

# Long non-coding RNA (lncRNA) CASC9/microRNA(miR)-590–3p/sine oculis homeobox 1 (SIX1)/NF- $\kappa$ B axis promotes proliferation and migration in breast cancer

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## ABSTRACT

Long non-coding RNA (lncRNA)–microRNA–mRNA signaling axes have recently been shown to have a key role in the development of breast cancer (BC). In this study, we investigated how the cancer susceptibility candidate 9 (CASC9) gene affects the cell growth, invasion, migration, and apoptosis of BC cells. The levels of microRNA-590-3p (miR-590-3p), CASC9, and the sine oculis homeobox 1 (SIX1) gene were determined through qRT-PCR. We conducted cell counting kit-8 (CCK-8) assays to assess cell proliferation, transwell assays to detect cell migration/invasion, and flow cytometry to evaluate cell apoptosis. StarBase v2.0 was used to predict interactions between miR-590-3p and SIX1 or CASC9, and dual-luciferase reporter assays were used to verify these predictions. CASC9 protein was overexpressed in BC cells and tissues, while CASC9 knockdown inhibited BC cell growth, invasion, and migration and promoted apoptosis. Additionally, we verified that CASC9 competes for binding with miR-590-3p. Moreover, SIX1 was determined to be a target of miR-590-3p, and SIX1 expression was inhibited by miR-590-3p overexpression. CASC9 enhanced BC development by downregulating miR-590-3p and upregulating SIX1 during the activation of the NF- $\kappa$ B pathway. These data suggest that the CASC9/miR-590-3p/SIX1/NF- $\kappa$ B axis is involved in breast cancer progression, providing insight into the function of CASC9 in breast cancer development.

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## 1. Introduction

Breast cancer (BC) is a common malignancy with high incidence in women [1]. Recurrence and metastasis after treatment pose severe threats to survival [2]. BC therapeutics mainly include surgery, chemotherapy, and radiation, but the mechanisms underlying BC development remain unclear [3]. Gene therapy has recently become a hot topic in the fields of cancer diagnosis and treatment. The characterization of tumor suppressor genes and oncogenic genes and their involvement in BC has helped improve the diagnosis and treatment of BC.

Long non-coding RNAs (lncRNAs) are RNAs with a length of over 200 nucleotides that do not encode functional proteins [4]. Several lncRNAs have been reported to participate in tumorigenesis and cancer progression. H19, the first lncRNA discovered, is abnormally expressed in various cancers including BC [5–9]. H19, the Let-7 microRNA

(miRNA), and LIN28 form a double-negative feedback loop, which plays a key role in enhancing BC cell growth and differentiation [10]. In addition, NEAT1 is positively correlated with breast cancer stage, such that low NEAT1 levels predict good prognosis whereas high NEAT1 levels predict poor prognosis [11]. The ANCR lncRNA is expressed at low levels in BC cells, which effectively inhibits tumorigenesis and distant metastasis [12]. CDKN2B-AS1 promotes the progression of breast cancer through the miR-122-5p/STK39 axis [13]. CASC9 was reported to promote malignancy in ovarian cancer, colorectal cancer (CRC), lung cancer, esophageal squamous cell carcinoma (ESCC), and BC [14–18]. Although the role of CASC9 in BC development has been studied, the underlying molecular mechanisms have not been fully elucidated.

lncRNAs have been suggested to act as competing endogenous RNAs (ceRNAs) that sponge

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miRNAs [19]. CASC9 has been reported to act as a ceRNA to target the tumor suppressor miR-125a-3p and regulate neuregulin-1 (NRG1) to promote hemangioma endothelial cell invasion and migration [20]. Moreover, CASC9 positively regulates checkpoint kinase 1 (CHK1) by sponging the miR-195/497 cluster to promote BC cell growth [21]. miR-590 has been identified as a tumor suppressor in different cancers including BC [22–26]. We previously identified miR-590-3p as a downstream target of CASC9 in relation to BC [27].

SIX1 has been identified as an oncogene in various cancers [28–30]. In BC, SIX1 induces the epithelial-mesenchymal transition (EMT) [31]. In addition, SIX1 was found to upregulate vascular endothelial growth factor-C (VEGF-C) expression to induce lymphangiogenesis and metastasis in BC mouse models [32]. Recent data suggest that several miRNAs including miR-362, miR-186-5p, and miR-188 target the 3' untranslated regions (3' UTRs) of the SIX1 mRNA to induce its degradation and inhibit its translation in several cancers [33–35]. Through bioinformatic analysis, we discovered putative miR-590-3p binding sites in the SIX1 3' UTR. Considering that miR-590-3p is a downstream target of CASC9, we reasoned that miR-590-3p might represent the 'bridge' for CASC9 and SIX1 in BC. Furthermore, SIX1 overexpression has been previously suggested to activate the NF- $\kappa$ B pathway [36]. Thus, CASC9 promotes BC progression by regulating miR-590-3p and modulating the SIX1/NF- $\kappa$ B axis. The present study explored CASC9 expression in BC cells and tissues, while exploring its roles in BC cell growth, invasion, migration, and apoptosis, and the underlying mechanisms. We hypothesized that the CASC9 lncRNA affects BC cell proliferation, migration, and invasion via the miR-590-3p/SIX1 axis.

## 2. Materials and methods

### 2.1 Patients and sample collection

From January 2018 to December 2019, 42 tissue specimens were collected from patients with BC who underwent BC surgery at Shangqiu Central Hospital (Henan, China). All samples were

pathologically confirmed to be without preoperative radiotherapy, chemotherapy, or any other treatment. The tissue samples were immersed in liquid nitrogen after removal. Our experimental procedures were approved by the Research Ethics Committee of Shangqiu Central Hospital. This study was performed in accordance with the Declaration of Helsinki. The participants provided informed consent for participation.

### 2.2 Cell culture

Normal breast epithelial cells (MCF-10A) and BC cells (MDA-MB-468, MCF7, and MDA-MB-231) were provided by the BeNa Culture Collection (Beijing, China) and cultured in high-glucose (HG) DMEM containing 10% fetal bovine serum (FBS). MDA-MB231 and MCF7 cells were cultivated in RPMI 1640 medium containing 10% FBS. Each sample was incubated at 37°C under 5% CO<sub>2</sub> conditions [37].

### 2.3 Cell line transfection

si-CASC9, miR-590-3p mimics, and corresponding negative controls (NCs) were obtained from GenePharma (Shanghai, China). Cells ( $6 \times 10^5$ /well) were seeded into 6-well plates and the above plasmids, a pcDNA3.1 empty vector, and a pcDNA3.1-SIX1 vector were separately transfected into MDA-MB231 and MCF7 cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA), following previously established protocols [27].

