

## Original Article

# SiRNA targeting LATS2 promotes proliferation and invasion in breast cancer cells by regulating the Hippo pathway

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**Abstract:** Large tumor suppressor kinase 2 (LATS2) is an AGC kinase of the NDR family of kinases. It is a tumor suppressor of the LATS family, and plays a significant role in centrosome duplication, maintenance of mitotic fidelity, and genomic stability. It has been investigated for possible tumor suppressing functions. However, it is unclear whether LATS2 is involved in breast tumor cells growth. **Methods:** In order to estimate the effects of small interfering RNA (siRNA) targeting LATS2 on the proliferation, expression, invasion, migration and tumorigenicity abilities of breast cancer cells, siRNA targeting LATS2 (LATS2-siRNA) and negative control siRNA were transfected into MDA-MB-231 and MCF-7 cells. The mRNA levels of LATS2 in the transfected cells were estimated by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), and the protein levels of LATS2 and its downstream gene YAP (Yes-associated protein) in these cells were evaluated by western blot analysis. The growth, migration and invasion abilities of the transfected cells were measured by MTT, colony formation, wound healing and transwell chamber assay, respectively. Flow cytometry was also used to detect the roles of endogenous LATS2 in breast cancer cells. **Results:** LATS2 mRNA expression was reduced after transfection with LATS2-siRNA, and protein expression had a similar trend, while the expression of YAP was upregulated. Transfection of LATS2-siRNA promotes breast cancer cells proliferation and migration, inhibits cellular apoptosis, meanwhile less cells were arrested at G0/G1 phase. **Conclusions:** The transfection of LATS2-siRNA into breast cancer MDA-MB-231 and MCF-7 cells suppressed the expression of LATS2 in these cells, and promoted their proliferation, invasion, migration and disrupted the cell cycle by regulating the Hippo pathway. Therefore, targeting LATS2 may be an efficient approach to control breast cancer.

**Keywords:** Breast cancer, LATS2, siRNA

## Introduction

Breast cancer is one of the most common female malignant tumors which is a leading cause of cancer mortality worldwide each year [1]. In the United States, breast cancer was expected to account for nearly 30% of all new cancer cases among women and it has been the number one cause of cancer death among women [2]. Traditional methods on breast cancer treatment are surgery, chemotherapy, radiotherapy and medicine for different subtypes [3]. The causes and pathogenesis of breast cancer are poorly understood, while genetic mutations have been demonstrated to be causative of the tumorigenesis in breast cancer. So it is essential to develop more effec-

tive methods for early diagnosis and treatment.

Small interfering RNA (siRNA) is a kind of small molecular RNA (21-25 nucleotides), it is processed by Dicer (RNAase III family specific for double stranded RNA enzyme) and causes the complementary target mRNA silencing directly [4]. siRNA has got more attention as a more specific and efficient approach to cancer therapy in the last few years. The mechanism of siRNA mediated cancer therapy is to downregulate the mutant cancer relevant transcripts in heterozygous cancer cell models [5].

LATS2 (large tumor suppressor kinase 2) is an AGC kinase of the NDR family of kinases. It is a

tumor suppressor of the LATS family, and plays a significant role in centrosome duplication, maintenance of mitotic fidelity, and genomic stability [6]. LATS2 inhibits cell growth at the G1/S transition by down-regulating cyclin E/CDK2 kinase activity [7]. As an upstream regulator in the Hippo pathway, LATS2 can regulate its downstream gene YAP (Yes-associated protein). Specifically, phosphorylated and activated LATS2 can phosphorylate transcription coactivators YAP, leading to the YAP cytoplasm retention by 14-3-3 protein or degradation [8, 9]. Even though LATS2 is considered to be a regulating factor that is important in controlling organ size and tumor progression by regulating cellular proliferation and promoting apoptosis [10], its role in breast cancer has not been well illustrated. To better explore the role of LATS2 in breast cancer, siRNA was transfected in breast cancer cell line MDA-MB-231 and MCF-7 to downregulate the expression of LATS2. LATS2 mRNA expression was reduced after transfection, and LATS2 protein expression had a similar trend. In addition, we verified the changes of cellular function after transfection of LATS2-siRNA. Our present study demonstrated that the knockdown of LATS2 has an acceleration effect on breast cancer cell proliferation and migration by regulating the Hippo pathway. Targeting LATS2 may be an efficient approach to treat breast cancer.

