

PVT1-derived miR-1207-5p promotes breast cancer cell growth by targeting STAT6

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Key words

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Accumulating evidence indicates that ectopic expression of non-coding RNAs are responsible for breast cancer progression. Increased non-coding RNA *PVT1*, the host gene of microRNA-1207-5p (*miR-1207-5p*), has been associated with breast cancer proliferation. However, how *PVT1* functions in breast cancer is still not clear. In this study, we show a *PVT1*-derived microRNA, *miR-1207-5p*, that promotes the proliferation of breast cancer cells by directly regulating *STAT6*. We first confirm the positive correlated expression pattern between *PVT1* and *miR-1207-5p* by observing consistent induced expression by estrogen, and overexpression in breast cancer cell lines and breast cancer patient specimens. Moreover, silence of *PVT1* also decreased *miR-1207-5p* expression. Furthermore, increased *miR-1207-5p* expression promoted, while decreased *miR-1207-5p* expression suppressed, cell proliferation, colony formation, and cell cycle progression in breast cancer cell lines. Mechanistically, a novel target of *miR-1207-5p*, *STAT6*, was identified by a luciferase reporter assay. Overexpression of *miR-1207-5p* decreased the levels of *STAT6*, which activated *CDKN1A* and *CDKN1B* to regulate the cell cycle. We also confirmed the reverse correlation of *miR-1207-5p* and *STAT6* expression levels in breast cancer samples. Therefore, our findings reveal that *PVT1*-derived *miR-1207-5p* promotes the proliferation of breast cancer cells by targeting *STAT6*, which in turn controls *CDKN1A* and *CDKN1B* expression. These findings suggest *miR-1207-5p* might be a potential target for breast cancer therapy.

Breast cancer is by far the most common malignancy in women, causing approximately 400 000 deaths annually worldwide.⁽¹⁾ Chemoresistance and the development of distant metastasis are the major challenges in the treatment of breast cancer. MicroRNAs (miRNAs) are small, non-coding RNAs (18–23 nt in length) that downregulate the expression of target genes by blocking the translation or degradation of mRNAs.⁽²⁾ MicroRNAs play important roles in several biological processes, including cell growth, differentiation, and development.^(3–5) MicroRNAs that are dysregulated in breast cancer are of special interest, because in many cases, they are responsible for tumorigenesis by targeting and downregulating cancer-related genes. For example, *miR-34c* downregulation in breast cancer cells upregulates *GIT1*, which in turn promotes the migration and invasion of breast cancer.⁽⁶⁾ Overexpression of *miR-1229* promotes cell proliferation and tumorigenicity and activates Wnt/ β -catenin signaling in breast cancer.⁽⁷⁾ Thus, the identification of markers that would predict a patient's responsiveness to treatment, as well as track tumor progression and establish potential target therapies, is becoming increasingly important.^(8,9)

PVT1 is a long non-coding RNA located in the 8q24 gene desert, which spans a genome interval of more than 300 kb.^(10–12) An increased copy number and overexpression of *PVT1* associate with many types of cancers, including breast

and ovarian cancers, acute myeloid leukemia, and Hodgkin's lymphoma.^(13–17) However, how *PVT1* functions in breast cancer progression is still not clear. *PVT1* produces a wide variety of spliced non-coding RNAs, as well as a cluster of six annotated miRNAs (*miR-1204*, *miR-1205*, *miR-1206*, *miR-1207-5p*, *miR-1207-3p*, and *miR-1208*).⁽¹⁸⁾ Recently, there has been increased interest in the role of *miR-1207-5p* in cancer. For example, Chen *et al.*⁽¹⁹⁾ reported decreased *miR-1207-5p* expression in gastric cancer after regulation by the target gene human telomerase reverse transcriptase (*hTERT*), which was unrelated to patient age, sex, or survival time. Lü *et al.*⁽²⁰⁾ reported that *LncRNA BC032469* could competitively bind to *miR-1207-5p* to regulate *hTERT* expression and promote tumor suppression. Yang *et al.*⁽²¹⁾ showed that *miR-1207-5p* decreased the target gene *STOML-2* in esophageal cancer, which was related to the differentiation status and pathological stage of the tumor, as well as to the presence/absence of lymph node metastasis. Wu *et al.*⁽²²⁾ reported that increased expression of *miR-1207-5p* in ovarian cancer promoted the transformation of tumor cells to stem cells by activating the Wnt/ β -catenin pathway. However, the role of *miR-1207-5p* in breast cancer, especially whether *miR-1207-5p* mediates its host *PVT1*'s function in breast cancer, remains unknown.

In this study, we show that *PVT1*-derived *miR-1207-5p* promotes cell proliferation of breast cancer cells by directly

regulating *STAT6*, which in turn activates *CDKN1A* and *CDKN1B* to control cell cycle. Moreover, correlated expression of *PVT1*, *miR-1207-5p*, and *STAT6* were confirmed in breast cancer specimens.

Materials and Methods

Tissue samples and cell lines. Normal and primary breast cancer tissues harvested from 50 patients were collected with informed consent and confirmed by pathologists of the Tianjin Medical University General Hospital (Tianjin, China) and the ethics committee of the institute. Specimens were obtained during surgery and stored in the Department of Pathology of Tianjin Medical University General Hospital; they were formalin fixed and embedded in paraffin following the standard methods. All clinical data of patients such as age, tumor stage, and estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 status were available and reviewed. The human breast cancer cell lines and 293T cells were obtained from the Chinese Academy Medical Science (Beijing, China). T-47D, MDA-MB-231, and BT-474 were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂. MCF-7, SKBR3, and 293T cells were cultured in DMEM (Gibco) supplemented with the same factors as described for T-47D, MDA-MB-231, and BT-474 cells. MCF-10A cells were cultured in M-171 medium supplemented with mammary epithelial growth factors (Invitrogen/Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂. Each cell line was authenticated by inspecting the cell morphology and monitoring contamination by other cell types.

Ectopic expression and knockdown of *PVT1* in T-47D cells. The *PVT1* level was upregulated by treating T-47D cells with 10 nM estradiol (Solarbio Science and Technology, Beijing, China) for 24 h. Ethanol served as the vehicle control. The *PVT1* level was knocked-down in T-47D cells by siRNA targeting the 5'-CAGCCATCATGATGGTACT-3' sequence of cDNA and the non-silencing siRNA oligonucleotide that does not target any known mammalian gene was used as a negative control (Sangon Biotech, Shanghai, China). The cells were cultured in 6-well plates for 24 h, followed by transfection of target and control siRNAs with Lipofectamine 3000 (Invitrogen/Life Technologies) for 24 h. Thereafter, the cells were used for RNA extraction or functional assays.