### 2.4 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) and quantified. The PrimeScript™ RT Kit (Takara, Dalian, China) was used to reverse transcribe 1  $\mu$ g of RNA into cDNA. SYBR Green PCR Master Mix Kit (Invitrogen, Thermo Fisher Scientific, Inc.; cat. no. 4,309,155) was used in qRT-PCR reactions run on the ABI 7500HT Real-Time PCR machine (Takara Bio, Shiga, Japan) according to established protocols [27]. The thermocycling conditions were as follows: 10 min at 94°C followed by 40 cycles of 30 s at 94°C, 34 s at 54°C, and 30 s at 72°C. The

**Table 1.** The primer sequences.

Gene	Position	Sequence (5' to 3')
CASC9	Forward	CAGGTAATCTCAGCAGTCAT
	Reverse	ACATCCACAGGTCTCCAA
GAPDH	Forward	CAAGGTCATCCATGACAACCTTG
	Reverse	GTCCACCACCCCTGTTGCTGTAG
miR-590-3p	Forward	TAATTTTATGTATAAGCTAGT
	Reverse	GCAGGGTCCGAGGTATTC
U6	Forward	TCCGATCGTGAAGCGTTC
	Reverse	GTGCAGGGTCCGAGGT
SIX1	Forward	AAGGTGAGTGGTGTATTGC
	Reverse	GAATGCTGTGAAGAGATAGTG
si-CASC9 <sup>1#</sup>		AUGAACAUCCACAAACACCAA
si-CASC9 <sup>2#</sup>		UAAUAAUUUCUUGAUAGUGCCA
si-CASC9 NC		GAAUCCUACUUUCACAGCCAU
miRNA-590-3p mimics		TAATTTTATGTATAAGCTAGT
miRNA-590-3p mimics NC		CTAGTCACTATATAGGAGCTG
si-SIX1		GCUUGUUUCUGGAGUUGUUUG
si-SIX1 NC		UCCUUUAGGGUGCGAUGGUUU

$2^{-\Delta\Delta C_t}$  approach was utilized to calculate relative transcript levels, with *U6* and *GAPDH* serving as reference genes for miR-590-3p and CASC9/SIX1, respectively. The sequences of all primers used in this study are presented in Table 1.

### 2.5 Cell Counting Kit-8 (CCK-8) assay

A CCK-8 assay was conducted to measure cell proliferation. In brief, after digestion, transfected MCF7 and MDA-MB231 cells ( $3 \times 10^5$ /well) were inoculated into 96-well plates and incubated under 5% CO<sub>2</sub> at 37°C for 1, 2, 3, 4, and 5 days. After adding 10  $\mu$ L CCK-8 solution (Dojindo Chemical, Japan), the cells were cultured for 2 h. Finally, the optical density (OD) was determined at 490 nm and the absorbance was measured in triplicate [38].

### 2.6 Cell migration and invasion assay

Transfected cells ( $1.0 \times 10^6$ ) were prepared in a cell suspension and added to the top of a Transwell chamber filled with serum-free medium (1 mL), while the bottom chamber was filled with 10% FBS in 600  $\mu$ L DMEM (Sangon Biotech Co., Ltd.). After 24 h, cells in the top chamber were collected and subjected to 95% ethanol fixation, 15 min of 0.05% crystal violet staining, and observation and enumeration using an optical microscope (Nikon, Tokyo, Japan) at 100 $\times$  magnification. For the invasion assays, transwell

chambers were coated with Matrigel (Millipore, USA) [38].

### 2.7 Western blotting (WB)

ter 24 h of transfection, MCF7 and MDA-MB231 cells were collected and lysed in RIPA buffer to isolate total protein, which was quantified using a BCA assay (Beyotime Biotechnology, Shanghai, China). Next, total protein samples (30  $\mu$ g) were separated on a 10% SDS-PAGE gel, followed by the transfer of the proteins to PVDF membranes (Beyotime Biotechnology, Shanghai, China). Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 2 h at room temperature. Membranes were washed with TBST, developed using ECL and a gel imaging system (Bio-Rad) and assessed by One software (Bio-Rad, USA) [38].

### 2.8 Luciferase reporter assay

The pmirGLO Dual-Luciferase Reporter Gene Detection System (Promega, Madison, WI, USA) was used to synthesize SIX1- and CASC9-binding sites for miR-590-3p in the corresponding 3' UTR. These constructs were inserted into the pmirGLO luciferase expression vector to generate a pmirGLO/CASC9 vector and a pmirGLO/SIX1-UTR vector. The mutant sequence was synthesized to generate the pmirGLO/CASC9-M and pmirGLO/SIX1-M-UTR vectors using the same method. We used Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.) to transfect the pmirGLO/CASC9 and pmirGLO/SIX1-UTR wild-type vectors, pmirGLO/CASC9-M, pmirGLO/SIX1-M-UTR vectors, and miR-590-3p mimics, together with the corresponding NC, into MCF7 and MDA-MB231. The cells were harvested 48 h after transfection. The fluorescence value of the sea kidney served as the endogenous control, as suggested by the manufacturer. The fluorescence ratio (firefly to sea kidney) was determined to evaluate the relative activity of the reporter gene [27].

## 2.9 Construction of the pCDH-Flag-SIX1 recombinant plasmid

SIX1 was amplified for 34 cycles (5 min of pre-denaturation at 95°C, 30 s of denaturation at 95°C, 30 s of annealing at 62°C, 60 s of extension at 72°C, and 7 min of extension at 72°C). PCR products were visualized by gel electrophoresis, and Not I and BamHI were used to digest the amplicons and the pCDH-Flag vector. T4 DNA ligase was used to ligate the amplicons into the pCDH-flag vector at 16°C for 30 minutes. The resulting vectors were transformed into *Escherichia coli* DH5 $\alpha$  cells and cultured on LB plates containing 100  $\mu$ g/ml ampicillin [39].

## 2.10 Flow cytometry

Transfected cells were digested using trypsin, centrifuged, and harvested. Cells were rinsed twice with PBS, resuspended in 70% ethanol, and fixed at 4°C for 30 min. Next, the fixed cells were washed twice with PBS and incubated in AnnexinV-FITC and propidium iodide (PI, Jiancheng, Nanjing, China) for 1 h at 4°C, at which point quantifications were performed [40].

## 2.11 Bioinformatic analysis

We searched the GEPIA database (<http://gepia.cancer-pku.cn/>) to analyze SIX1 expression in BC samples [41]. The LncBase Predicted v.2 ([http://carolina.imis.athena-innovation.gr/diana\\_tools/web/index.php?r=lncbasev2%2Findex-predicted](http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted)) [42] and TargetScan 7.2 databases ([http://www.targetscan.org/vert\\_72/](http://www.targetscan.org/vert_72/)) [43] were used to search for miR-590-3p binding sites in CASC9 and the SIX1 3' UTR. Regarding the search for miR-590-3p binding sites, the LncBase Predicted v.2 database was used to input the location of CASC9 (chr8:75,120,409–75,352,327) and then identify binding sites for miRNAs, including miR-590-3p.

## 2.12 Statistical analysis

SPSS24.0 software was used to perform all statistical analyses. Data are displayed as the mean  $\pm$  standard deviation (SD). Significant differences between groups were identified using Student's *t*-tests or ANOVA followed by Tukey's post-hoc test.