### Materials and methods

#### Cell lines

The human breast cancer cell lines MDA-MB-231, MCF-7, MDA-MB-436, HCC1937 and non-malignant breast epithelial cell line MCF-10A were acquired from Chinese Academy of Sciences in Shanghai. The breast cancer cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Enpromise, China). MCF-10A cells were cultured in Mammary Epithelial Basal Medium (MEBM) (Cambrex). All these cells were incubated at 37°C in a humidified chamber supplemented with 5% CO<sub>2</sub>.

#### Transfection assay

LATS2 siRNA and its NC were chemosynthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells (1×10<sup>6</sup>) were added into each well of a 6-well plate and cultured with DMEM medium without serum and antibiotics.

LATS2 siRNA and its NC were transfected at working concentrations using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions as the confluency of breast cancer cells reached 30-50%. After incubation for 4-5 h, DMEM medium was replaced by DMEM with 10% FBS, and all the cells were incubated at 37°C in a CO<sub>2</sub> incubator for specific time prior to further testing.

#### RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

According to the manufacturer's protocol, total RNA was extracted from the cells or tissues using TRIzol (Invitrogen, Carlsbad, CA, USA). For detection of LATS2 expression, the primers used were as follows: LATS2 forward, 5'-CTGGAATTCGAAGTGTGAGCAAGGTGATG-3', reverse, 5'-ACGACTAGTGACTTGAGTATGCCACTCAC-3'; β-actin forward, 5'-CATGTACGTGCTATCCAGGC-3', reverse, 5'-CTCCTTAATGTCACGCACGAT-3'. The PCR parameters for relative quantification were as follows: 2 min at 95°C, followed by 40 cycles of 45 sec at 57°C and 45 sec at 72°C. The relative expression was evaluated following the relative quantification equation, 2<sup>-ΔΔCt</sup>. Each sample was tested in triplicate.

#### Western blotting

Breast cancer cell lines, MDA-MB-231 and MCF-7 were divided into two groups: siRNA group, the negative control group. They were cultured and transfected with LATS2-siRNA as described above. Cells were washed twice with PBS. Then, total proteins were extracted from cell cultures using RIPA buffer (Beyotime, Shanghai, China). Then the cells were collected and centrifuged for 30 min at 4°C (Eppendorf 5804R, Eppendorf Biotech, Germany). Supernatants were collected and the protein amounts were measured using the BCA method. Protein samples were denatured with 5× SDS loading buffer (Beyotime) at 100°C for 10 min. Total protein was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime) and transferred to a 0.45-µm nitrocellulose membrane (Beyotime). The membrane was incubated at 4°C with primary antibodies against LATS2 (1:1,000; Bioworld Technology, China), YAP (1:1,000; Bioworld Technology, China) and β-actin (1:1,000; Bioworld Technology, China). After washing with PBST (Shanghai Engineering co), the membranes were incubated with secondary antibodies for 60 min. At last, immune-

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reactive protein bands were detected with Odyssey Scanning system.

### *Cell proliferation assay (MTT assay)*

Cell proliferation was detected using an MTT assay kit (Sigma, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, the transfected cells ( $2 \times 10^3$  cells/well) were seeded into 96-well culture plates (BD Biosciences, Franklin Lakes, NJ, USA) and incubated overnight at 37°C in 5% CO<sub>2</sub>. Cell proliferation was assessed at 24, 48, 72 and 96 h following addition of 0.5 mg/ml MTT (Sigma) solution. After a 4-h incubation, the medium was replaced by 150 µl dimethylsulfoxide (DMSO; Sigma). After 10 min of agitation (100 rpm), the optical density (OD) at 490 nm was determined with microplate reader (BioTek). Each sample was tested with six replicates. All experiments were performed in biological triplicate.