RNA extraction and quantitative real-time PCR. To isolate miRNAs, total RNA was extracted from cells or tissues using TRIzol Reagent (TaKaRa Bio, Shiga, Japan). Total RNA was eluted with RNase-free water and stored at 80°C. DNase was used to remove contaminating genomic DNA. The concentration of RNA was determined by spectrophotometry using a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA, USA). To quantify the *PVT1*, *MYC*, *pS2*, *STAT6*, *CDKN1A*, and *CDKN1B* expression levels, total RNA was reverse transcribed to cDNA using an oligo-dT primer and the FastQuant RT Kit (Tiangen Biotech, Beijing, China). For *miR-1207-5p*, total RNA was reverse transcribed using a specifically designed stem-loop primer (Guangzhou RiboBio, Guangzhou, China). Quantitative real-time PCR (qPCR) was carried out using the SYBR-Green PCR Master Mix (TaKaRa Bio) in a Fast Real-Time PCR 7500 System (Life Technologies). The mature *miR-1207-5p* DNA sequence was used to design the forward primer, and the 3' universal primer (Guangzhou RiboBio) was used as the reverse primer. The

human U6 mRNA was amplified in parallel and served as the internal control. For mRNA detection, the gene-specific primers (Sangon Biotech) were as follows: *PVT1* (forward, 5'-GTCTCCCTATGGAATGTAAG-3'; reverse, 5'-AGTGTCTCCAGTAAAAGG-3'); *Myc* (forward, 5'-TGGTCGCCCTCC TATGTTG-3'; reverse, 5'-CCGGGTGCGAGATGAACTC-3'); *pS2* (forward, 5'-GCGCCCTGGTCTGGTGTCCAT-3'; reverse, 5'-GAAACCACAATTCTGTCTTTTCAC-3'); *STAT6* (forward, 5'-CCTCGTCACCAGTTGCTT-3'; reverse, 5'-TCCA GTGCTTTCTGCTCC-3'); *CDKN1A* (forward, 5'-CGACTGTG ATGCGCTAATGG-3'; reverse, 5'-AGAAGATCAGCCGGCG TTTG-3'); and *CDKN1B* (forward, 5'-GTCAAACGTAAAC AGCTGAAT-3'; reverse, 5'-TGCATAATGCTACATCCAA CG-3'). *GAPDH* (forward, 5'-GGAGCGAGATCCCTCCAA AAT-3'; reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3') served as the internal control. The qPCR reactions were undertaken at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 34 s. The ΔC_t was calculated by subtracting the C_t of the U6 or *GAPDH* mRNA from the C_t of the *miR-1207-5p* or target mRNA. The $\Delta\Delta C_t$ was then calculated by subtracting the ΔC_t of the control from the ΔC_t of the treatment group. The fold-change of the miRNA or mRNA was calculated using the $2^{-\Delta\Delta C_t}$ method.

Transfection with *miR-1207-5p* mimic or *miR-1207-5p* inhibitor. Overexpression or knockdown of *miR-1207-5p* was carried out by transfection with the *miR-1207-5p* mimic or *miR-1207-5p* inhibitor (Guangzhou RiboBio), respectively. The cells were plated in 6-well plates for 24 h and then transfected for 48 h with the *miR-1207-5p* mimic (50 nM) or *miR-1207-5p* inhibitor (100 nM) using Lipofectamine 3000 (Invitrogen/Life Technologies). In parallel experiments, the cells were transfected with mimic and inhibitor negative controls (Guangzhou RiboBio). Thereafter, the cells were used for RNA/protein extraction or functional assays.

MicroRNA target prediction. We used TargetScan (<http://www.targetscan.org/>) to predict the target genes interacting with *miR-1207-5p*. The *miR-1207-5p* sequence was obtained from the miRBase webpage (<http://www.mirbase.org/>), and the 3'-UTR sequence of the target mRNA was downloaded from TargetScan.

Knockdown of *STAT6* expression. *STAT6* expression was silenced by siRNA as described elsewhere.⁽²³⁾ Small interfering RNA oligonucleotide duplexes were chemically synthesized by GenePharma (Shanghai, China) based on the following sequences: *STAT6*-specific siRNA: 5'-AGACCU-GUCCAUCGCUCATT-3' (sense), 5'-UGAGCGAAUGGACAGGUCUTT-3' (anti-sense), NC: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense), 5'-ACGUGACACGUUCGGA-GAATT-3' (anti-sense). The knockdown of *STAT6* was confirmed by Western blot analysis.

Luciferase reporter activity assay. To generate the pMIR-RB-3'-UTR construct, the *STAT6* 3'-UTR containing the target sites of *miR-1207-5p* and its mutant form were cloned into the pMIR-Report luciferase vector (Guangzhou RiboBio) between the *Hind*III and *Sac*I sites. Approximately 1×10^5 human breast cancer cells were seeded in 12-well plates and cultured for 24 h. Then 2 µg pMIR-control or recombinant pMIR report plasmid and 50 nM *miR-1207-5p* mimic or negative control were cotransfected into cells. The activity of firefly luciferase was measured using the Dual-Luciferase Reporter Assay (Beyotime, Jiangsu, China). The relative luciferase activity was normalized to the firefly luciferase activity.