Correlations were determined using Pearson's correlation analysis. Statistical significance was set at  $p < 0.05$ .

## 3. Results

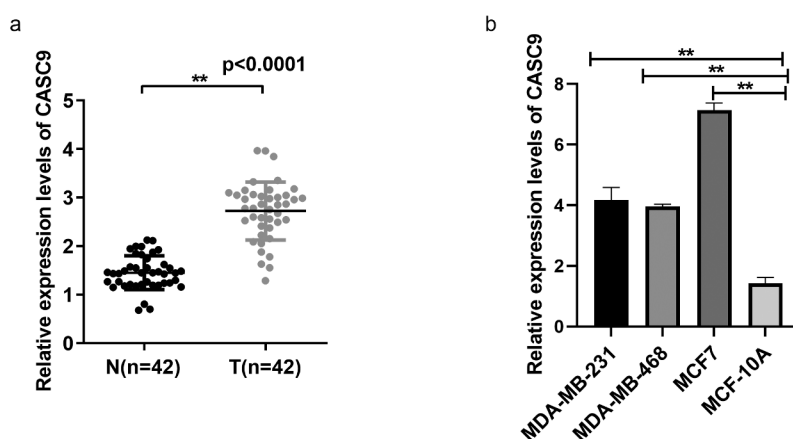
In this study, we explored the role of CASC9 in BC. A series of assays led to the discovery that CASC9 promotes BC progression by regulating the miR-590-3p/SIX1/NF- $\kappa$ B axis. Our findings highlight the functional roles of CASC9 in BC, providing new insights into BC pathogenesis.

### 3.1 CASC9 is upregulated in BC tissues and cells

We first interrogated CASC9 expression in 42 pairs of BC and non-carcinoma tissues by qRT-PCR. CASC9 levels were significantly increased in BC samples compared to the levels in control samples (Figure 1a). CASC9 levels were examined in normal breast epithelial MCF-10A cells and BC cells (MDA-MB-231, MDA-MB-468, and MCF7) by qRT-PCR. CASC9 was found to be highly expressed in MDA-MB231, MCF7, and MDA-MB-468 cell lines (Figure 1b), suggesting that CASC9 is closely related to BC progression. CASC9 expression was the highest in the MCF7 and MDA-MB231 cell lines, therefore these cell lines were selected for the subsequent experiments.

### 3.2 CASC9 knockdown suppresses BC cell proliferation and promotes apoptosis

siRNA-CASC9 was used to downregulate CASC9, and qRT-PCR was used to confirm that CASC9 expression was lower in the siRNA-CASC9<sup>1#</sup> group than in the siRNA-CASC9<sup>2#</sup> group (Figure 2a). Therefore, siRNA-CASC9<sup>1#</sup> was used for further experiments. CCK8 and flow cytometry assays were used to investigate the function of CASC9 in BC development. CASC9 knockdown significantly reduced BC cell proliferation (Figure 2b and c) and increased apoptosis in MCF7 and MDA-MB231 cells (Figure 2d). These results suggest that CASC9 promotes BC cell proliferation and inhibits cell apoptosis.



**Figure 1.** CASC9 significantly up-regulated within BC cells and tissues.

(A) CASC9 level in 42 BC tissues relative to non-carcinoma samples. (B) CASC9 expression level within BC cells (MDA-MB-468, MCF-7, MDA-MB-231) and MCF-10A cells.  $**P < 0.01$ .

### 3.3 CASC9 knockdown suppresses BC cell migration and invasion

Transwell migration and invasion experiments revealed that, compared to the NC group, the number of invading and migrating BC cells markedly decreased in response to CASC9 knockdown (Figure 3a and b), suggesting that CASC9 promotes BC cell migration and invasion.

### 3.4 miR-590-3p is a target of CASC9

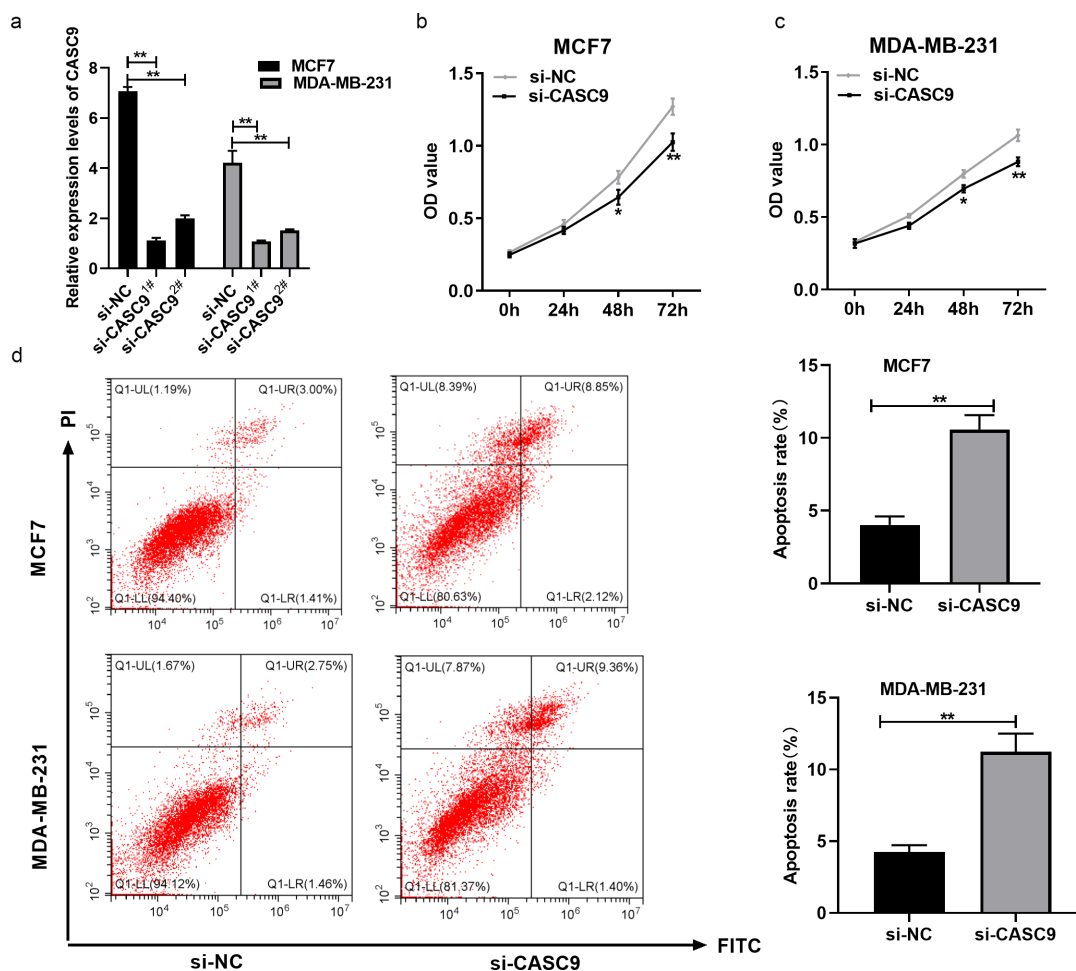
To further explore the molecular mechanism underlying the role of CASC9 in the development and progression of BC, putative CASC9-binding sites were predicted using LncBase v.2. Interestingly, bioinformatic analysis revealed that miR-590-3p was a potential target of CASC9 (Figure 4a). A physical interaction between miR-590-3p and CASC9 was observed in MDA-MB231 and MCF7 cells using a luciferase reporter assay (Figure 4b and c). In addition, we revealed that knockdown of CASC9 markedly upregulated miR-590-3p expression in MCF7 and MDA-MB231 cells relative to the NC (Figure 4d). We also measured miR-590-3p expression in BC tissues and cells, and found that miR-590-3p expression was decreased in BC cells and tissues in a manner that was directly proportional to CASC9 levels in BC tissue samples (Figure 4e-g).

