Long Noncoding RNA CAMTA1 Promotes Proliferation and Mobility of the Human Breast Cancer Cell Line MDA-MB-231 via Targeting miR-20b

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Breast cancer is a serious threat to women's physical and psychological health. Long noncoding RNA CAMTA1 (lncCAMTA1) was believed to be related with tumor progression, but its role in breast cancer is not clear. The human breast cancer cell line MDA-MB-231 was used to investigate the effect of lncCAMTA1 on cell viability, migration/invasion, and apoptosis. The expression of lncCAMTA1, miR-20b, and *VEGF* in MDA-MB-231 were measured after corresponding transfections. Binding effects between lncCAMTA1 and miR-20b, miR-20b, and VEGF 3′-UTR were measured. The effects of miR-20b and VEGF on breast cancer cells were also assessed after transfections. The phosphorylation levels of the MAPK/ERK and JAK/STAT3 pathways were determined to assess the effect of VEGF. The results showed that lncCAMTA1 expression promoted cell viability and migration/invasion, while knockdown of lncCAMTA1 promoted cell apoptosis via binding with miR-20b. lncCAMTA1 negatively regulated miR-20b expression. VEGF was a target of miR-20b, leading to the modification of the phosphorylation levels of MAPK, ERK, JAK, STAT1, and STAT3. Our findings suggested that lncCAMTA1 might promote proliferation and mobility of human breast cancer cells via binding with miR-20b. VEGF was a direct target of miR-20b and regulated activation of the MAPK/ERK and JAK/STAT3 signaling pathways. Therefore lncCAMTA1 has potential as a novel cancer diagnostic marker and as a putative novel therapeutic target for breast cancer treatment.

Key words: Long noncoding RNA CAMTA1 (lncCAMTA1); MicroRNA-20b (miR-20b);
Vascular endothelial growth factor (VEGF); Janus kinase/stat (JAK/STAT) signaling pathway;
Mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK)

INTRODUCTION

Breast cancer, as a malignant tumor, is a clinically heterogeneous disease that occurs in the breast glandular epithelial tissue. The incidence rate of breast cancer in males compared to females is 1:99[1]. Although breast cancer in situ is usually not fatal, the cancer cells lose their normal characteristics and easily metastasize and thus endanger a patient's life[2]. The normal breast is not an important life-sustaining organ, so surgery and adjuvant chemotherapy are still the main choices for treating breast cancer[3]. However, because of the different inducements, the clinical characteristics of different disease subtypes, as well as surgery and medication effects, the detection and treatment of breast cancer are still challenging and can adversely affect the survival of patients[4]. Therefore, breast cancer has become a big threat to women's health and life span[5]. It is of paramount importance to understand the underlying molecular mechanisms of tumorigenesis, progression, invasion, and metastasis, as well

as the development of novel diagnostic and therapeutic strategies for treating breast cancer[6–8].

Long noncoding RNAs (lncRNAs) are a group of noncoding RNAs defined as transcripts more than 200 nucleotides (nt) in length absent of protein-coding potential[9]. lncRNAs have been shown to be involved in fundamental biological mechanisms via their ability to regulate gene expression[10,11]. Some lncRNAs have been found to be aberrantly expressed in multiple cancers, including breast cancer. Some cancer-related lncRNAs may have potential as diagnostic indicators and antitumor targets[12,13]. Research has demonstrated that lncRNAs regulate cancer cell proliferation, apoptosis, metastasis, etc.[14–17]. Therefore, the identification of breast cancer-related lncRNAs, which can be used in clinical prevention and treatment, is very important. The lncRNA CAMTA1, so called because it associates with the calmodulin-binding transcription activator 1 promoter (lncCAMTA1), was recently identified and found to be

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upregulated in both hepatocarcinoma cells and hepatocarcinoma stem cells. Research showed that lncCAMTA1 promoted the proliferation of liver cancer cells and tumor progression[18]. However, the potential role and regulation mechanisms of lncCAMTA1 in breast cancer cells are still unclear.

Diverse gene regulation effects are caused by interactions between lncRNAs and RNA, DNA, and protein[19]. MicroRNAs (miRNAs) are one kind of noncoding RNAs closely related with organism development and various diseases[20]. miRNAs, acting as oncogene or tumor suppressors, could regulate gene expression in multiple cancers[21]. lncRNA was believed to have an miRNA titration sponge-like effect that can regulate miRNA expression[22]. Research about the interaction among mRNA, miRNA, and lncRNA might be conducive to understanding more about the mechanism and pathogenesis of breast cancer and thus could contribute to clinical treatment.

In this study, we identified the role of lncRNA CAMTA1 in breast cancer cells and investigated underlying molecular mechanisms of lncRNA, miRNA, and mRNA in human breast cancer cells by in vitro functional experiments. The potential regulatory signaling mechanisms were also explored by assessing the activity of the related signaling pathways.

MATERIALS AND METHODS

Cell Culture

The human breast cancer cell line MDA-MB-231 was obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, P.R. China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, San Francisco, CA, USA) in a humidified incubator with 5% CO_2 at 37°C. The culture medium was changed every 2–3 days. After 80% confluence, cell subculture was performed to select stable cell lines for the next analysis experiments.

RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from the corresponding treated cells using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of the RNA were evaluated by modified formaldehyde agarose gel electrophoresis and OD_{260}/OD_{280} absorbance ratio detection with a spectrophotometer (Genova Nano, BIBBY, UK). The One-Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, P.R.

China) was used for qPCR analysis to test the expression levels of lncCAMTA1. The expression level of miR-20b was measured using TaqMan MicroRNA Reverse Transcription Kit and TaqMan custom small RNA Assay (Applied Biosystems, Foster City, CA, USA) combined with TaqMan Universal Master Mix II for qPCR. For vascular endothelial growth factor (VEGF), RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Biotechnology) was used. The expression of lncCAMTA1, miR-20b, and VEGF genes were normalized to U6 small nuclear RNA (snRNA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in each sample, respectively. Fold changes were calculated by the $2^{-\Delta\Delta CT}$ method, and the data were analyzed with Real-Time StatMiner (Integromics, Granada, Spain)[23].

Transfection and Generation of Stably Transfected Cell Lines

The special short hairpin RNA (shRNA) against human lncCAMTA1 was ligated into the pGPU6/green fluorescent protein (GFP)/Neo plasmid (GenePharma, Shanghai, P.R. China), referred to as sh-lncCAMTA1 for knockdown of lncCAMTA1 expression levels in cells, and the empty vector was used as a negative control (sh-NC). For overexpression transfection, full-length lncCAMTA1 or VEGF sequences were respectively constructed into the pEX-2 plasmid (GenePharma) as pEX-CAMTA1, or pEX-VEGF. The empty vectors were used as negative control. Special small interfering RNA (siRNA) against VEGF and siRNA negative control (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for VEGF knockdown. For miRNA transfection, miR-20b mimics, inhibitors, and their respective scramble control sequences referred to as NC were synthesized (Life Technologies Corporation, Frederick, MD, USA). All transfections were performed using Lipofectamine 3000 reagent (Life Technologies Corporation) according to the manufacturer's protocol. The highest transfection efficiency occurred at 48 h; thus, 72 h posttransfection was considered as the harvest time in the subsequent experiments. The stably transfected cells were selected using the culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA).