Cell proliferation assay. Cells were seeded in a 96-well plate at 5×10^3 cells per well and cultured at 37°C for an

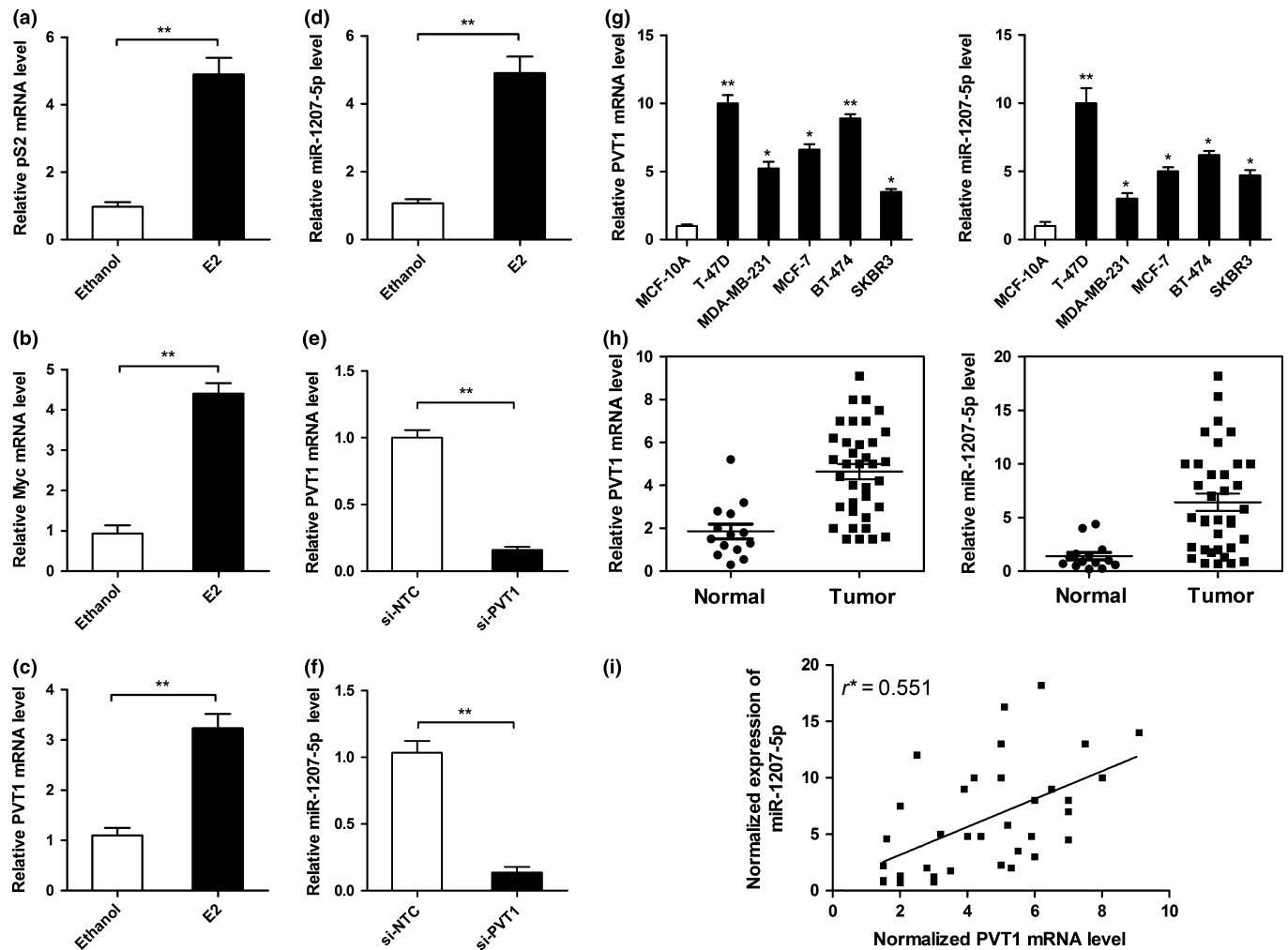


Fig. 1. Correlation between *miR-1207-5p* and *PVT1* expression levels. (a–c) Levels of *pS2* (a), *Myc* (b), and *PVT1* (c) in the indicated cells treated with ethanol (vehicle control) or estradiol (E2) were measured by quantitative real-time PCR (qPCR). The levels of *pS2*, *Myc*, and *PVT1* were normalized to the GAPDH level. (d) *miR-1207-5p* levels in the indicated cells treated with E2 was measured by qPCR. The *miR-1207-5p* level was normalized to the U6 snRNA level. (e, f) Levels of *PVT1* (e) and *miR-1207-5p* (f) in T-47D cells transfected with siRNA targeting *PVT1* (si-*PVT1*) or negative control (si-NTC) were measured by qPCR. The *PVT1* level was normalized to the GAPDH level. The *miR-1207-5p* level was normalized to the U6 snRNA level. Data are presented as mean \pm SEM ($n = 3$). (g) Levels of *PVT1* (left panel) and *miR-1207-5p* (right panel) in MCF-10A and breast cancer cell lines (T-47D, MDA-MB-231, MCF-7, BT-474, and SKBR3). Data are presented as mean \pm SEM ($n = 3$). (h) Levels of *PVT1* (left panel) and *miR-1207-5p* (right panel) in non-cancerous and cancerous tissues by qPCR ($P < 0.01$ for *PVT1*; $P < 0.05$ for *miR-1207-5p*). (i) Pearson's correlation analysis indicated the positive correlation between the expression of *PVT1* and *miR-1207-5p* in cancerous human breast tissues ($r^* = 0.551$). $*P < 0.05$; $**P < 0.01$.

additional 1–4 days in medium supplemented with 10% CCK-8 (Solarbio Science and Technology Co., Ltd.) of the total volume until color conversion. Thereafter, the absorbance at 450 nm was measured using an ELISA plate reader. This assay was carried out independently at least three times.

Colony formation assay. Cells transfected with the *miR-1207-5p* mimic or *miR-1207-5p* inhibitor were seeded at 1×10^3 cells per 60-mm culture dish. Fourteen days later, after Wright–Giemsa staining, the number of colonies was counted.

Cell cycle. For cell cycle analysis, detached cells were fixed overnight at 4°C in 70% ethanol, stained with propidium iodide, and analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and ModFit 3.0 software (Verity Software House, Topsham, ME, USA).