### *Colony formation assay*

Five hundred cells of the MDA-MB-231/siRNA or NC and MCF-7/siRNA or NC cells were seeded in a 6-well plate in complete medium 4 h after transfection. The medium was replaced every 3 days. After 7-10 days, or when the colonies were visible, the culture was terminated and the plates were washed twice in phosphate buffered saline (PBS) after removing the complete medium. Then the colonies were fixed by 95% ethanol for 10 min, dried and stained with 0.1% crystal violet solution for 10 min. At last, each plate was washed for three times by water, and the number of colonies was counted only if the well contained > 50 cells. The experiment was performed three times.

### *Wound healing assay*

In the *in vitro* wound healing assay, cells were cultured in 6-well plates until the cell confluence reached about 90%. Then the plates were washed in PBS after making a scratch in each well using a sterile pipette tip. Wound healing was observed under a light microscope and images were captured at the same view at 0, 12, 24 and 48 h after scratching to observe the process of wound healing. The experiments were repeated twice and representative photographs are shown.

### *Transwell invasion assay*

A transwell invasion assay was performed by using CHENICON Cell Invasion Assay Kit 2767

(Chemicon, USA). Cells ( $5 \times 10^4$  cells/Transwell) were plated in the top chamber of Transwells with a Matrigel (2 mg/ml)-coated membrane containing 8 mm diameter pores in 200 µl serum-free DMEM. The lower chambers were filled with 500 µl of DMEM containing 10% FBS. After 48 h of incubation, the membrane was stained with 0.1% crystal violet and observed under a microscope after removing the Matrigel and cells in the upper chambers. Five fields were randomly selected from each membrane, and the number of cells penetrating the membrane was counted at a magnification of  $\times 200$ . The invasion ability was described as the number of invading cells. Each experiment was carried out in triplicate. Membrane-binding crystal violet was dissolved with 400 µl 33% glacial acetic acid, and then absorbance at 573 nm was measured using microplate reader.

### *Cell cycle assay*

24 hours after transfection, cells were trypsinized and centrifuged at 1000 rpm for 5 min, followed by two washes in cold PBS. Then cells were fixed in 70% ice-cold ethanol at 4°C for 24 h. A total of 250 µl 0.05 g/l propidium iodide (PI) staining solution was added into each sample and incubated for 30 min at room temperature, and cell cycle distribution was analyzed using flow cytometry (FACSCanto™ II, BD Biosciences).

### *Apoptosis assay*

Cells transfected with LATS2-siRNA and negative control were incubated in six-well plates for 24 h. Cells were subsequently stained with fluorescein (FITC)-conjugated Annexin V and propidium iodide (FITC-Annexin V/PI) (BD Biosciences, San Diego, CA, USA), the rate of apoptosis was detected on a flow cytometry (FACSCanto™ II, BD Biosciences).

### *Statistical analysis*

Data were presented as the means  $\pm$  standard deviation (SD) from at least three independent experiments. The Students t test was used to evaluate the differences between each group in SPSS 20.0 software. Differences were considered significant for *P*-values < 0.05.