Cell Viability Assay

Cell viability was assessed using trypan blue dye exclusion. Briefly, the human breast cancer cell line MDA-MB-231 cells (1×10^5 /well) were seeded into 24-well plates and cultured until attachment. The cells were then treated with trypsin and stained by trypan blue dye. The viable cells were counted using cell counting chambers. The number of viable cells in each group was used to assess the relative cell viability.

Apoptosis Assay

Cell apoptosis analysis was performed using propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated annexin V stain and followed by flow cytometry. In brief, cells after corresponding administration were washed twice by cold phosphate-buffered saline (PBS; Sigma-Aldrich) and then stained with PI/FITC–annexin V in the presence of 50 μg/ml RNase A (Sigma-Aldrich). Afterward, cells were incubated for 1 h at room temperature in the dark. Flow cytometry analysis was performed by FACScan (Beckman Coulter, Brea, CA, USA) to differentiate apoptotic cells (annexin V$^+$ and PI$^-$) from necrotic cells (annexin V$^+$ and PI$^+$). Data were analyzed using the FlowJo software.

Migration and Invasion Assay

Cell migration was determined using a modified 24-well migration chamber Millicell Hanging Cell Culture insert with polyethylene terephthalate (PET) 8-μm membranes (Millipore, Bedford, MA, USA). The invasion behavior of cells after administration was determined using BD BioCoat™ Matrigel™ Invasion Chambers (8-μm pore-size polycarbonate filters; BD Biosciences, San Jose, CA, USA) supplemented with cell culture matrix. In brief, after being treated for the indicated condition, cells (total of 5×10^4) were suspended in 200 μl of serum-free DMEM and seeded on the upper compartment of chambers, and 600 μl of complete culture medium was added into the lower chamber. Then chambers were incubated for 48 h (37°C, 5% CO_2). The nontraversed cells were removed from the upper surface of the filter carefully with a cotton swab. Traversed or invaded cells on the lower side of the filter were fixed with methanol and then stained with crystal violet solution. The relative migration or invasion was calculated by cell counting.

Dual-Luciferase Reporter Assay

The fragment from lncCAMTA1 containing the predicted miR-20b binding site was amplified by PCR and then cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to form the reporter vector lncCAMTA1 wild type (lncCAMTA1-wt). The putative binding sites were point mutated as lncCAMTA1 mutated type (lncCAMTA1-mt) using Quick-change® Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA). The putative binding sites in the 3′-untranslated region (3′-UTR) of *VEGF* were generated by PCR, and luciferase reporter construct *VEGF*-wt or *VEGF*-mt was cotransfected with miR-20b or scramble control to assess luciferase activity. The vectors and miR-20b mimic or scramble control were cotransfected into MDA-MB-231 cells using Lipofectamine 300, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's information.

Western Blot Analysis

All protein samples used for Western blotting were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, P.R. China) supplemented with protease inhibitor (Roche, Guangzhou, P.R. China). The samples were quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Western blot systems were established using the Bio-Rad Bis-Tris Gel system (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Polyvinylidene difluoride (PVDF) membranes were incubated with the primary antibodies (at a dilution of 1:1,000): VEGF (sc7269), B-cell lymphoma 2 (Bcl-2; sc7382), Bcl-2-associated X protein (Bax; sc493), caspase 3 (sc377567), caspase 9 (sc17784), cleaved caspase 3 (sc22171), cleaved caspase 9 (sc22182), mitogen-activated protein kinase (MAPK; sc7972), phosphorylated (p)-MAPK (sc101759), extracellular signal-regulated kinase (ERK; sc135900), p-ERK (sc81492), janus kinase 1 (JAK1; sc136225), STAT1 (sc34524), p-STAT1 (sc71791), STAT3 (sc293151), p-STAT3 (sc56747), and GAPDH (sc293335) (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Subsequently, the rinsed membranes were incubated with secondary antibodies (Santa Cruz) tagged with horseradish peroxidase (HRP) for 1 h at room temperature. Then the membranes were transferred into the Bio-Rad ChemiDoc™ XRS system and covered by 200 μl of Immobilon Western Chemiluminescent HRP Substrate (Millipore). The blot signals were captured and quantified using Image Lab™ Software (Bio-Rad, Shanghai, P.R. China).

Statistical Analysis

Each experiment was repeated at least three times using three independent patient samples. The data of the results were presented as the mean±standard deviation (SD). Statistical analyses were performed using GraphPad 6.0 software (GraphPad, San Diego, CA, USA). The statistically significant difference was determined using a one-way analysis of variance (ANOVA) for multiple comparison or Student's *t*-test between two groups. A value of $p < 0.05$ was considered to indicate a statistically significant result.