Western blot analysis. Western blot analysis was carried out using anti-STAT6 (1:2000), anti-CDKN1A (1:1000), anti-CDKN1B (1:1000), and anti- β -actin (1:1000) antibodies (all

Abcam, Cambridge, MA, USA). The cells were detached with a cell lifter and lysed in RIPA lysis buffer (Solarbio Science and Technology). Approximately 25 μ g protein was resolved on 12% T SDS–polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies at room temperature for 2 h, washed with Tris-buffered saline containing 0.1% Tween-20, and incubated with species-compatible secondary antibodies conjugated to HRP (1:10 000). The signals were visualized by enhanced chemiluminescence (Amersham Life Sciences, Little Chalfont, UK), and the individual intensities of the protein bands were quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data were expressed as the mean \pm SEM from at least three independent experiments. For real-time PCR experiments, as well as the CCK-8, plate colony

Table 1. Clinicopathologic characteristics and expression of miR-1207-5p in breast cancer

Characteristic	Cases	miR-1207-5p		P-value
		Low	High	
Age, years				
≤55	11	5	6	0.888
>55	25	12	13	
Tumor stage				
I	13	7	6	0.549
II–III	23	10	13	
N stage				
0	20	11	9	0.296
1–4	16	6	10	
ER status				
Positive	26	14	12	0.199
Negative	10	3	7	
PR status				
Positive	20	8	12	0.332
Negative	16	9	7	
HER-2 status				
Positive	32	16	16	0.345
Negative	4	1	3	

ER, estrogen receptor; HER-2, human epidermal growth factor receptor-2; PR, progesterone receptor.

formation, and luciferase reporter assays, differences between two groups were analyzed by two-tailed Student's *t*-test. The Wilcoxon signed-rank test was used to compare the *miR-1207-5p* level in non-cancerous and cancerous breast tissues. The χ^2 -test was used to analyze the association of *miR-1207-5p* expression level with clinicopathological characteristics. Pearson's correlation was used to identify correlations among the *miR-1207-5p*, *PVT1*, and *STAT6* expression levels. Differences were considered statistically significant for *P*-values less than 0.05 (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Results

Correlation between miR-1207-5p and PVT1 expression levels. *PVT1* gives rise to *miR-1207-5p*⁽¹⁸⁾ which prompted us to investigate the relationship between *PVT1* and *miR-1207-5p*. In a previous study, estradiol was found to increase Myc expression, resulting in the binding of Myc to the E-box regions of *PVT1* and in the activation of transcription.⁽²⁴⁾ These findings suggest that there may be an estradiol–Myc–*PVT1* regulatory axis in hormone-responsive cancer cells. Compared with the vehicle control (ethanol), the *PVT1* level increased after treating the breast cancer cell line T-47D with estradiol for 24 h. The effects of estrogen were validated by a significant increase (*P* < 0.01) in the level of *ps2*, an estradiol-responsive gene, at 1–24 h after treatment (Fig. 1a). Quantitative PCR showed that *PVT1* and *Myc* expression also increased in estrogen-treated T-47D cells and that the *miR-1207-5p* level increased significantly compared with that of the control (*P* < 0.01, Fig. 1b–d). We also silenced *PVT1* by siRNA, which significantly reduced *miR-1207-5p* expression in T-47D cells (*P* < 0.01; Fig. 1e,f). The *miR-1207-5p* level was not affected after transfection with the control siRNA. Moreover, we investigated *PVT1* and *miR-1207-5p* expression in T-47D, MDA-MB-231, MCF-7, BT-474, and SKBR3 cell lines; the results were compared with those of the human

mammary epithelial cell line MCF-10A (Fig. 1g). *PVT1* and *miR-1207-5p* expression was higher in cancer cell lines than in the MCF-10A cell line. To further investigate the relationship between the two genes, the levels of *PVT1* and *miR-1207-5p* were quantified in non-cancerous and cancerous human breast tissues (Fig. 1h). *PVT1* and *miR-1207-5p* expression was significantly higher in cancerous breast tissues than in non-cancerous tissues (*P* < 0.01 for *PVT1*; *P* < 0.05 for *miR-1207-5p*). In addition, there was a positive correlation in expression tendency for *PVT1* and *miR-1207-5p* (Fig. 1i). Next, we analyzed the association between *miR-1207-5p* expression and the clinicopathologic status of breast cancer patients (Table 1). We found that *miR-1207-5p* levels were not correlated with age, tumor stage, lymph node stage, or estrogen receptor, progesterone receptor, or human epidermal growth factor receptor 2 among breast cancer patients.

miR-1207-5p promotes breast cancer cell proliferation. To investigate the role of *miR-1207-5p* in breast cancer cell proliferation, T-47D, MCF-7, and MDA-MB-231 cells were transfected with the *miR-1207-5p* mimic or *miR-1207-5p* inhibitor and their respective controls. *miR-1207-5p* expression in cells transfected with the *miR-1207-5p* mimic was significantly higher (*P* < 0.001) than in the control, whereas *miR-1207-5p* expression in cells transfected with the *miR-1207-5p* inhibitor was significantly lower (*P* < 0.01) than in the control (Fig. 2a–c). Cells were counted for four consecutive days by the CCK-8 assay. Increased *miR-1207-5p* expression promoted cell proliferation, whereas decreased *miR-1207-5p* expression suppressed cell proliferation (Fig. 2d–f). These data show that overexpression of *miR-1207-5p* promotes breast cancer cell proliferation.

miR-1207-5p promotes breast cancer cell colony formation. To investigate the effects of *miR-1207-5p* in breast cancer cell colony formation, T-47D (Fig. 2g), MCF-7 (Fig. 2h), and MDA-MB-231 (Fig. 2i) cells were transfected with the *miR-1207-5p* mimic or *miR-1207-5p* inhibitor and their respective controls. The cells were cultured for 14 days to allow colony formation. Compared with their respective controls, the number of colonies increased in cells transfected with the *miR-1207-5p* mimic, whereas the number of colonies decreased in cells transfected with the *miR-1207-5p* inhibitor. These data indicate that *miR-1207-5p* can regulate colony formation in breast cancer cells.