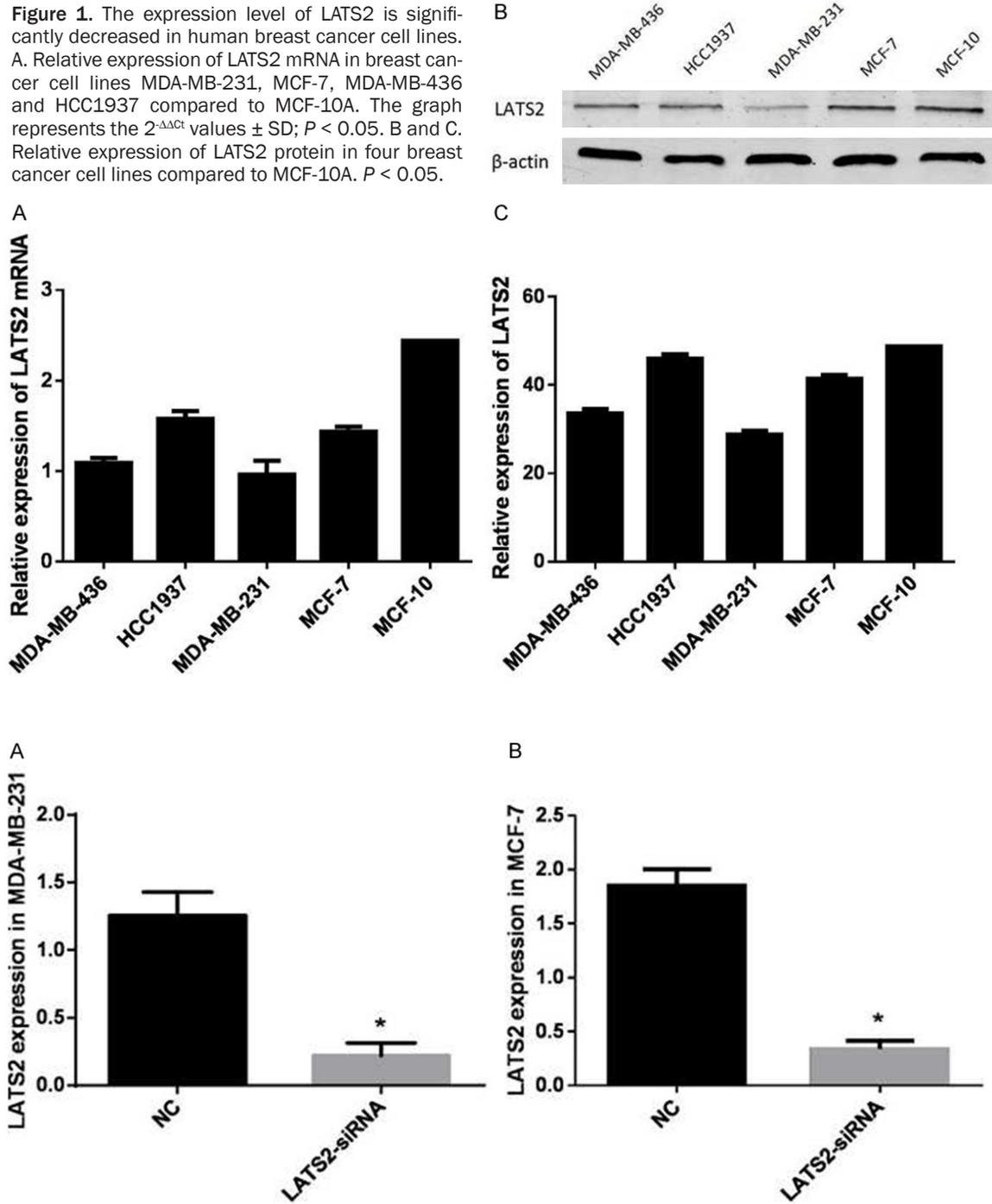
## **Results**

### *LATS2 is downregulated in human breast cancer cell lines*

We detected the LATS2 expression in human breast cancer cell lines MDA-MB-231, MCF-7,