RESULTS

lncCAMTA1 Promoted Cell Viability and Migration/Invasion

In order to verify the role of lncCAMTA1 in MDA-MB-231 cells, we overexpressed lncCAMTA1 or performed
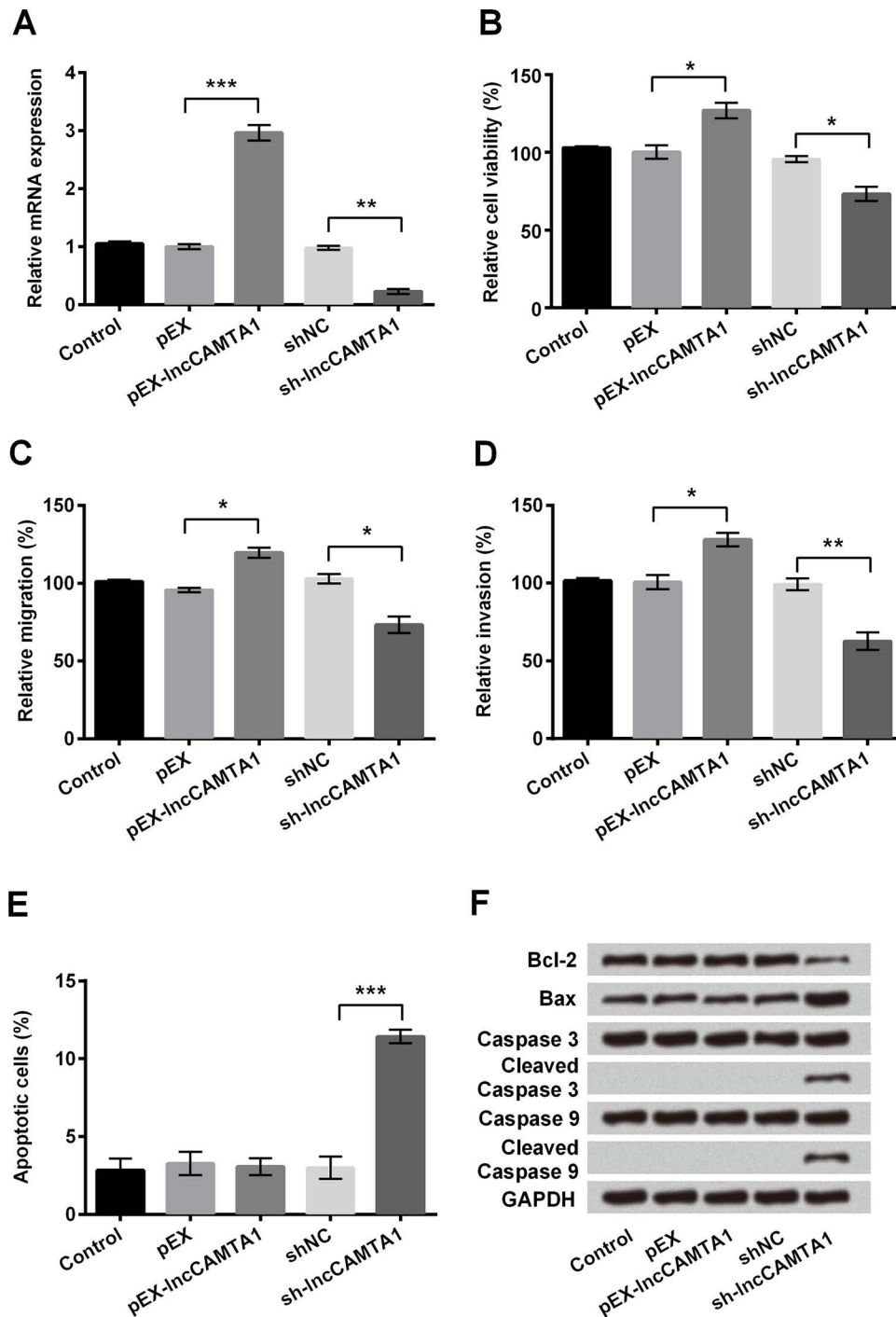
Figure 1. Long noncoding RNA CAMTA1 (lncCAMTA1) promoted MDA-MB-231 cell viability, migration, and invasion, while knockdown of lncCAMTA1 promoted apoptosis. Human breast cancer cell line MDA-MB-231 cells were transfected with recombinant vector pEX-lncCAMTA1, lncCAMTA1 short hairpin RNA (shRNA) (sh-CAMTA1), or negative control (sh-NC). (A) lncCAMTA1 expression levels in MDA-MB-231 cells after transfection were measured by quantitative polymerase chain reaction (qPCR). (B) Relative cell viability was measured by trypan blue dye exclusion. (C) Relative number of migrated cells based on the Transwell assay. (D) Relative number of invasive cells based on the invasion assay. (E) Relative number of apoptotic cells as measured by flow cytometry. (F) Protein expression levels of apoptotic-related factors measured by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acted as internal control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to appropriate control as shown. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

knockdown of lncCAMTA1 expression by shRNA trans-fection in cells, respectively. lncCAMTA1 expression was significantly increased by pEX-LncCAMTA1 transfec-tion ($p<0.001$), while sh-lncCAMTA1 transfection inhib-ited lncCAMTA1 expression in cells ($p<0.01$) (Fig. 1A). Therefore, transfection could effectively alter lncCAMTA1 levels in MDA-MB-231 cells.

The results of the trypan blue dye exclusion showed that the cell viability of pEX-CAMTA1-treated MDA-MB-231 cells was obviously promoted compared with the control group ($p<0.05$), and sh-lncCAMTA1 trans-fection significantly inhibited cell viability compared with the sh-NC group ($p<0.05$) (Fig. 1B). Migration and invasion assay results showed that the pEX-CAMTA1-transfected cells showed increased migration and inva-sion compared with the corresponding control ($p<0.05$), while sh-lncCAMTA1 transfection reduced cell migra-tion and invasion compared to the sh-NC group ($p<0.05$ or $p<0.01$) (Fig. 1C and D). These results suggested that lncCAMTA1 knockdown suppressed MDA-MB-231 cell viability, migration, and invasion, and pEX-CAMTA1 induced lncCAMTA1 overexpression accompanied by promotion of cell viability, migration, and invasion.

Knockdown of lncCAMTA1 Promoted Apoptosis of Breast Cancer Cells

In addition, we also assessed cell apoptosis after transfection. Sh-lncCAMTA1 transfection significantly increased apoptosis of MDA-MB-231 cells compared with the sh-NC group ($p<0.001$) (Fig. 1E). The expressions of apoptosis-related factors were measured by Western blotting. After sh-lncCAMTA1 transfection, expression of Bcl-2 was decreased, while Bax, cleaved caspase 3, and cleaved caspase 9 expressions were all increased, suggesting that knockdown of lncCAMTA1 regulated apoptosis-related protein expressions and promoted cell apoptosis in MDA-MB-231 cells (Fig. 1F).
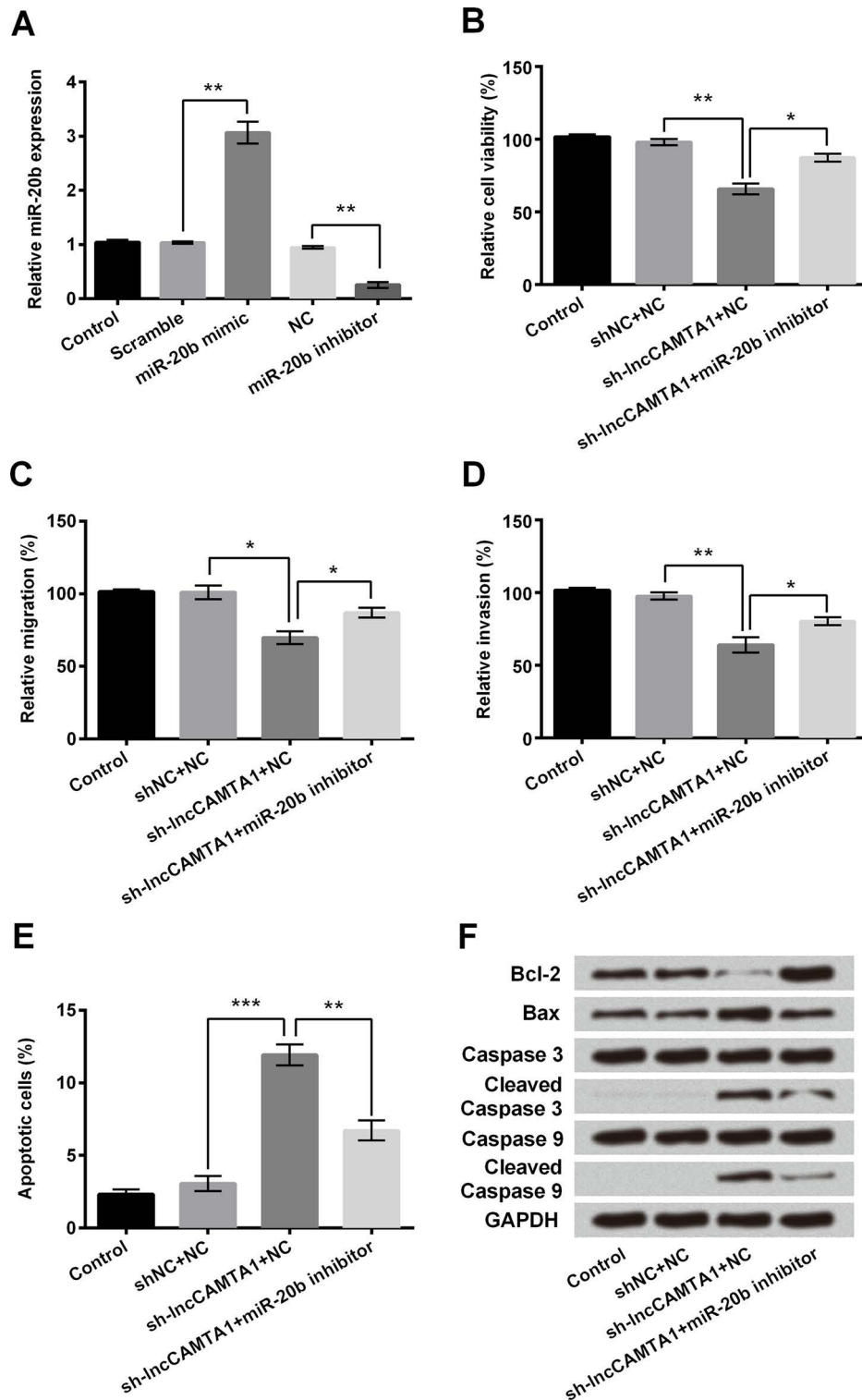
lncCAMTA1 Negatively Regulated miR-20b Expression via Binding Effect