Effects of miR-1207-5p overexpression on the cell cycle in breast cancer cells. To better understand the role of *miR-1207-5p* in the proliferation of breast cancer cells, the cell cycle of T-47D, MDA-MB-231, and MCF-7 was analyzed by flow cytometry. Compared with the control, the percentage of breast cancer cells at G₂ phase increased after transfection with the *miR-1207-5p* mimic (Fig. 3), whereas the percentage of cells at G₂ phase decreased after transfection with the *miR-1207-5p* inhibitor. These data illustrate that *miR-1207-5p* regulates breast cancer cell proliferation by arresting the cell cycle.

miR-1207-5p regulates STAT6 expression by directly targeting the 3'-UTR of STAT6 mRNA. To investigate the role of *miR-1207-5p* in cell proliferation and cell cycle progression, the computational algorithm (TargetScan) was used to search for potential *miR-1207-5p* target genes. Among these candidate target genes, *STAT6* was identified by the algorithm and selected for further study. We transfected T-47D, MCF-7, and MDA-MB-231 cells with the *miR-1207-5p* mimic or *miR-1207-5p* inhibitor. By Western blot analysis, the *miR-1207-5p* mimic significantly reduced the *STAT6* protein levels in the three cell lines, whereas the *miR-1207-5p* inhibitor increased

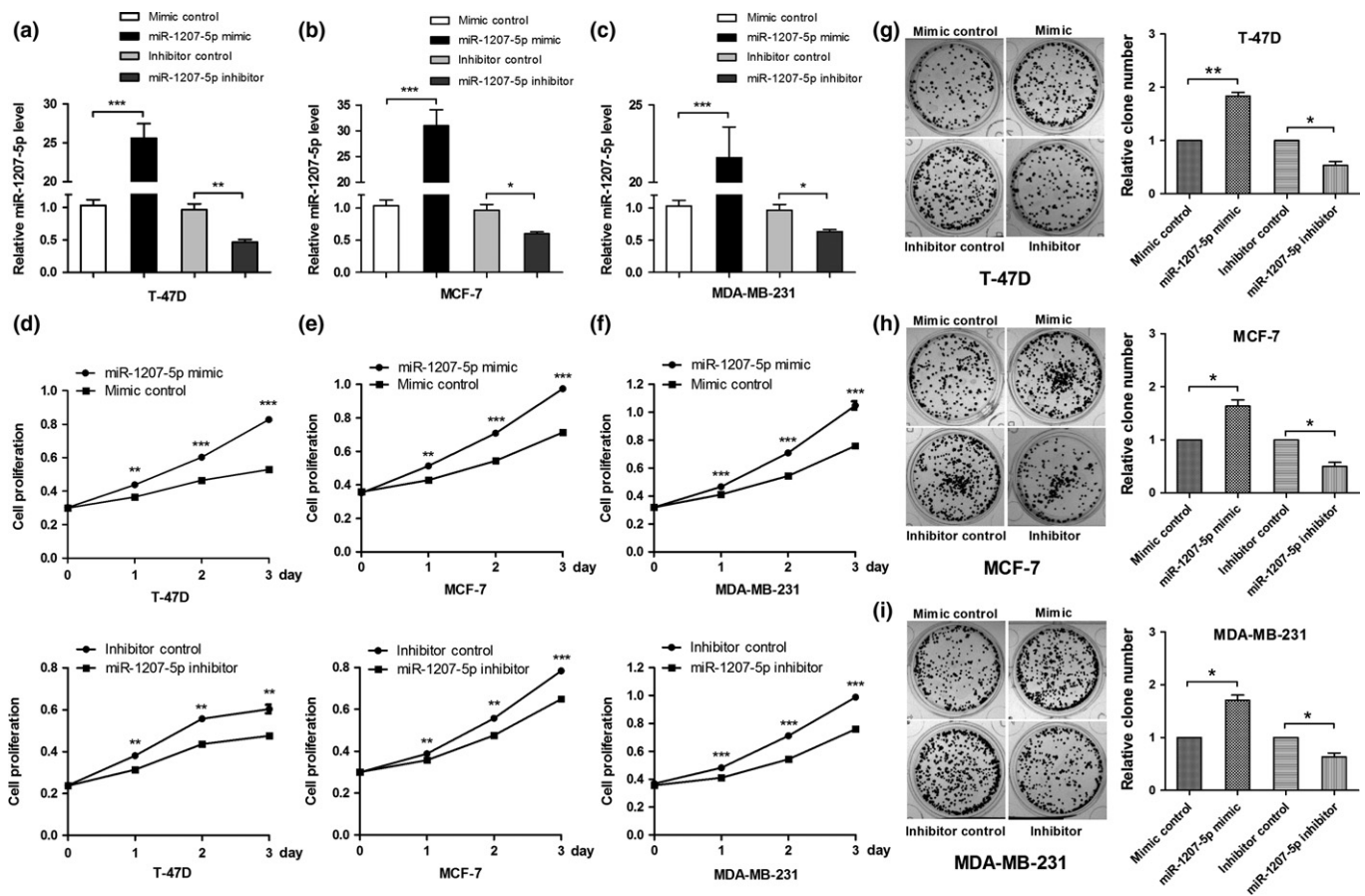


Fig. 2. *miR-1207-5p* promotes breast cancer cell proliferation and colony formation. (a–c) *miR-1207-5p* levels in T-47D (a), MCF-7 (b), and MDA-MB-231 (c) cells transfected with the *miR-1207-5p* mimic or *miR-1207-5p* inhibitor was quantified by quantitative real-time PCR. The *miR-1207-5p* level was normalized to the U6 snRNA level. (d–f) Proliferation of T-47D (d), MCF-7 (e), and MDA-MB-231 (f) cells after transfection with the *miR-1207-5p* mimic or *miR-1207-5p* inhibitor was analyzed using the CCK-8 assay. *miR-1207-5p* overexpression promotes cell proliferation and *miR-1207-5p* inhibition suppresses cell proliferation. (g–i) Photomicrographs of Wright-Giemsa-stained T-47D (g), MCF-7 (h), and MDA-MB-231 (i) cell colonies 14 days after transfection with the *miR-1207-5p* mimic or *miR-1207-5p* inhibitor. The number of cell colonies was counted. This experiment was repeated at least three times with similar results; only one set of the representative results is shown. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the *STAT6* and protein levels (Fig. 4a). We also found that knockdown of *STAT6* expression, as confirmed by Western blot analysis (Fig. 4b), promoted the proliferation of T-47D, MDA-MB-231, and MCF-7 cells (Fig. 4c–e). Thereafter, we used a dual luciferase activity assay to verify *STAT6* as a target gene of *miR-1207-5p*. Bioinformatics analysis showed that there are three binding sites for *miR-1207-5p* in the 3'-UTR of *STAT6* (Fig. 4f). To further investigate the target sites, we used a pMIR-reporter system that utilized the 3'-UTR of *STAT6* containing the binding sites of *miR-1207-5p* (named pMIR-wt-1, pMIR-wt-2, and pMIR-wt-3) embedded in the pMIR-Report luciferase vector (Fig. 4g). As shown in Figure 4(h), only the pMIR-wt-1 luciferase level was decreased by *miR-1207-5p*. Then we generated some mutant sites (pMIR-mut-1) in pMIR-wt-1. Figure 4(i–k) showed that luciferase activity of pMIR-wt-1 was suppressed after transfection with *miR-1207-5p* mimic in breast cancer cells. In contrast, pMIR-mut-1 abolished the effect of *miR-1207-5p* on luciferase activity. To further validate *miR-1207-5p* and *STAT6* relationships, the levels of *miR-1207-5p* and *STAT6* were examined in non-cancerous and cancerous human breast tissues. Compared with the control, the levels of *miR-1207-5p* increased in cancerous breast tissues, as mentioned above, whereas the