### 3.5 CASC9 promotes malignancy of BC cells by decreasing miR-590-3p levels

To further explore whether CASC9 promoted malignancy by regulating miR-590-3p in BC, we co-transfected si-CASC9 and miR-590-3p mimics into BC cells. First, miR-590-3p mimics, together with the corresponding NCs, were transfected into MCF7 and MDA-MB231 cells to determine the transfection efficiency. The miR-590-3p mimics group was characterized by remarkably higher miR-590-3p mRNA levels compared to the NC group, demonstrating successful transfection (Figure 5a). In addition, we investigated whether CASC9 promotes the malignancy of breast cancer cells by interacting with miR-590-3p. To verify this hypothesis, we transfected miR-590-3p mimics and si-CASC9 in MCF7 and MDA-MB231 cells. We confirmed that CASC9 knockdown remarkably suppressed cell growth, invasion, and migration, but enhanced apoptosis. However, these effects were enhanced when cells were co-transfected with miR-590-3p mimics (Figure 5b-f). Based on these findings, CASC9 may promote the malignancy of BC cells by decreasing miR-590-3p.

### 3.6 SIX1 is a direct target of miR-590-3p in BC cells

TargetScan analyses predicted that SIX1 may be a direct target of miR-590-3p (Figure 6a). Luciferase



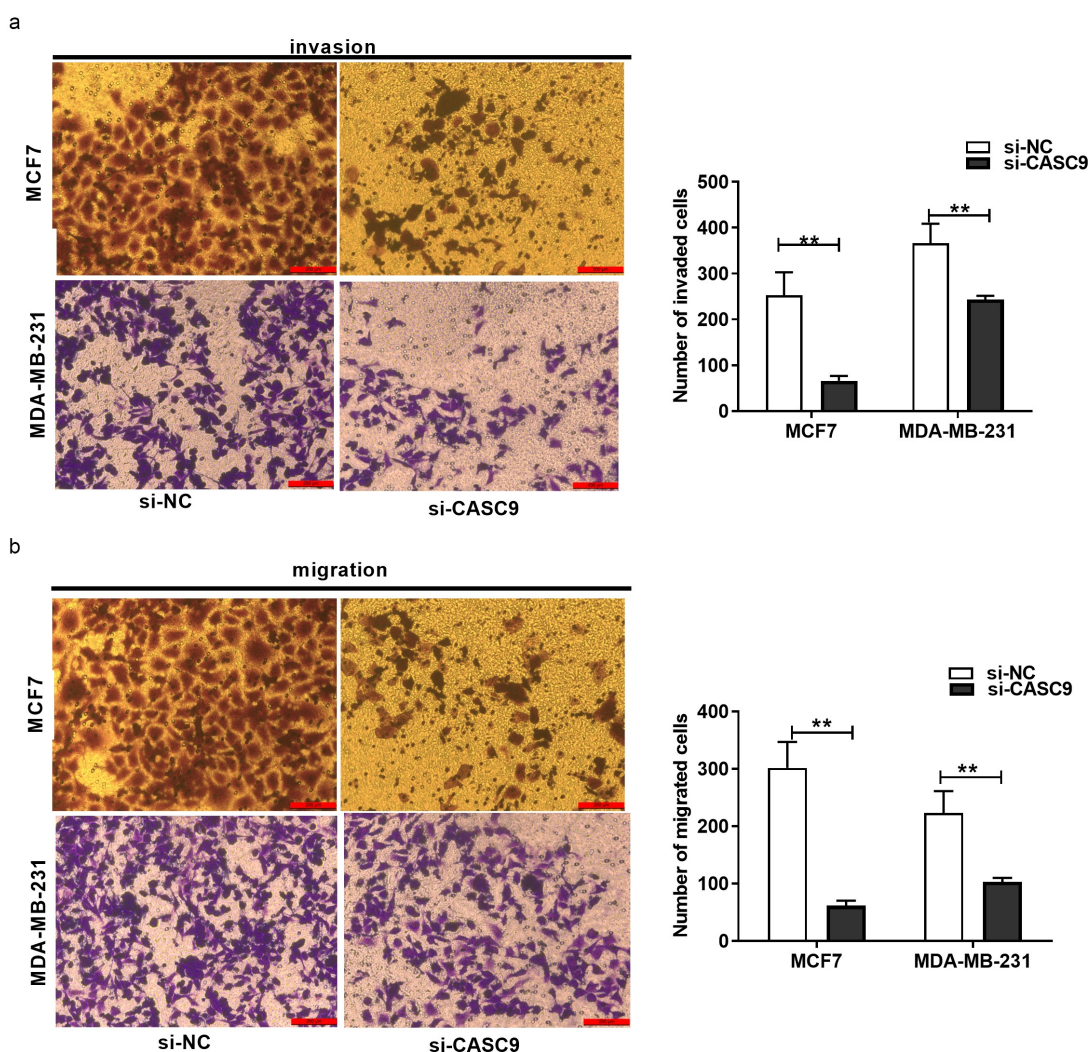
**Figure 2.** Knock-down of CASC9 inhibits BC cell growth but promotes their apoptosis.

(A) CASC9 levels within MDA-MB-231 and MCF7 cells subjected to si-CASC9- or si-NC transfection were analyzed via qRT-PCR. (B) CCK-8 assay analysis of cell proliferation abilities in MCF-7 cells subjected to si-CASC9 or si-NC transfection. (C) CCK-8 assay was conducted for detecting MDA-MB-231 cell proliferation subjected to si-CASC9 or si-NC transfection. (D) Flow cytometry analysis of cell apoptosis abilities of MCF-7 and MDA-MB-231 cells subjected to si-CASC9- or si-NC transfection. \* $P < 0.05$ , \*\* $P < 0.01$ .

reporter assays verified that miR-590-3p directly interacted with CASC9 in MCF7 and MDA-MB231 cells (Figure 6b and c). In addition, qRT-PCR assays validated that miR-590-3p overexpression markedly decreased SIX1 expression (Figure 6d). Additionally, SIX1 levels were increased in BC patient tumor samples compared to normal samples, as evidenced by the analysis of The Cancer Genome Atlas database using GEPIA (Figure 6e). SIX1 was highly expressed in BC tissues (figure 6f) in a manner that was indirectly proportional to miR-590-3p expression (Figure 6g) and positively correlated with CASC9 levels in BC tissue samples (Figure 6h). These findings suggest that SIX1 is directly targeted by miR-590-3p.