miR-20b has been reported to accumulate in tumor cells and is speculated to be related with tumor develop-ment[24]. The potential binding site for miR-20b was found on the sequence of lncCAMTA1 by prediction (Fig. 2A). Then we explored the role of miR-20b in breast cancer cells based on the above studies. Dual-luciferase activity analysis showed that miR-20b mimic cotransfection with lncCAMTA1 significantly decreased luciferase activ-ity of the lncCAMTA1-wt group ($p<0.01$) (Fig. 2B), suggesting the existence of a binding effect between miR-20b and lncCAMTA1. Moreover, the expression level of miR-20b in lncCAMTA1-transfected cells was
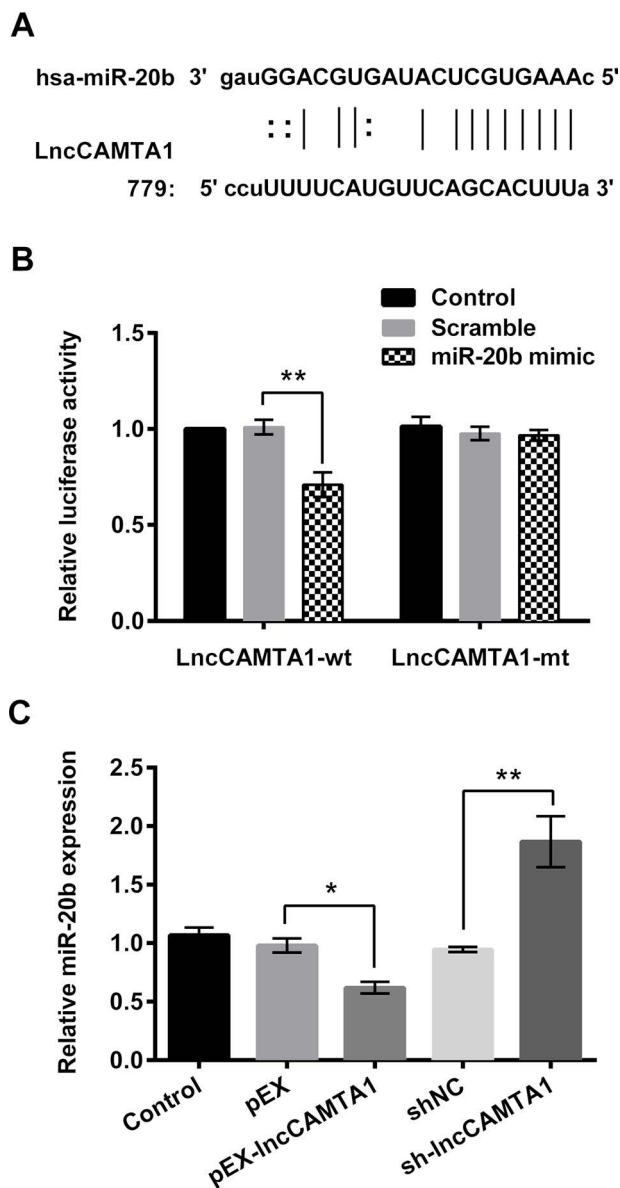
Figure 2. lncCAMTA1 binds with microRNA-20b (miR-20b) and negatively regulates miR-20b expression. (A) The poten-tial binding sites of miR-20b on the sequence of lncCAMTA1. (B) Relative luciferase activity of MDA-MB-231 cells after being cotransfected with lncCAMTA1 wild type (wt) and miR-20b mimic. lncCAMTA1 mutant type (mt) was used as positive control. (C) Relative expression levels of miR-20b in MDA-MB-231 cells that been transfected with recombinant vector (pEX-lncCAMTA1) or lncCAMTA1 shRNA (sh-CAMTA1). *$p<0.05$, **$p<0.01$, compared to appropriate control as shown.

measured. Expression of miR-20b in pEX-CAMTA1-transfected cells was obviously decreased ($p<0.05$), and sh-lncCAMTA1 transfection significantly increased miR-20b expression compared with the corresponding nega-tive control ($p<0.01$) (Fig. 2C). These results suggested

Figure 3. Effect of lncCAMTA1 on MDA-MB-231 cells via negative regulation of miR-20b expression. Human breast cancer cell line MDA-MB-231 cells were transfected with lncCAMTA1 shRNA (sh-CAMTA1) and/or miR-20b inhibitor. (A) Expression levels of miR-20b were measured by qPCR after miR-20b mimic or miR-20b inhibitor transfection in MDA-MB-231 cells. (B) Trypan blue dye exclusion results for the relative viability of cells after corresponding treatment. (C) Relative number of migrated cells based on the Transwell assay. (D) Relative number of invasive cells based on the invasion assay. (E) Relative number of apoptotic cells measured by flow cytometry. (F) Protein expression levels of apoptotic-related factors measured by Western blotting. GAPDH acted as internal control. $*p<0.05$, $**p<0.01$, $***p<0.001$.

that lncCAMTA1 might negatively regulate miR-20b expression via a binding effect in MDA-MB-231 cells.