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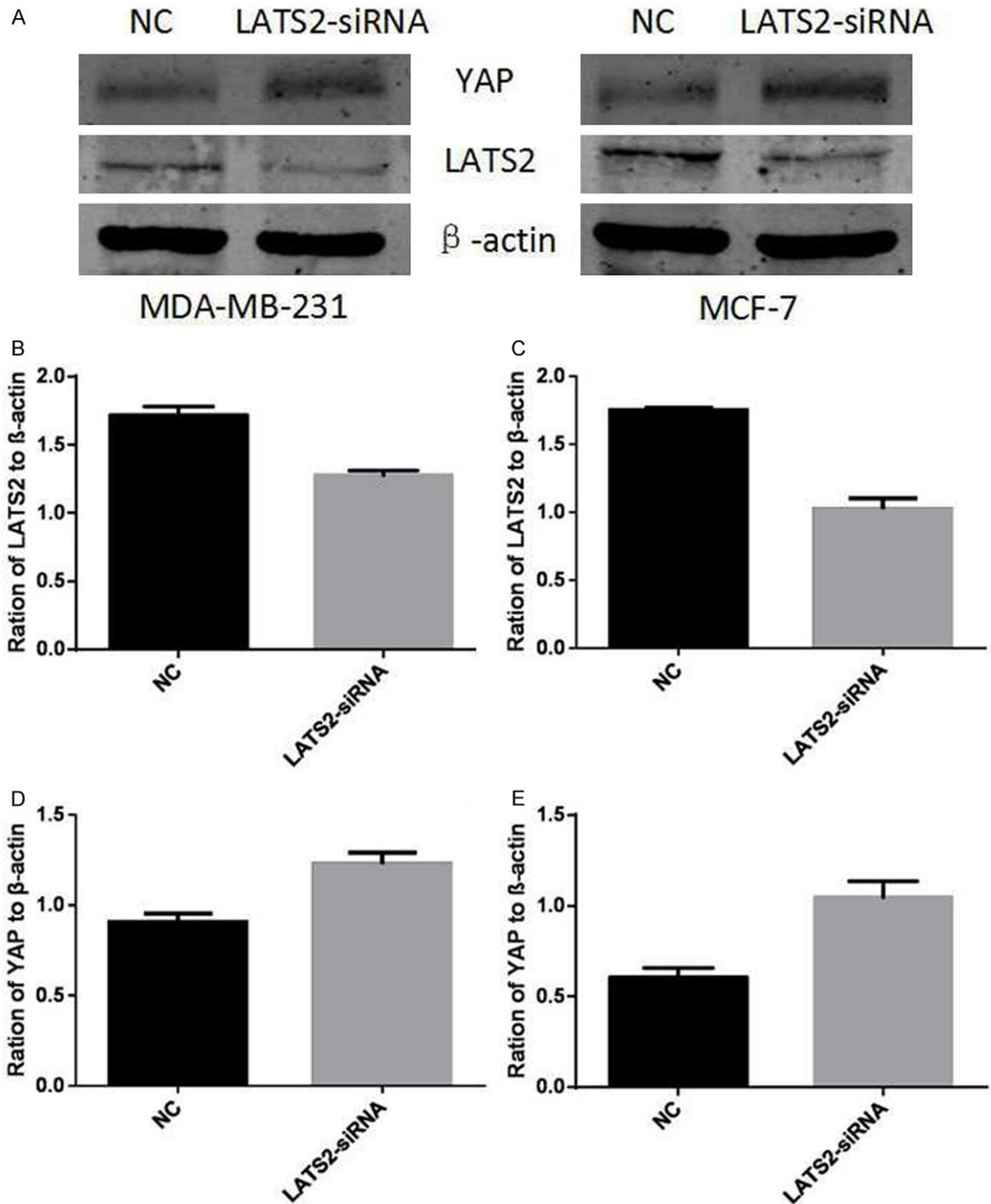
**Figure 1.** The expression level of LATS2 is significantly decreased in human breast cancer cell lines. A. Relative expression of LATS2 mRNA in breast cancer cell lines MDA-MB-231, MCF-7, MDA-MB-436 and HCC1937 compared to MCF-10A. The graph represents the  $2^{-\Delta\Delta Ct}$  values  $\pm$  SD;  $P < 0.05$ . B and C. Relative expression of LATS2 protein in four breast cancer cell lines compared to MCF-10A.  $P < 0.05$ .



**Figure 2.** The expression level of LATS2 mRNA is decreased after LATS2-siRNA transfection in both MDA-MB-231 and MCF-7 cells. A. LATS2 expression in MDA-MB-231 cells detected by qRT-PCR. B. LATS2 expression in MCF-7 cells detected by qRT-PCR. Data represents the  $2^{-\Delta\Delta Ct}$  values  $\pm$  SD;  $P < 0.05$ .

MDA-MB-436, HCC1937 and non-malignant breast epithelial cell line MCF-10A. The mRNA levels of LATS2 in these cells were estimated by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), and the protein levels were evaluated by western blot anal-

ysis. As shown in **Figure 1**, all breast cancer cell lines expressed lower levels of LATS2 compared with the levels in the non-malignant breast epithelial cell line MCF-10A. These results indicated that LATS2 is downregulated in breast cancer.