### 3.7 CASC9 promotes the malignancy of BC cells by decreasing miR-590-3p levels and upregulating SIX1

As previously mentioned, CASC9 binds to miR-590-3p, while miR-590-3p targets SIX1 to exert its function in BC cells. To further explore whether CASC9 promoted the malignancy of BC cells by regulating the miR-590-3p/SIX1 axis in BC, si-SIX1 (si-NC) and pcDNA-SIX1 (pcDNA3.1) were transfected into MCF7 and MDA-MB231 cells. qRT-PCR analysis demonstrated that SIX1 mRNA levels were decreased in response to si-SIX1, while SIX1 mRNA levels



**Figure 3.** Knock-down of CASC9 suppresses breast cancer cell invasion and migration.

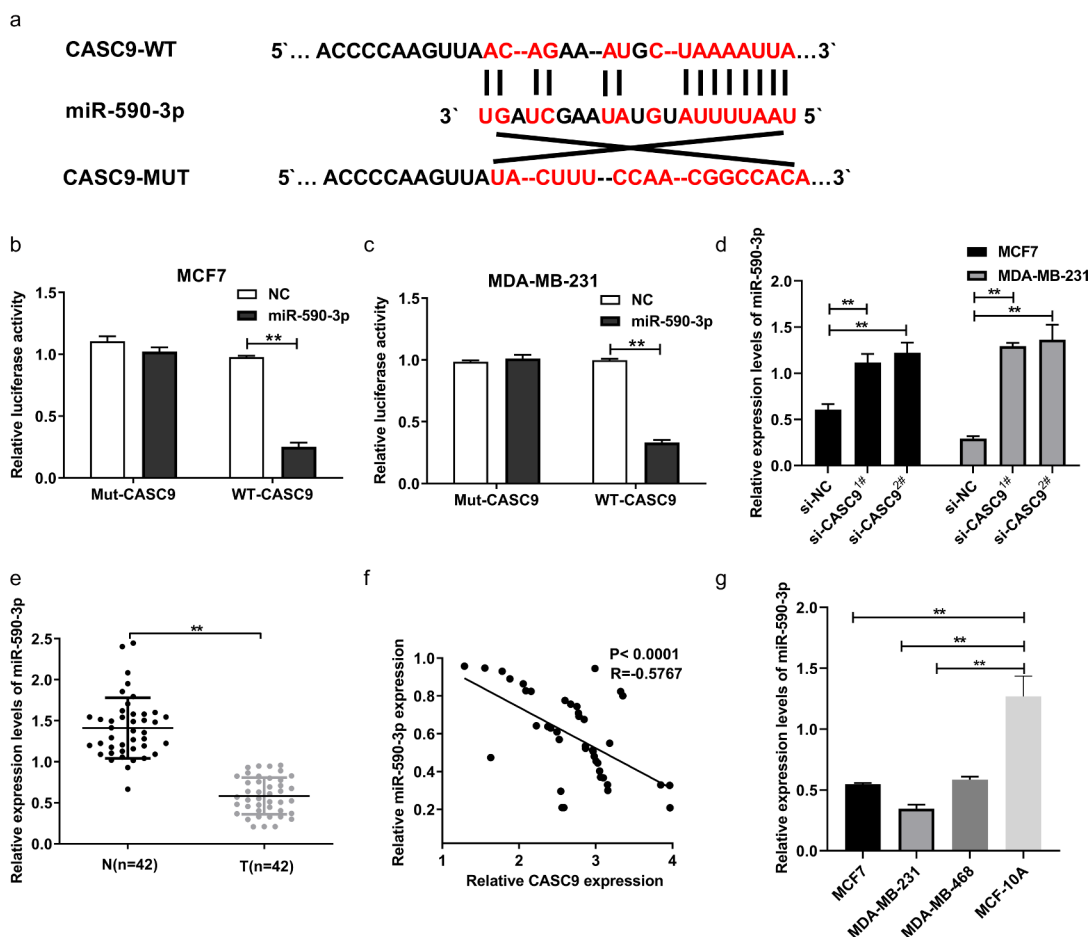
(A) Transwell assays analysis of MDA-MB-231 and MCF-7 cell migration after si-CASC9- or si-NC transfection. (B) Transwell assays analysis on the invasion of the above two cell lines after si-CASC9- or si-NC transfection.  $**P < 0.01$ .

increased in response to pcDNA-SIX1, indicating successful transfection (Figure 7a and b). A series of functional assays demonstrated that transfection of si-CASC9 markedly suppressed BC cell proliferation, migration, and invasion, which markedly increased after miR-590-3p mimic co-transfection, while co-transfection with pcDNA-SIX1 restored these effects (Figure 7c-f). Moreover, CASC9 knock-down increased BC cell apoptosis, which was markedly increased by co-transfection of a miR-590-3p mimic, an effect that was abolished by SIX1 overexpression (Figure 7g). These observations suggest that CASC9

enhances BC malignancy by decreasing miR-590-3p expression and upregulating SIX1.

### 3.8 The CASC9/miR-590-3p/SIX1/NF- $\kappa$ B axis is involved in BC progression

SIX1 silencing was reported to inhibit NF- $\kappa$ B activation [36]. We discovered that CASC9 knock-down decreased the levels of related proteins such as SIX1, p65, matrix metalloproteinase 9 (MMP9), and B-cell lymphoma 2 (BCL2), while miR-590-3p overexpression enhanced these effects and SIX1 overexpression reversed these effects



**Figure 4.** miR-590-3p as a CASC9 candidate target.

(A) miR-590-3p binding sites for CASC9. (B) CASC9 mut or WT luciferase reporter plasmid was co-transfected in MCF-7 cells with miR-590-3p mimics or corresponding NC for luciferase assays. (C) CASC9 mut or WT luciferase reporter plasmid was co-transfected into MDA-MB-231 cells with miR-590-3p mimics or corresponding NC for luciferase assays. (D) miR-590-3p levels in MDA-MB-231 and MCF-7 cell lines subjected to si-CASC9 or si-NC transfection. (E) miR-590-3p levels within 42 BC tissue samples relative to matched non-carcinoma samples. (F) Correlation of CASC9 levels with miR-590-3p levels within BC tissues. (G) miR-590-3p levels within normal breast epithelial MCF-10A cells and BC cells (MDA-MB-231, MDA-MB-468, MCF7). \*\* $P < 0.01$ .