Effect of lncCAMTA1 on Breast Cancer Cells via Binding With miR-20b
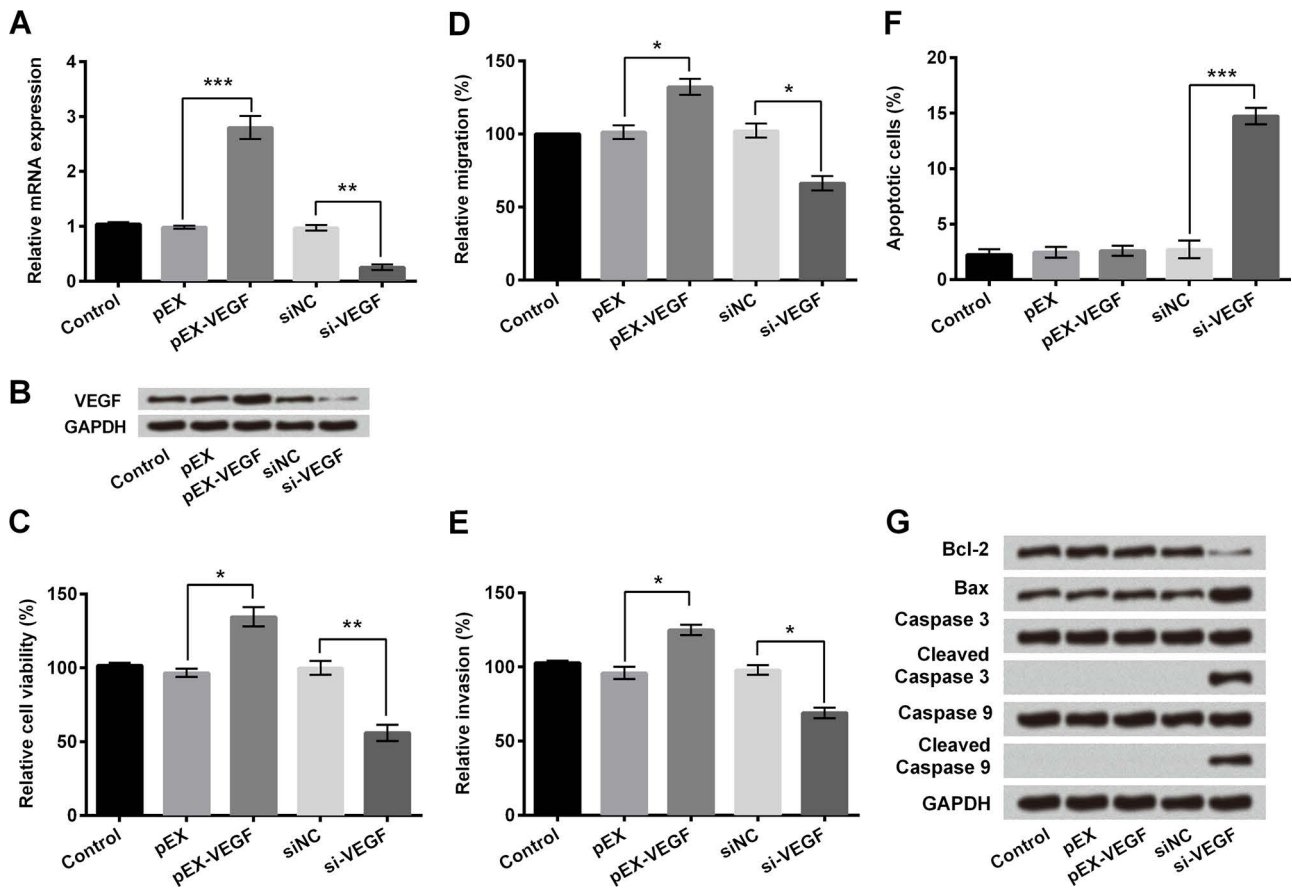
The MDA-MB-231 cells were transfected with miR-20b mimic or inhibitor to investigate the effect of miR-20b on lncCAMTA1 regulation. Following miR-20b mimic transfection, miR-20b expression was significantly increased and expression was decreased after miR-20b inhibitor transfection compared with the corresponding scramble control, respectively ($p < 0.01$) (Fig. 3A). Then MDA-MB-231 cells were transfected with sh-lnc CAMTA1 and/or miR-20b inhibitor. Cell viability analysis showed that cell viability was increased after miR-20b inhibitor transfection compared with sh-lncCAMTA1 administration alone ($p < 0.05$) (Fig. 3B). Migration and invasion of cells after miR-20b inhibitor coadministration with sh-lncCAMTA1 were also increased compared with sh-lncCAMTA1 alone ($p < 0.05$) (Fig. 3C and D). The apoptosis assay showed that sh-lncCAMTA1 administration increased the relative number of apoptotic cells ($p < 0.001$), and miR-20b inhibitor transfection suppressed the increased number of apoptotic cells compared with sh-lncCMATA1 administration alone ($p < 0.01$) (Fig. 3E). sh-lncCAMTA1 coadministration with the miR-20b inhibitor increased expression of Bcl-2, which was decreased by sh-lncCAMTA1 alone (Fig. 3F). Expression of Bax, cleaved caspase 3, and cleaved caspase 9 were all decreased by the miR-20b inhibitor, compared with lncCAMTA1 knockdown alone. All these results suggested that the effect of lncCAMTA1 knockdown on cell proliferation and apoptosis might be via regulation of miR-20b.

miR-20b Regulated VEGF Expression via Targeting the 3′-UTR of VEGF to Affect Breast Cancer Cells