STAT6 level decreased ($P < 0.01$, Fig. 4l). Figure 4(m) also showed that there was a negative correlation in expression tendency for *miR-1207-5p* and *STAT6*. These results show that *miR-1207-5p* can regulate *STAT6* expression by directly targeting the 3'-UTR of the *STAT6* mRNA in breast cancer cells.

***miR-1207-5p* regulates the cell cycle in T-47D cells through *CDKN1A* and *CDKN1B*.** To determine how *miR-1207-5p* regulates the cell cycle in T-47D cells, we measured the levels of the cell cycle-dependent kinase inhibitors *CDKN1A* and *CDKN1B*, which are activated by *STAT6* in breast cancer cells.⁽²⁵⁾ By Western blot analysis and qPCR, *miR-1207-5p* overexpression decreased *CDKN1A* and *CDKN1B* mRNA and protein levels, whereas *miR-1207-5p* inhibition increased *CDKN1A* and *CDKN1B* mRNA and protein levels (Fig. 5). These results indicate that *miR-1207-5p* regulates the cell cycle in T-47D cells via *CDKN1A* and *CDKN1B*.

Discussion

There has been little research on the function of PVT1 in breast cancer. Zhang *et al.*⁽²⁶⁾ found that frequent mutation of rs13281615 was significantly associated with cell proliferation by influencing PVT1 expression in breast cancer. Guan

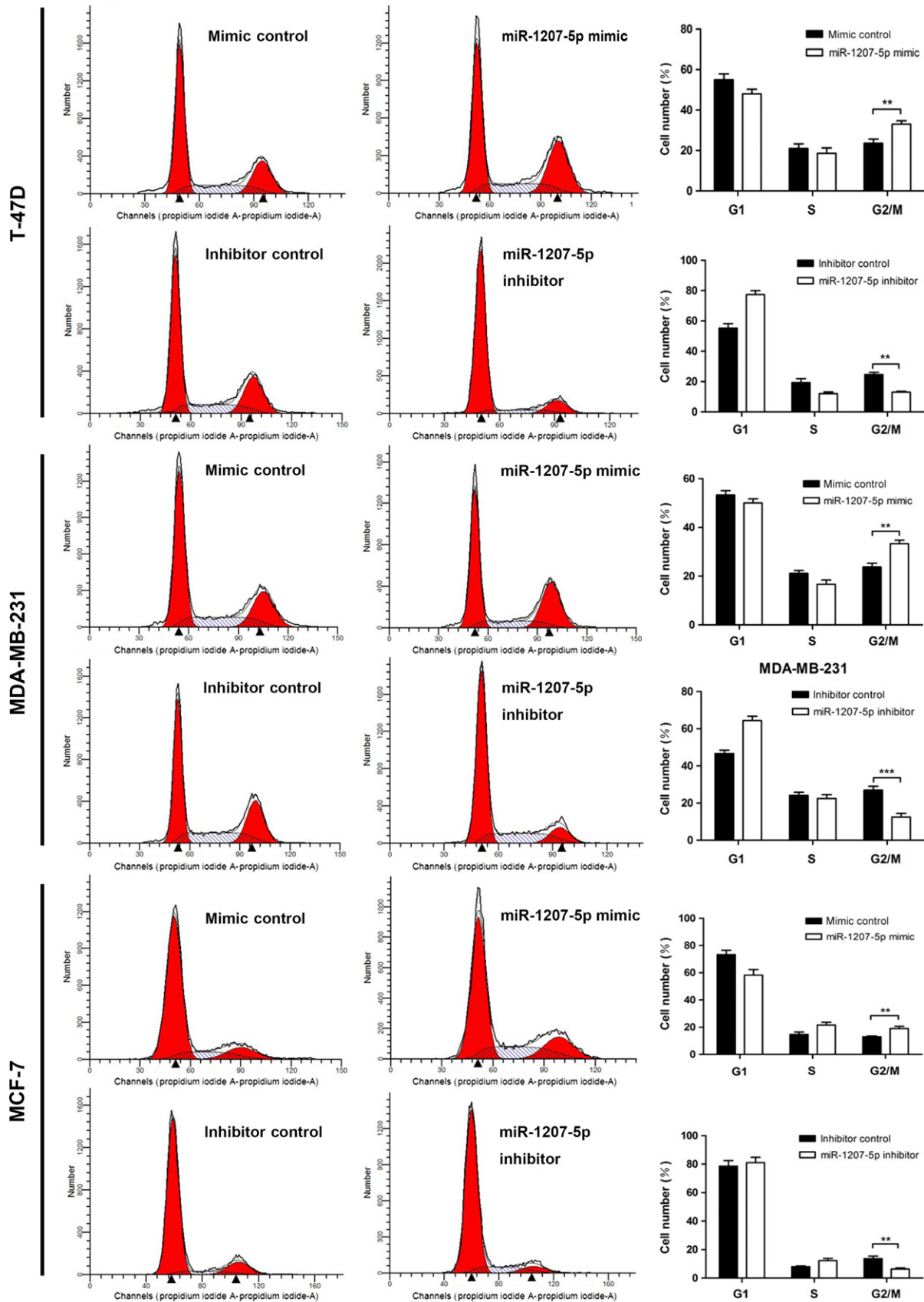


Fig. 3. Effects of *miR-1207-5p* on the cell cycle in T-47D, MDA-MB-231, and MCF-7 breast cancer cells, analyzed by flow cytometry. The line graphs and histograms show the population of cells in G₁, S, and G₂ phase of the groups transfected with *miR-1207-5p* mimic or inhibitor. S, G₂, and G₁ show the percentages of cells in S, G₂, and G₁ phase, respectively.