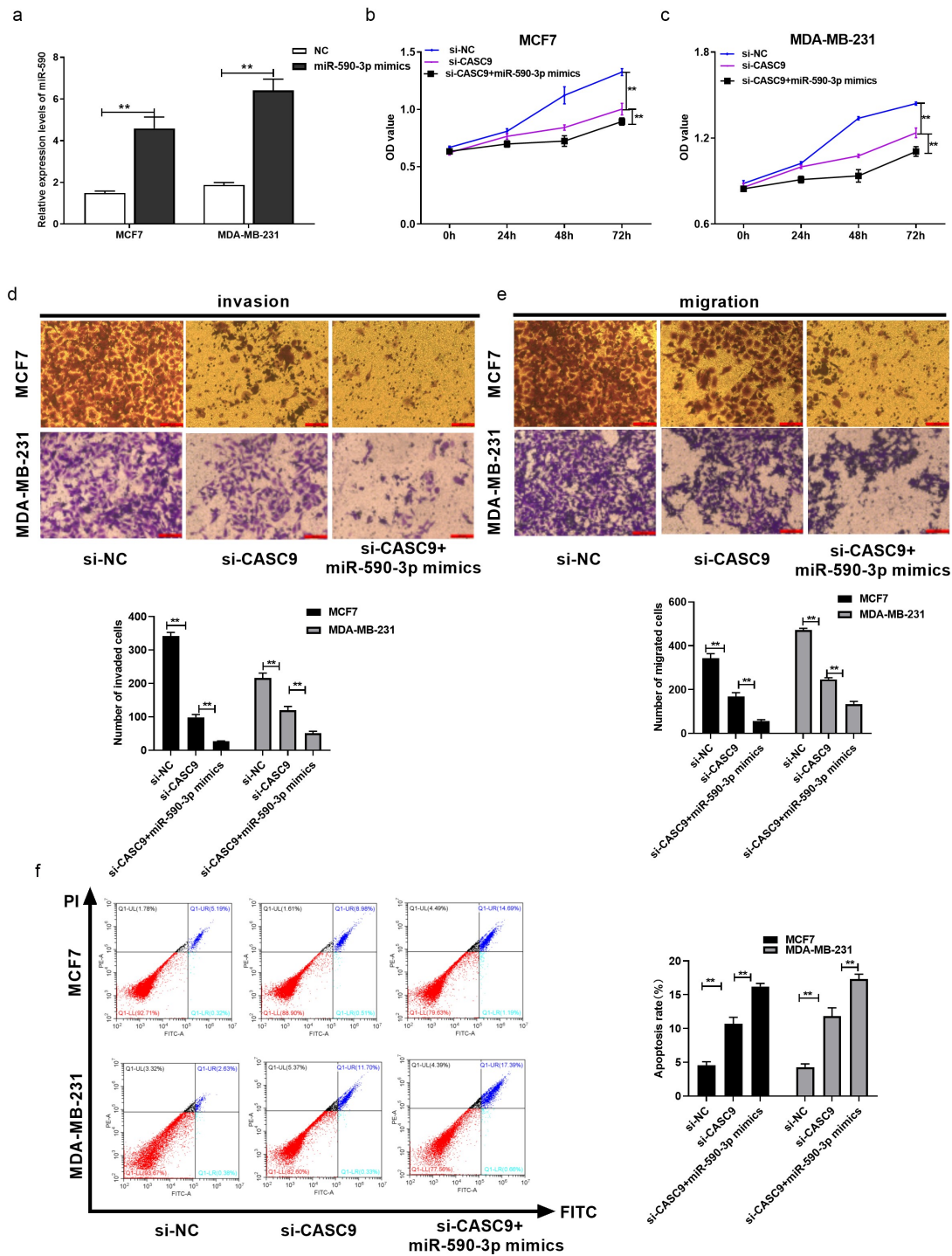
(Figure 8a-b). These findings confirm that the CASC9/miR-590-3p/SIX1/NF- $\kappa$ B axis is involved in BC progression.

#### 4. Discussion

lncRNAs are endogenous, single-stranded, non-coding RNAs with lengths of over 200 nucleotides [4]. They participate in epigenetic regulation via chromatin remodeling, transcriptional regulation, and post-transcriptional regulation [44]. lncRNAs also function in regulating cell growth, differentiation, neural development, immune homeostasis,

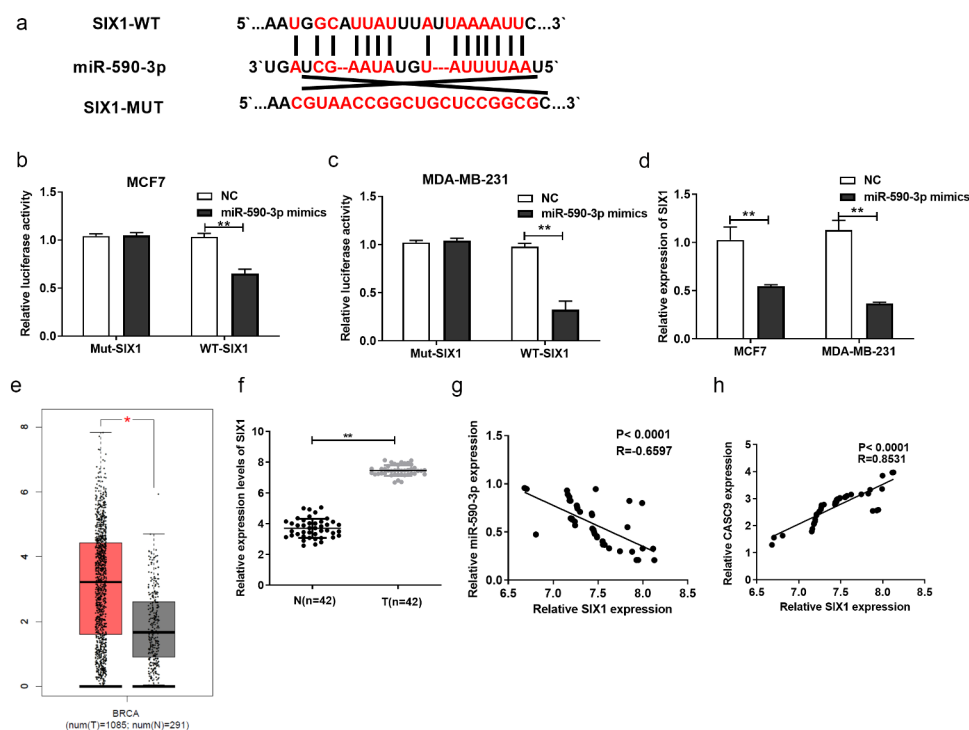
tumorigenesis, and other biological processes [45–49]. Several studies have examined the functions of lncRNAs in BC [8,11,13]. For instance, high expression of the VCAN-AS1 lncRNA was detected in BC, which promotes the malignancy of BC by regulating the miR-106a-5p-regulated signal transducer and activator of transcription 3/hypoxia-inducible factor-1 $\alpha$  pathway [37]. The BCAR4 lncRNA is overexpressed in BC cells and tissues, which promotes BC cell proliferation by suppressing p16 expression [50]. The CASC9 oncogene is highly expressed in esophageal squamous cell carcinoma (ESCC), ovarian cancer, BC,





