B expression were more common in patients with breast cancer, and were associated with a larger tumor size or higher grade, which are poor prognostic factors in breast cancer. Various genes that are involved in breast cancer invasion have been found to be regulated by NF- κ B, including cell adhesion molecules, inflammatory cytokines and MMPs (23-25). Abnormal activation of NF- κ B can promote breast cancer cell proliferation, invasion and angiogenesis, and reduce cell sensitivity to apoptotic stimuli through regulating downstream target genes including MMPs or intercellular adhesion molecule 1, therefore facilitating the survival of tumor cells (26). Studies have confirmed that small interfering RNA against NF- κ B can simultaneously inhibit the growth and suppress the distant metastasis of MDA-MB-231 and MCF-7 cells (27).

There have been multiple reports of increased levels of MMPs in breast cancer, and high expression of MMP-7 and MMP-9 has been reported to be associated with an unfavorable prognosis for patients with breast cancer (28-30). A meta-analysis also demonstrated that polymorphisms in the promoter regions of MMP-7 and MMP-9 may be associated with metastasis in breast cancer (31). Studies have demonstrated that MMP-7 is involved in the invasion process of MDA-MB-231 cells, and the endogenous long non-coding RNA urothelial cancer associated 1 (non-protein coding) can significantly reduce the number of invading cells via inhibition of MMP-7 (32). Other studies have demonstrated that common MMP-7 genetic polymorphisms are significant determinants of survival in Chinese patients with breast cancer (33). Overexpression of MMP-9 induced by the inflammatory cytokine interleukin-1 β can promote MCF-7 cell metastasis, and the inhibition of MMP-9 can inhibit breast cancer cell metastasis (34-37). Further studies indicated that MMP-9 is regulated by the NF- κ B pathway, and that inhibition of MMP-9 via the NF- κ B signaling pathway can suppress the MCF-7 cell invasion ability (38-41). In the present study, the expression of MMPs in MDA-MB-231 cells was affected by BCSC-1 overexpression. The results indicated that MMP-7 and MMP-9 were reduced significantly by BCSC-1 overexpression ($P < 0.05$). These data suggest that BCSC-1-mediated inhibition of breast cancer cell metastasis may be attributed to the down-regulation of MMP-7 and MMP-9.

OPN is a type of phosphorylated glycoprotein present in the extracellular matrix. It is synthesized and secreted by various

tissue cells, including tumor cells. OPN was first identified by Senger *et al* (42) in malignant transformed epithelial cells in 1979. An increasing number of studies have reported that OPN is overexpressed in a variety of malignant tumor types, and that it is closely associated with tumor cell metastasis and growth (43,44). Overexpression of OPN can promote tumor cell metastasis by stimulating proliferation, inducing the formation of new blood vessels and promoting tumor cell metastasis through binding CD44 or integrins (45-47). Previous study has also confirmed that OPN is associated with breast cancer. OPN is highly expressed in breast cancer tissues compared with in normal breast tissues, and the overall survival time of patients with breast cancer and high OPN expression is significantly lower than for those with low OPN expression (48).

A meta-analysis also indicated that OPN overexpression is a positive candidate prognostic biomarker for patients with breast cancer (49). Certain experiments have demonstrated a high level of OPN secretion in MDA-MB-435 cells, and a significant decrease in the metastasis capacity of tumor cells following inhibition of OPN expression. This indicates that OPN has an important role in the metastasis of breast cancer cells (50).

In the present study, lentivirus-mediated RNAi methods were used to knockdown BCSC-1 in MCF-7 cells that had high BCSC-1 expression. The results indicated that stable silencing of BCSC-1 in MCF-7 cells resulted in a higher capacity for metastasis. These results further confirmed a tumor suppressor function for BCSC-1 in breast cancer.

In conclusion, the results of the current study suggested that BCSC-1 is expressed at low levels in breast cancer tissues, and it can suppress human breast cancer cell metastasis by changing the expression of MMP7, MMP9, OPN, and the activity of the NF- κ B pathway, indicating that BCSC-1 may serve as a biomarker for the treatment of breast cancer in the future. The results of the present study provided novel insights into the role of BCSC-1 in breast cancer and improved the understanding of the mechanisms underlying the progression of breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JJ and CZ were responsible for experimental design and manuscript writing. DD and LC performed the histological

examination of the breast cancer tissue and were major contributors in writing the manuscript. YG and LW performed the western blot analysis and cell experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were approved by the Institutional Ethics Committee of Weifang Medical University (Weifang, China). All patients were required to provide written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare no that they have no competing interests.

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