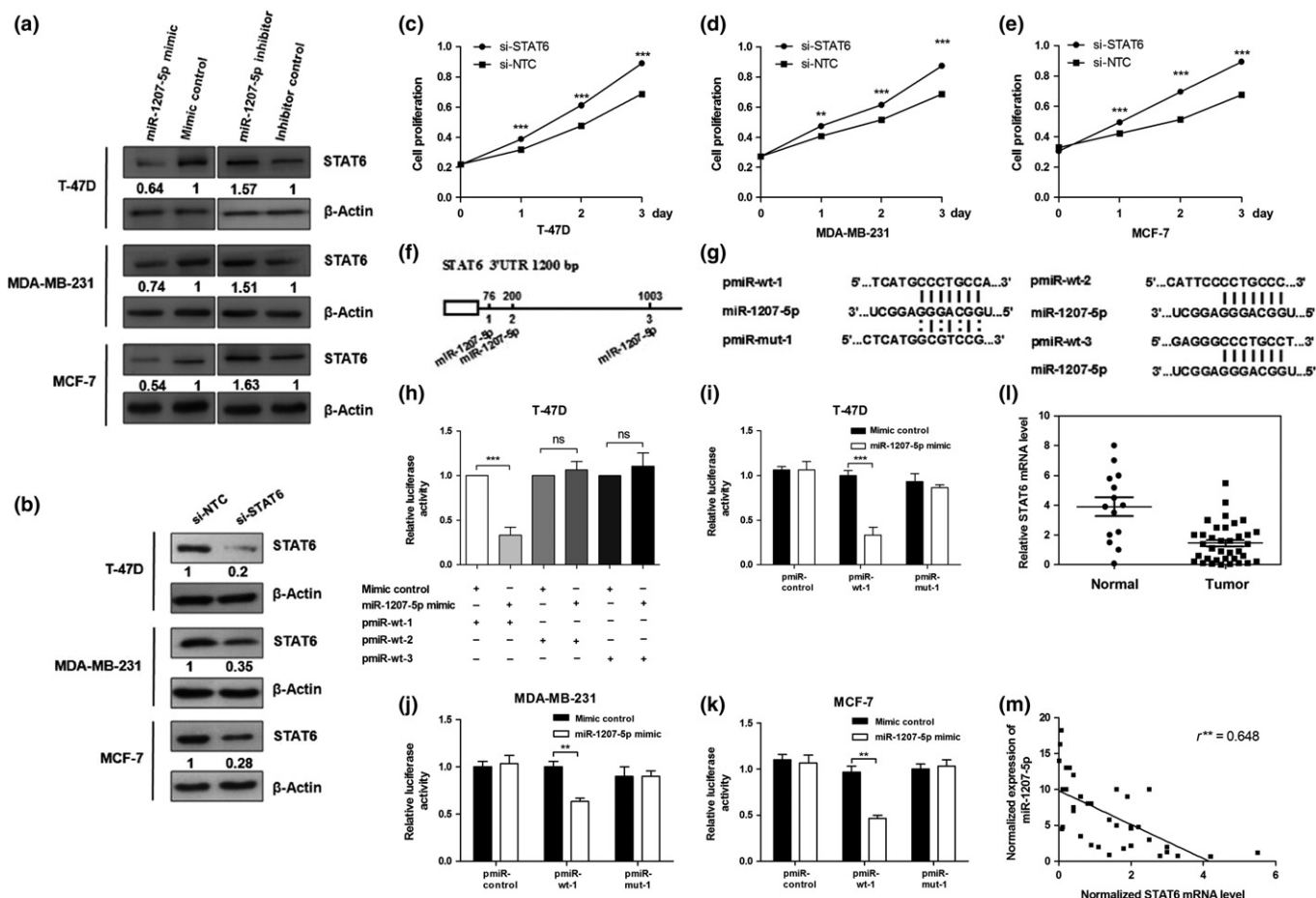


Fig. 4. *miR-1207-5p* directly targets *STAT6*. (a) Effects of transfection with *miR-1207-5p* mimic or *miR-1207-5p* inhibitor on the expression of *STAT6* protein in T-47D, MDA-MB-231, and MCF-7 breast cancer cells. *STAT6* protein levels were assessed by Western blot analysis. This experiment was repeated at least three times with similar results; only one set of the representative results is shown. The number beneath each protein band corresponds to the relative *STAT6* protein level. The intensity of the bands was quantified by ImageJ software. The *STAT6* level was normalized to the β -actin level. (b) Effects of *STAT6* knockdown by si-*STAT6* on the expression of *STAT6* protein in T-47D, MDA-MB-231, and MCF-7 cells. The *STAT6* level was normalized to the β -actin level. (c–e) T-47D (c), MDA-MB-231 (d), and MCF-7 (e) cell proliferation after transfected with si-*STAT6* or control was analyzed using the CCK-8 assay. (f) Three potential binding sites of *miR-1207-5p* in the 3'-UTR of *STAT6*. (g) Sequence of three binding sites, (wild-type, wt) and mutant (mut) sites. (h) T-47D cells were cotransfected with the *miR-1207-5p* mimic or the respective control and pMIR reporter plasmid containing the 3'-UTR (wt-1, wt-2, and wt-3) for 36 h. Data are presented as mean \pm SEM ($n = 3$). (i–k) T-47D (i), MDA-MB-231 (j), and MCF-7 (k) cells were cotransfected with *miR-1207-5p* mimic or respective control and pMIR-wt-1 or pMIR-mut-1. (l) Levels of *STAT6* in non-cancerous and cancerous tissues by quantitative real-time PCR ($P < 0.01$). (m) Pearson's correlation analysis indicated the negative correlation between the expression of *miR-1207-5p* and *STAT6* in cancerous human breast tissues ($r^{**} = -0.648$). $**P < 0.01$; $***P < 0.001$.