A previous study has reported that miR-20b is a putative regulator of VEGF[25]. Here we hypothesized that VEGF was a target of miR-20b in breast cancer cells. For this purpose, we performed qPCR and dual-luciferase activity analysis and found that cotransfection of miR-20b and VEGF 3′-UTR luciferase reporter vector resulted in a significant decrease in fluorescence intensity ($p < 0.05$) (Fig. 4A). In MDA-MB-231 cells, protein expression of VEGF was decreased after miR-20b mimic transfection, while the transfection of miR-20b inhibitor resulted in the upregulation of VEGF (Fig. 4B). miR-20b negatively regulated the mRNA expression of VEGF in MDA-MB-231 cells compared with the negative control group ($p < 0.05$) (Fig. 4C). These results suggested that miR-20b could directly target the 3′-UTR of VEGF and negatively regulate VEGF expression.
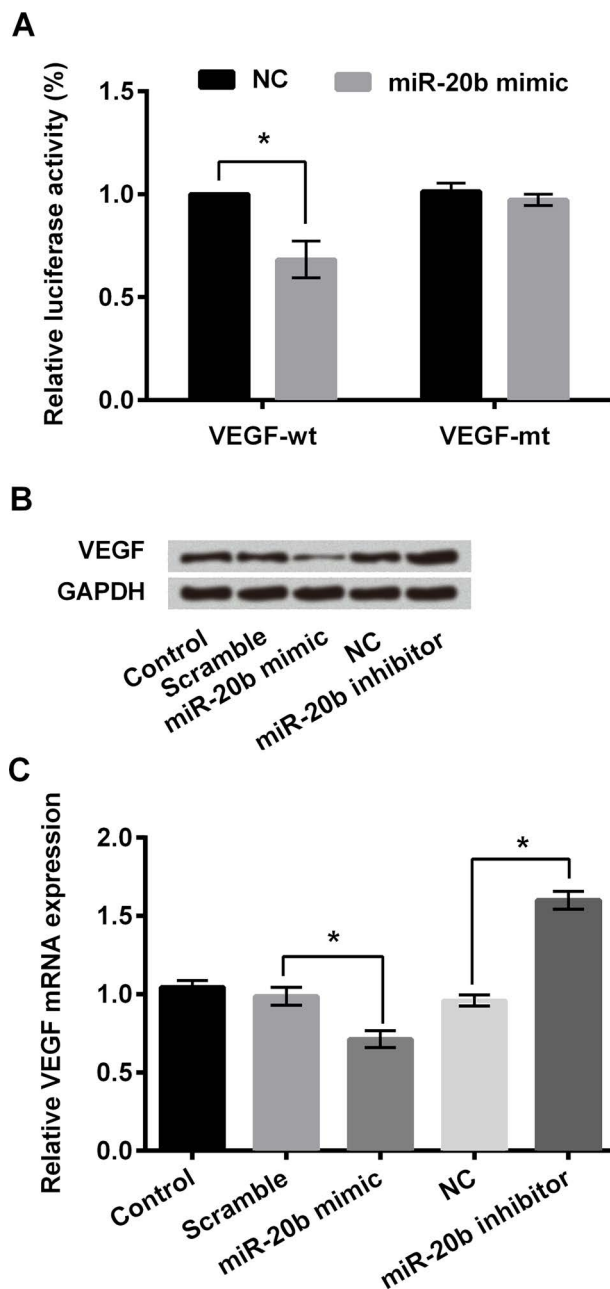
Figure 4. Vascular endothelial growth factor (VEGF) was the direct target of miR-20b in MDA-MB-231 cells. (A) Relative luciferase activity of MDA-MB-231 cells after cotransfection with VEGF-wt (wild type) and miR-20b mimic. VEGF-mt (mutant type) was used as positive control. (B) Protein expression levels of VEGF in MDA-MB-231 cells after miR-20b mimic or miR-20b inhibitor transfection as determined by Western blotting. (C) Relative mRNA expression level of VEGF in MDA-MB-231 cells transfected with miR-20b mimic or miR-20b inhibitor. GAPDH acted as internal control. *$p < 0.05$ compared to appropriate control as shown.

Figure 5. Overexpression of VEGF promoted MDA-MB-231 cell viability, migration, and invasion, and knockdown of VEGF promoted apoptosis. Human breast cancer cell line MDA-MB-231 cells were transfected with recombinant vector pEX-VEGF, VEGF siRNA (si-VEGF), or negative control. (A) The mRNA expression levels of VEGF were measured by qPCR. (B) Protein expression levels of VEGF in MDA-MB-231 cells after corresponding transfections were measured by Western blotting. (C) Trypan blue dye exclusion showed relative cell viability of MDA-MB-231 cells treated with pEX-VEGF or si-VEGF. (D) Relative number of migrated cells based on the Transwell assay. (E) Relative number of invasive cells based on invasion assay. (F) Relative number of apoptotic cells measured by flow cytometry. (G) Protein expression levels of apoptotic-related factors measured by Western blotting. GAPDH acted as internal control. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

*Effect of Differentially Expressed VEGF on Proliferation and Apoptosis of Breast Cancer Cells*

We further investigated the effect of VEGF on breast cancer cell proliferation and apoptosis. Expression of VEGF at the mRNA ($p < 0.01$ or $p < 0.001$) (Fig. 5A) and protein levels (Fig. 5B) after overexpression or knockdown in MDA-MB-231 cells were verified. Overexpression of VEGF in MDA-MB-231 cells significantly increased cell viability, migration, and invasion compared to the control group ($p < 0.05$), while knockdown of VEGF by si-VEGF transfection showed a significant decrease compared with the negative control ($p < 0.05$ or $p < 0.01$) (Fig. 5C–E). Flow cytometry showed that knockdown of VEGF in MDA-MB-231 cells increased the apoptotic number of tumor cells compared with the si-NC group ($p < 0.001$) (Fig. 5F), as well as altering protein expression

of apoptosis-related factors including decreased expression of Bcl-2 and upregulation of Bax, cleaved caspase 3, and cleaved caspase 9 (Fig. 5G). All these results suggested that miR-20b-mediated downregulation of VEGF was required for MDA-MB-231 cell proliferation and apoptosis.

To explore the underlying mechanism(s), we investigated the effect of VEGF on the activity of the MAPK/ERK and JAK/STAT3 signaling pathways in breast cancer cells in vitro. The expression of p-MAPK, p-ERK, p-JAK, p-STAT1, and p-STAT3 was decreased after VEGF knockdown in MDA-MB-231 cells, suggesting a suppressive effect of VEGF knockdown on the activation of the MAPK/ERK and JNK/STAT signaling pathways (Fig. 6A and B). Based on all the above results, we inferred that the effect of VEGF on breast cells, which was regulated by lncCAMTA1-mediated miR-20b expression, might

A

B
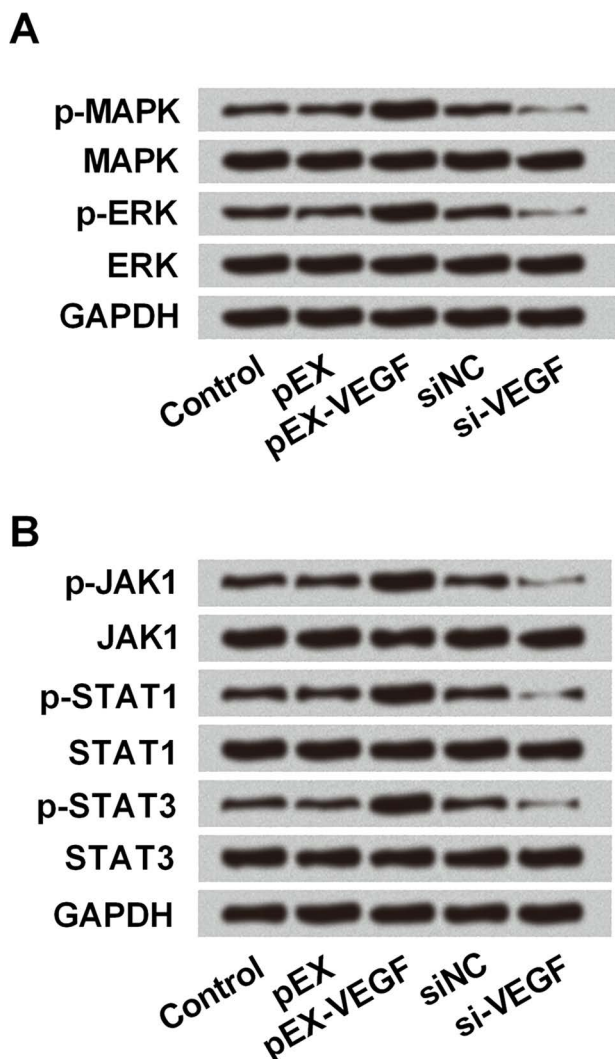
Figure 6. VEGF expression was positively related with activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and janus kinase/STAT3 (JAK/STAT3) signaling pathways. Human breast cancer cell line MDA-MB-231 cells were transfected with recombinant vector (pEX-VEGF), VEGF shRNA (si-VEGF), or negative control. Protein expression levels of the MAPK/ERK (A) and JAK/STAT3 (B) signaling pathways in MDA-MB-231 cells were measured by Western blotting after corresponding transfections. p-, phosphorylated. GAPDH acted as internal control.

be via modulating the activation of the MAPK/ERK and JNK/STAT3 signaling pathways.