**Figure 5.** CASC9 promotes malignant behavior of BC cells through reducing miR-590-3p.

(A) miR-590-3p levels within MDA-MB-231 and MCF7 cells subjected to miR-590-3p mimics or mimics-NC transfection were measured via qRT-PCR assay. (B) MCF-6 cell proliferation subjected to miR-590-3p mimics (or NC) and/or si-CASC9 (or si-NC) transfection detected via CCK-8 assay. (C) MDA-MB-231 cell proliferation subjected to miR-590-3p mimics (or NC) and/or si-CASC9 (or si-NC) transfection measured by CCK-8 assay. (D) Transwell assays of MDA-MB-231 and MCF7 cell migration following miR-590-3p mimics (or NC) and/or si-CASC9 (or si-NC) transfection. (E) Transwell assays analysis of cell invaded abilities within MDA-MB-231 and MCF7 cells subjected to miR-590-3p mimics (or NC) and/or si-CASC9 (or si-NC) transfection. (F) Flow cytometry analysis of cell apoptosis abilities within the above two cell lines following miR-590-3p mimics (or NC) and/or si-CASC9 (or si-NC) transfection.  $**P < 0.01$ .



**Figure 6.** SIX1, a direct miR-590-3p target within BC cells.

(A) SIX1 binding sites in miR-590-3p. (B) SIX1 mut or WT luciferase reporter plasmid was co-transfected in MCF-7 cells with miR-590-3p mimics or corresponding NC for luciferase assays. (C) SIX1 mut or WT luciferase reporter plasmid was co-transfected into MDA-MB-231 cells with miR-590-3p mimics or corresponding NC for luciferase assays. (D) SIX1 levels within MCF-7 and MDA-MB-231 cells after NC or miR-590-3p mimics transfection. (E) The expression level of SIX1 in BC tumor samples and normal samples predicted by GEPIA website. (F) SIX1 expression within 42 BC samples relative to matched non-carcinoma samples. (G) Correlation of SIX1 level with miR-590-3p level within BC tissues. (H) The correlation analysis between SIX1 expression and CASC9 level within BC samples. \*\* $P < 0.01$ .

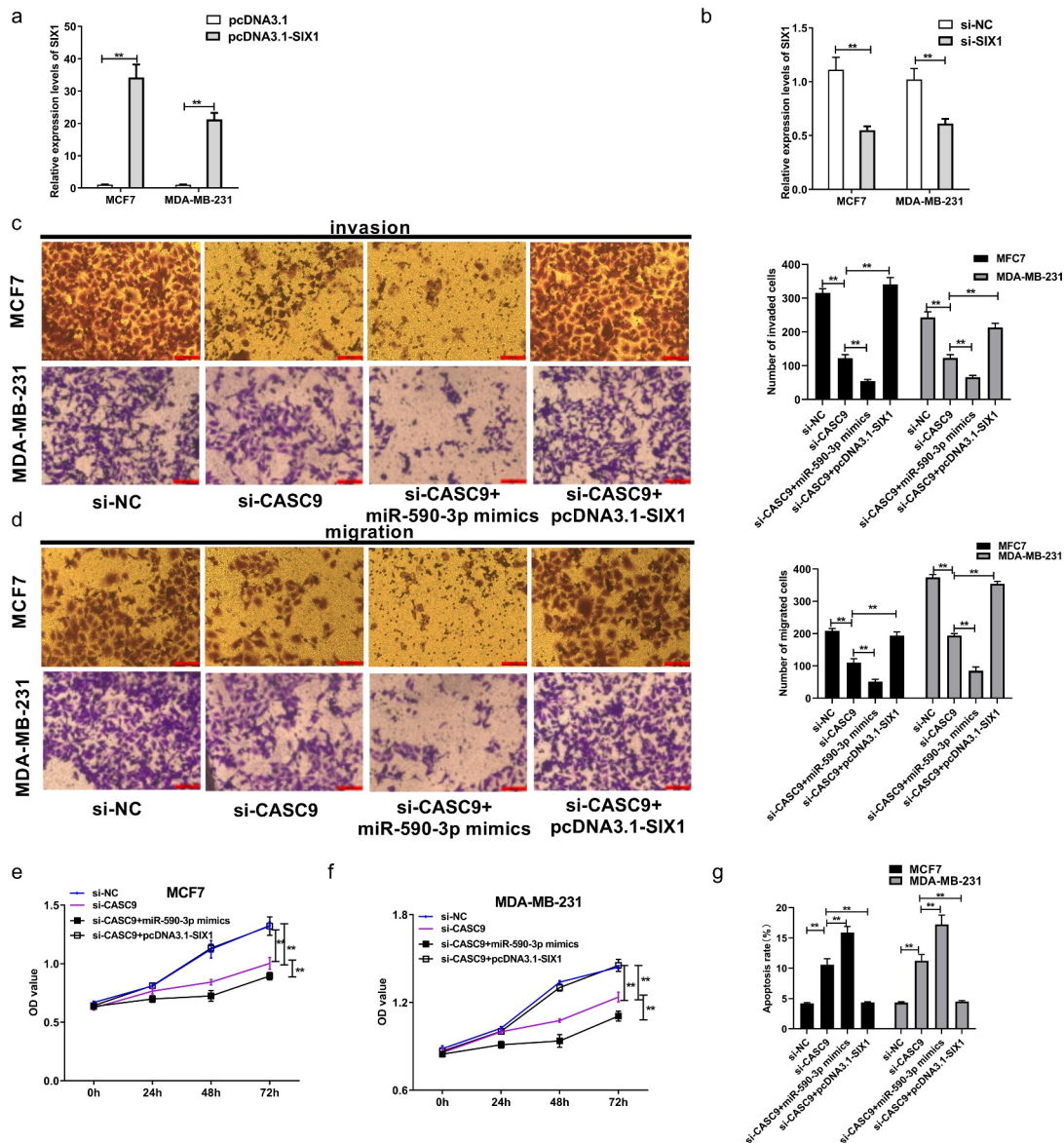
lung cancer, papillary thyroid cancer, and bladder cancer [14–17,51,52]. The CASC9 lncRNA positively regulates CHK1 to promote the growth and survival of BC cells by sponging the miR-195/497 cluster. Zhang et al. showed that CASC9 promotes BC cell metastasis by mediating the miR-215/Twist-related protein 2 signal transduction pathway in BC [53]. Shao et al. reported that CASC9 enhanced the growth and survival of BC cells by sponging the miR-195/497 cluster [21]. However, the effect of CASC9 in BC had not yet been investigated. Understanding its role in BC may shed light on the development and progression of BC.

The present study discovered that the CASC9 lncRNA was highly expressed in BC tissues and cells, indicating a possible regulatory role for CASC9 in BC progression. To explore the role of CASC9 in BC, we silenced CASC9 in MCF7 and

MDA-MB231 BC cells. Furthermore, we demonstrated that low expression of CASC9 remarkably suppressed cell growth, migration, and invasion, but enhanced apoptosis.

lncRNAs can act as molecular sponges by binding to miRNAs to inhibit the miRNA-mediated silencing of target mRNAs. TargetScan analysis indicated that miR-590-3p might be a binding partner of CASC9. Bioinformatic predictions and dual-luciferase reporter assays revealed that SIX1 is a target of miR-590-3p. Thus, the upregulation of SIX1 in BC tissues was confirmed. Thus, in this study, we characterized the CASC9/miR-590-3p/SIX1 regulatory network.