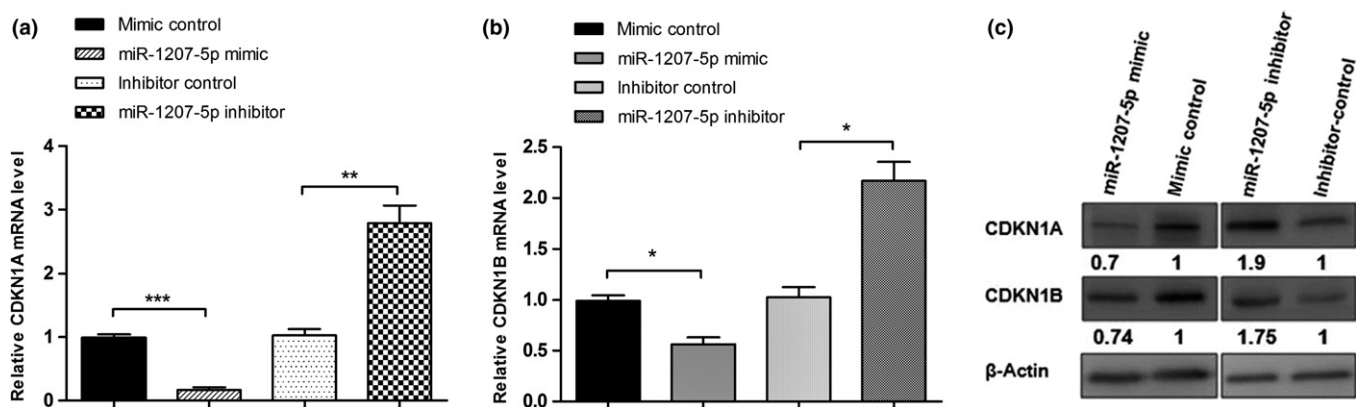


Fig. 5. *miR-1207-5p* regulates the cell cycle in T-47D breast cancer cells through *CDKN1A* and *CDKN1B*. (a,b) *CDKN1A* (a) and *CDKN1B* (b) mRNA levels were quantified by quantitative real-time PCR after *miR-1207-5p* overexpression and inhibition. (c) Protein levels of *CDKN1A* and *CDKN1B* after *miR-1207-5p* overexpression and inhibition by Western blot analysis. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

et al.⁽¹⁶⁾ reported that amplification of PVT1 contributed to the pathophysiology of ovarian and breast cancer. Huppi *et al.*⁽¹⁸⁾ reported that PVT1 introns produced a cluster of six miRNAs, namely, *miR-1204*, *miR-1205*, *miR-1206*, *miR-1207-5p*, *miR-1207-3p*, and *miR-1208*. However, it is not known how PVT1 and PVT1-derived miRNAs regulate breast cancer progression. Their study is important, because it provides new insights on breast cancer progression, and if it can be halted. In the current study, we investigated the relationship between PVT1 and PVT1-derived *miR-1207-5p* and their involvement in human breast cancer. We speculated that PVT1 might act a host gene by regulating *miR-1207-5p* expression and then influencing breast cancer progression. Among the six annotated miRNAs, we found that *miR-1207-5p* was the most abundantly expressed miRNA in different breast cancer cells (Fig. S1). Our results first showed that there was a significant positive correlation between *miR-1207-5p* expression and that of its host gene, PVT1, in breast cancer tissues and different breast cancer cells, suggesting that the expression of one is dependent on that of the other.

Several investigators have shown coexpression of intronic miRNAs that act similarly on their host genes; however, other studies have suggested that their expression level is opposite to that of their host genes.⁽²⁷⁾ Interestingly, Alvarez *et al.*⁽²⁸⁾ reported no change in *miR-1207-5p* expression after a decrease in the PVT1 level. *miR-1207-5p* could also increase the levels of transforming growth factor- β 1, plasminogen activator inhibitor 1, and fibronectin 1, but in a manner that was independent of PVT1 in normal human mesangial cells. These authors hypothesized that *miR-1207-5p* expression and regulation might be independent of the host gene. A change in the epigenetics of tumor cells might explain these differences in PVT1 and *miR-1207-5p* in tumor and other cells, which alludes to their different roles in different cell types and diseases. The relationship between intronic miRNAs and host genes is complicated, and at this point, additional research is required.

In this study, we found that abnormal *miR-1207-5p* expression influenced the proliferation, colony formation and cell cycle progression of breast cancer cells. Based on these results and bioinformatics, we predicted *STAT6* to be a target gene of *miR-1207-5p*. Results from the luciferase activity assay also illustrated that *miR-1207-5p* downregulates *STAT6* expression by directly binding to its 3'-UTR. *STAT6* belongs to the *STAT*

transcription factor family, which contains seven members (*STAT 1–4*, 5a, 5b, and 6), and they can be activated by an array of hormones, cytokines, and growth factors.⁽²⁹⁾ *STATs* dissociate from the receptor after activation and then translocate to the nucleus, activating the transcription of their target genes by binding to cognate DNA response elements.^(30,31) For example, *STAT6* is an important member of the JAK–*STAT* pathway that can be activated by interleukin 4 (IL4) to activate the transcription of downstream genes. In breast cancer cells, *STAT6* mediates IL4-induced growth inhibition.⁽³²⁾ Papageorgis *et al.*⁽³³⁾ found that targeting interleukin 13 receptor subunit α 2 (IL13R α 2) activated the *STAT6*–tumor protein p63 pathway, which suppressed breast cancer lung metastasis. Wei *et al.*⁽²⁵⁾ showed that *STAT6* directly interacted with the transcription factor specificity protein 1, thereby activating the promoter activities of the cell cycle-dependent kinase inhibitors *CDKN1A* and *CDKN1B* in human breast cancer cells. This inhibited cyclin-dependent kinase activity at the G₁ phase and retinoblastoma protein phosphorylation, which maintained cells at this phase of the cell cycle. There are other genes regulating the cell cycle, such as *CDKN2A*, *CDKN2B*⁽³⁴⁾ and *TP53*⁽³⁵⁾ that require further research. In our study, a decreased *STAT6* level after *miR-1207-5p* overexpression downregulated the levels of *CDKN1A* and *CDKN1B* in T-47D, MCF-7, and MDA-MB-231 cells, whereas an increased *STAT6* level after *miR-1207-5p* inhibition upregulated their protein levels.

In summary, PVT1-derived *miR-1207-5p* controls the cell cycle suppressors *CDKN1A* and *CDKN1B* by affecting the expression of the target gene *STAT6*, thereby regulating cell proliferation and inducing breast cancer. These results suggest that *miR-1207-5p* might be a potential target for breast cancer therapy.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Relative expression of six miRNA in breast cancer cells.