DISCUSSION

The incidence of breast cancer has increased year by year and tends to be occurring at a younger age[26]. In order to avoid distal metastasis, surgery and adjuvant chemotherapy were commonly recommended, even if followed with great physical and mental harm to the patients[1].

Therefore, early diagnosis and timely treatment are critical for breast cancer therapy. In recent years, the pivotal role of lncRNAs in gene regulation and disease control has received considerable attention. lncCAMTA1 is a recently discovered lncRNA that was found to regulate cell proliferation and tumor progression in liver cancer[18]. In this study, we performed the preliminary study about the role of lncCAMTA1 in breast cancer cells. In vitro experiments in which knockdown of lncCAMTA1 in MDA-MB-231 cells was shown to suppress cell viability, migration, and invasion, while promoting cell apoptosis, suggest that lncCAMTA1 was involved in breast cancer cell proliferation and apoptosis. lncCAMTA1 might therefore be a potential therapeutic target for human breast cancer treatment.

lncRNAs are involved in cellular behavior that controls cell apoptosis, cell growth, and cell function via the regulation of gene expression at the transcriptional, post-transcriptional, and epigenetic levels[10]. lncRNAs regulate transcription through a variety of mechanisms, including interacting with RNA-binding proteins, or acting as a coactivator of transcription factors, as well as repressing a major promoter of their target genes[27]. Thus, lncRNAs act as oncogenic or tumor suppressors that may be involved in the progression of many tumors[28,29]. Our in vitro experimental results demonstrated that lncCMATA1 could bind with miR-20b and negatively regulate its expression. miR-20b was found to be aberrantly expressed in several tumor types and has been suggested as a potential molecular marker for the prognosis of multiple tumors[30,31]. Previous research suggested a novel role of miR-20b in breast cancer brain metastasis, which might be developed as a prognostic and/or therapeutic target[32]. In this study, we found that the aberrantly expressed miR-20b was related with breast cancer cell viability, migration, and invasion, as well as apoptosis in vitro. It suggested that lncCAMTA1 might regulate human breast cancer cell proliferation and apoptosis via regulating miR-20b expression.

The miRNAs, as a kind of noncoding RNA, also act as an oncogene or suppressor via regulating its target gene expression in cancers[33]. In our study, we further detected the target gene of miR-20b in human breast cancer cell line MDA-MB-231. We found that miR-20b could directly target the 3′-UTR of VEGF and thus negatively regulate the expression of VEGF in breast cancer cells in vitro. VEGF is a key regulator of physiological angiogenesis during embryogenesis and reproductive functions[34]. It was also implicated in pathological angiogenesis, which is associated with malignancies[35]. We found that in the human breast cancer cell line MDA-MB-231 cells, overexpression of VEGF could promote cell viability, migration, and invasion, while knockdown of VEGF promoted cell apoptosis. It suggested that expression of

VEGF, which is regulated by miR-20b in breast cancer cells, affected breast cancer cell proliferation. Combined with the effect of lncCAMTA1 on breast cancer cells, via regulating miR-20b expression, our results suggested an interactive network among lncCAMTA1, miR-20b, and VEGF in MDA-MB-231 cells.

In our study, we also found that the effect of VEGF on breast cancer cells in vitro might be related to the activation of the MAPK/ERK and JAK/STAT3 signaling pathways. Researchers have investigated the cross-talk between transcription factors STAT3 and ERK and demonstrated that they could affect each other's gene expression by phosphorylation interactions[36]. In breast cancer cells, the inhibition of JAK could inhibit the invasion and metastasis of human breast cancer cells via inhibition of the cross-talk between the MAPK/ERK and JAK/STAT3 pathways[37]. Our results suggested that knockdown of lncCAMTA1 could upregulate miR-20b and then downregulate the expression of VEGF, thus suppressing activation of the MAPK/ERK and JAK/STAT3 signaling pathways, suggesting that lncCAMTA1 might affect breast cancer cells via the effect of VEGF on the MAPK/ERK and JAK/STAT3 signaling pathways.

In summary, this study revealed that lncCAMTA1 might affect breast cancer cells via binding with miR-20b, which was related with the effect of VEGF on the MAPK/ERK and JAK/STAT3 pathways. It suggested that further research on lncCAMTA1 as a promising biomarker might provide a new strategy for breast cancer treatment, and lncCAMTA1 could act as a potential therapy target. More in vivo research or application of animal models will help us to understand the exact role of lncCAMTA1 in breast cancer and further improve breast cancer clinical diagnosis and treatment.