The NF- $\kappa$ B pathway is essential to immunity, and its importance in cancer development has been extensively studied, especially in relation to tumor cell proliferation, survival, and metastasis. Multiple studies have shown that lncRNAs and

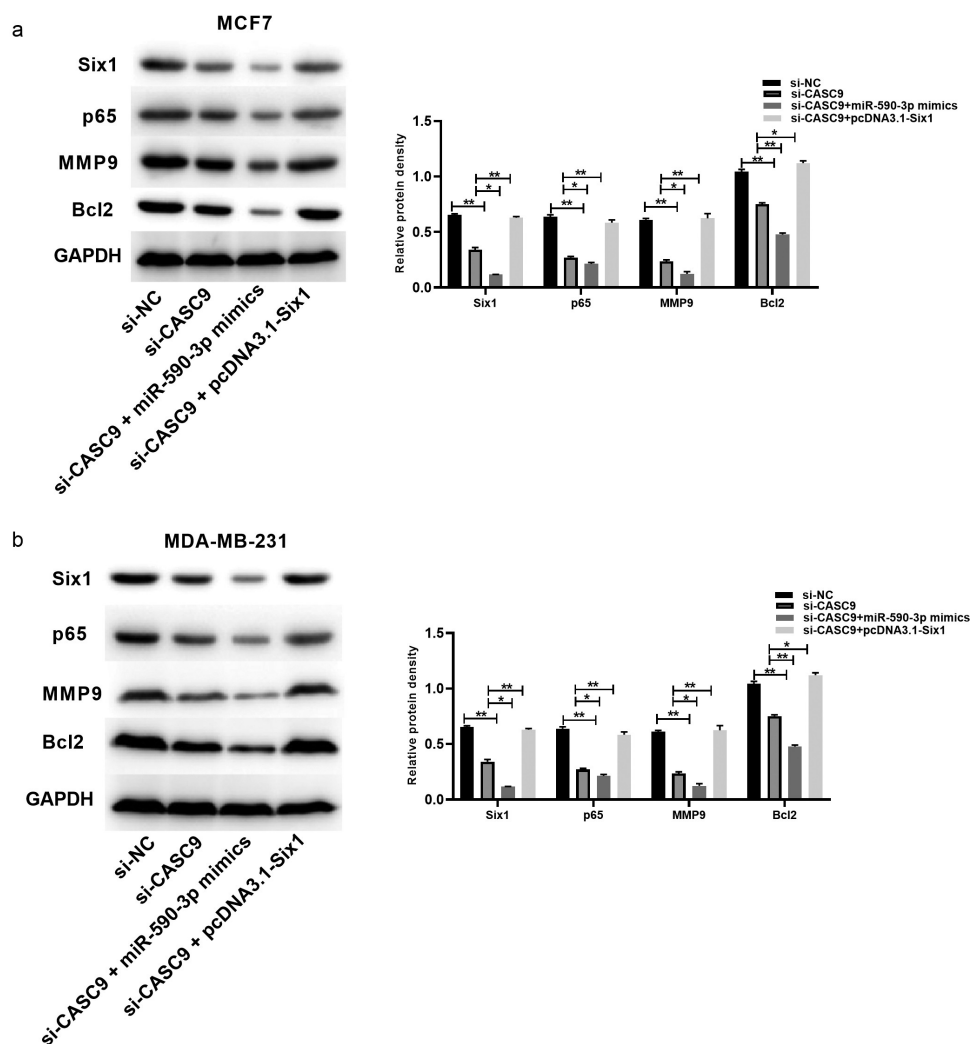


**Figure 7.** CASC9 promotes malignant behavior of BC cells by down-regulating miR-590-3p and up-regulating SIX1.

(A) SIX1 levels within pcDNA3.1-SIX1- or pcDNA3.1-transfected MDA-MB-231 and MCF7 cell lines measured through qRT-PCR. (B) SIX1 levels within MDA-MB-231 and MCF7 cells following si-SIX1 or si-NC transfection measured by qRT-PCR. (C) Transwell assays analysis of migration abilities within the above two cell lines. (D) Transwell assays analysis of invaded abilities within the above two cell lines. (E) Viability of MCF7 cells measured through CCK-8 assay. (F) Viability of MDA-MB-231 cells detected through CCK-8 assay. (G) MDA-MB-231 and MCF7 cell apoptosis examined through flow cytometry.  $**P < 0.01$ .

several protein-coding genes regulate the NF- $\kappa$ B signaling pathway [54–56]. As reported by Yang et al., SIX1 knockdown dramatically suppressed NF- $\kappa$ B activation [36]. Han et al. reported that the DNAJC3-AS1/miR-214-3p axis affected NF- $\kappa$ B activation via the regulation of LIVIN expression, which further impacted the malignant phenotype of colorectal cancer cells [56]. Gao et al. demonstrated that the inhibition of XIST promoted U2OS cell death via the activation of the

NF- $\kappa$ B/PUMA pathway [55]. Here, we examined the levels of the NF- $\kappa$ B protein p65, apoptosis-associated protein Bcl2, and MMP9. SIX1, p65, MMP9, and Bcl2 protein levels were reduced by CASC9 knockdown, and miR-590-3p overexpression enhanced these effects. Thus, the CASC9/miR-590-3p/SIX1/NF- $\kappa$ B axis is involved in BC progression. However, there are some limitations to our study. For example, an electrophoretic mobility shift assay was not performed to confirm



**Figure 8.** CASC9/miR-590-3p/SIX1/NF- $\kappa$ B pathway related to BC progression.

(A) Protein levels within MCF7 cells detected through WB assay. (B) Protein levels within MDA-MB-231 cells detected through WB assay. All bars stand for mean  $\pm$  SD for 3 individual assays. \* $P$  < 0.05, \*\* $P$  < 0.01.

the involvement of the NF- $\kappa$ B signaling pathway. In addition, RNA immunoprecipitation and RNA pull-down assays were not performed to confirm the relationship between CASC9, miR-590-3p, and SIX1.

## 5. Conclusion

In this study, we established that CASC9 regulates BC tumorigenesis by modulating the SIX1/NF- $\kappa$ B signaling pathway via miR-590-3p. Thus, CASC9 may represent an attractive therapeutic target for the prevention and treatment of BC.

## Highlights

- (1) CASC9 was up-regulated in BC.
- (2) CASC9 promoted proliferation, migration, invasion and inhibited apoptosis of BC cells.
- (3) CASC9 regulated SIX1 by sponging miR-590-3p.
- (4) CASC9/miR-590-3p/SIX1 regulated BC through NF- $\kappa$ B signaling.

## Disclosure statement

No conflicts of interest was reported by the author(s).

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## Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

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