REFERENCES

1. Dimitrov G, Odzhakov F, Baitchev G. Breast cancer and its impact in society at a glance. Int J Surg Med. 2015; 1(1):7–11.
2. Matsunaga S, Shuto T, Kawahara N, Suenaga J, Inomori S, Fujino H. Gamma knife surgery for metastatic brain tumors from primary breast cancer: Treatment indication based on number of tumors and breast cancer phenotype. J Neurosurg. 2010;113(Suppl 12):65–72.
3. Kümmel S, Holtschmidt J, Loibl S. Surgical treatment of primary breast cancer in the neoadjuvant setting. Br J Surg. 2014;101(8):912–24.
4. Yaghjyan L, Colditz GA, Collins LC, Schnitt SJ, Rosner B, Vachon C, Tamimi RM. Mammographic breast density and subsequent risk of breast cancer in postmenopausal women according to tumor characteristics. J Nati Cancer Inst. 2011;103(15):1179–89.
5. Yang RJ, Huang LH, Hsieh YS, Chung UL, Huang CS, Bih HD. Motivations and reasons for women attending a breast self-examination training program: A qualitative study. BMC Womens Health 2010;10(1):23.
6. Zhao SG, Shilkrut M, Speers C, Liu M, Wilderromans K, Lawrence TS, Pierce LJ, Feng FY. Development and validation of a novel platform-independent metastasis signature in human breast cancer. PLos One 2015;10(5):e0126631.
7. Rahim F, Hajizamani S, Mortaz E, Ahmadzadeh A, Shahjahani M, Shahrabi S, Saki N. Molecular regulation of bone marrow metastasis in prostate and breast cancer. Bone Marrow Res. 2014;2014(4):405920.
8. Mukhopadhyay KD, Elkahloun AG, Hinck AP, Yoon K, Cornell JE, Shu L, Yang J, Sun L. Abstract P1-05-18: Determining the molecular mechanism of the breast cancer-induced brain metastasis and a role of a novel pan-TGF-β inhibitor as a potential therapy for brain metastasis in a mouse xenograft model. Cancer Res. 2012;72 (24 Supplement):P1-05-18.
9. Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG. The GENCODE v7 catalog of human long noncoding RNAs: Analysis of their gene structure, evolution, and expression. Genome Res. 2012;22(9):1775–89.
10. Maass PG, Luft FC, Bähring S. Long non-coding RNA in health and disease. J Mol Med. 2014;92(4):337–46.
11. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. Annu Rev Biochem. 2012;81(1):145–66.
12. Chen X, Yang J, Qian L, Cao T. Aberrantly expressed mRNAs and long non-coding RNAs in patients with invasive ductal breast carcinoma: A pilot study. Mol Med Rep. 2015;11(3):2185–90.
13. Zhang Z, Zhou C, Chang Y, Zhang Z, Hu Y, Zhang F, Lu Y, Zheng L, Zhang W, Li X. Long non-coding RNA CASC11 interacts with hnRNP-K and activates the WNT/β-catenin pathway to promote growth and metastasis in colorectal cancer. Cancer Lett. 2016;376(1):62–73.
14. Ni B, Yu X, Guo X, Fan X, Yang Z, Wu P, Yuan Z, Deng Y, Wang J, Chen D. Increased urothelial cancer associated 1 is associated with tumor proliferation and metastasis and predicts poor prognosis in colorectal cancer. Int J Oncol. 2015;47(4):1329–38.
15. Wan L, Sun M, Liu GJ, Wei CC, Zhang EB, Kong R, Xu TP, Huang MD, Wang ZX. Long noncoding RNA PVT1 promotes non-small cell lung cancer cell proliferation through epigenetically regulating LATS2 expression. Mol Cancer Ther. 2016;15(5):1082–94.
16. Liao T, Qu N, Shi RL, Guo K, Ma B, Cao YM, Xiang J, Lu ZW, Zhu YX, Li DS. BRAF-activated LncRNA functions as a tumor suppressor in papillary thyroid cancer. Oncotarget 2017;8(1):238–47.
17. Chen S, Shao C, Xu M, Ji J, Xie Y, Lei Y, Wang X. Macrophage infiltration promotes invasiveness of breast cancer cells via activating long non-coding RNA UCA1. Int J Clin Exp Pathol. 2015;8(8):9052–61.
18. Ding LJ, Li Y, Wang SD, Wang XS, Fang F, Wang WY, Lv P, Zhao DH, Wei F, Qi L. Long noncoding RNA lncCAMTA1 promotes proliferation and cancer stem cell-like properties of liver cancer by inhibiting CAMTA1. Int J Mol Sci. 2016;17(10):1617.
19. Hung T, Chang HY. Long noncoding RNA in genome regulation: Prospects and mechanisms. RNA Biol. 2010; 7(5):582–5.
20. Nd DG, Matkovich SJ. Menage a trois: Intimate relationship among a microRNA, long noncoding RNA, and mRNA. Circ Res. 2014;114(9):1362–5.

21. Shenouda SK, Alahari SK. MicroRNA function in cancer: Oncogene or a tumor suppressor? Cancer Metastasis Rev. 2009;28(3):369–78.

22. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell 2011;147(2):358–69.

23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25(4):402–8.

24. Li D, Ilnytskyy Y, Kovalchuk A, Khachigian LM, Bronson RT, Wang B, Kovalchuk O. Crucial role for early growth response-1 in the transcriptional regulation of miR-20b in breast cancer. Oncotarget 2013;4(9):1373–87.

25. Hua Z, Lv Q, Ye W, Wong CKA, Cai G, Gu D, Ji Y, Zhao C, Wang J, Yang BB. miRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. Plos One 2006; 1(1):e116.

26. Keramatinia A, Mousavijarrahi SH, Hiteh M, Mosavijarrahi A. Trends in incidence of breast cancer among women under 40 in Asia. Asian Pac J Cancer Prev. 2014;15(3):1387–90.

27. Yarmishyn AA, Kurochkin IV. Long noncoding RNAs: A potential novel class of cancer biomarkers. Front Genet. 2015;6:145.

28. Liu Q, Huang J, Zhou N, Zhang Z, Zhang A, Lu Z, Wu F, Mo YY. LncRNA loc285194 is a p53-regulated tumor suppressor. Nucleic Acids Res. 2013;41(9):4976–87.

29. Xu B, Shao Q, Xie K, Zhang Y, Dong T, Xia Y, Tang W. The long non-coding RNA ENST00000537266 and ENST00000426615 influence papillary thyroid cancer cell proliferation and motility. Cell Physiol Biochem. 2016; 38(1):368–78.

30. Xue TM, Tao LD, Zhang M, Xu GC, Zhang J, Zhang PJ. miR-20b overexpression is predictive of poor prognosis in gastric cancer. Onco Targets Ther. 2015;8:1871–6.

31. Ingwersen J, Menge T, Wingerath B, Kaya D, Graf J, Prozorovski T, Keller A, Backes C, Beier M, Scheffler M. Natalizumab restores aberrant miRNA expression profile in multiple sclerosis and reveals a critical role for miR-20b. Ann Clin Transl Neurol. 2015;2(1):43–55.

32. Ahmad A, Ginnebaugh KR, Sethi S, Wei C, Ali R, Mittal S, Sarkar FH. miR-20b is up-regulated in brain metastases from primary breast cancers. Oncotarget 2015;6(14):12188–95.

33. Schickel R, Boyerinas B, Park SM, Peter ME. MicroRNAs: Key players in the immune system, differentiation, tumorigenesis and cell death. Oncogene 2008;27(45):5959–74.

34. Ferrara N, Gerber HP, Lecouter J. The biology of VEGF and its receptors. Nat Med. 2003;9(6):669–76.

35. Delongchamps NB, Peyromaure M. The role of vascular endothelial growth factor in kidney and prostate cancer. Can J Urol. 2007;14(5):3669–77.

36. Fang XX, Jiang XL, Han XH, Peng YP, Qiu YH. Neuroprotection of interleukin-6 against NMDA-induced neurotoxicity is mediated by JAK/STAT3, MAPK/ERK, and PI3K/AKT signaling pathways. Cell Mol Neurobiol. 2013;33(2):241–51.

37. Yang B, Chuang H, Yang KD. Cross-talking between JAK/STAT3 and MAPK/ERK signaling transduction pathways on invasion and metastasis of the human breast cells. Cancer Res Prev Treat. 2009;36(4):293–7.