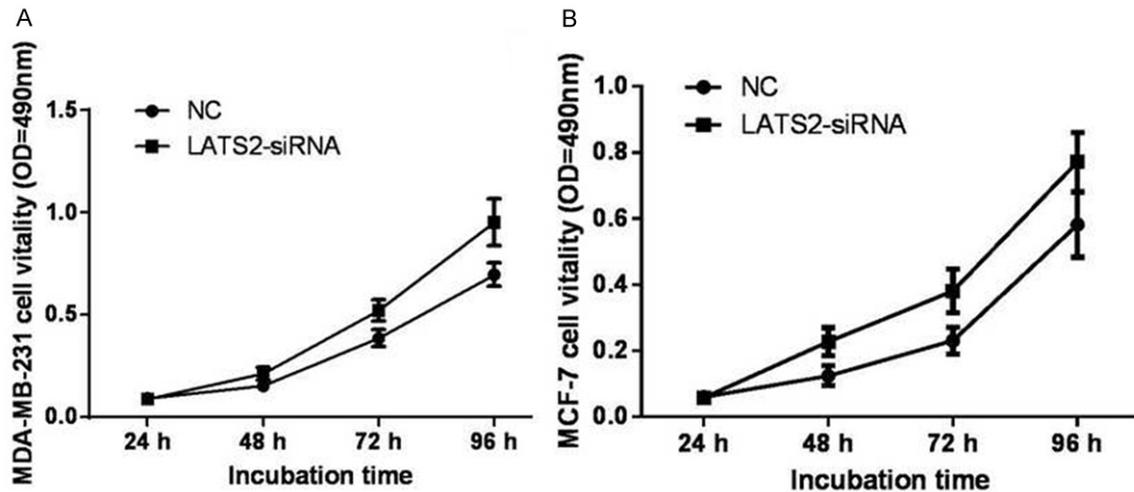


**Figure 3.** The LATS2 protein expression was obviously inhibited after LATS2-siRNA transfection in both MDA-MB-231 and MCF-7 cells while the YAP protein expression was upregulated. A. LATS2 and YAP protein level was detected by Western blot. B and C. Relative expression of LATS2 protein in MDA-MB-231 and MCF-7 cells. D and E. Relative expression of YAP protein in MDA-MB-231 and MCF-7 cells.  $P < 0.05$ .

*LATS2-siRNA transfection can inhibit the expression of LATS2 in breast cancer cells*

As two of the most representative breast cancer cell lines, MDA-MB-231 and MCF-7 cells

were selected in our following experiments. The LATS2-siRNA or negative control siRNA and the Lipofectamine 2000 were infected into MDA-MB-231 and MCF-7 cells. To explore the influence of siRNA on the endogenous expression



**Figure 4.** The cell viability was significantly improved in MDA-MB-231 and MCF-7 cell lines compared to the non-silencing control siRNA infected cell groups. A. MDA-MB-231 cell vitality detected by MTT. B. MCF-7 cell vitality detected by MTT.  $P < 0.05$ .

of LATS2. As shown in **Figure 2**, MDA-MB-231 and MCF-7 cells were successfully infected with the LATS2-siRNA verified by qRT-PCR. Furthermore, we use western blotting analysis to observe the gene expression levels of LATS2 and YAP following LATS2-siRNA infection. The LATS2 protein expression was obviously inhibited after LATS2-siRNA transfection in both MDA-MB-231 and MCF-7 cells while the YAP protein expression was upregulated (**Figure 3**,  $P < 0.05$ ). Therefore, we confirmed that the LATS2-siRNA is an effective method to reduce the abnormal expression level of LATS2 in breast cancer cells.

#### *LATS2-siRNA transfection promotes the proliferation of breast cancer cells in vitro*

To investigate the impact of LATS2-siRNA on the proliferation of breast cancer cells, we assessed cell proliferation at 24, 48, 72 and 96 h post-transfection using MTT assays. Inhibition rate was calculated as following: inhibition rate (%) = (OD value of the control group - OD value of experimental group) / OD value of control group  $\times 100\%$ . It could be clearly observed that the proliferation of both LATS2-siRNA infected in MDA-MB-231 and MCF-7 cells proliferation was time and dosage-dependent increased. The cell viability was significantly ( $P < 0.05$ ) improved in both cell lines compared to the non-silencing control siRNA infected cell groups (**Figure 4**). These results indicated that

LATS2 is closely related to the proliferation of breast cancer cells.

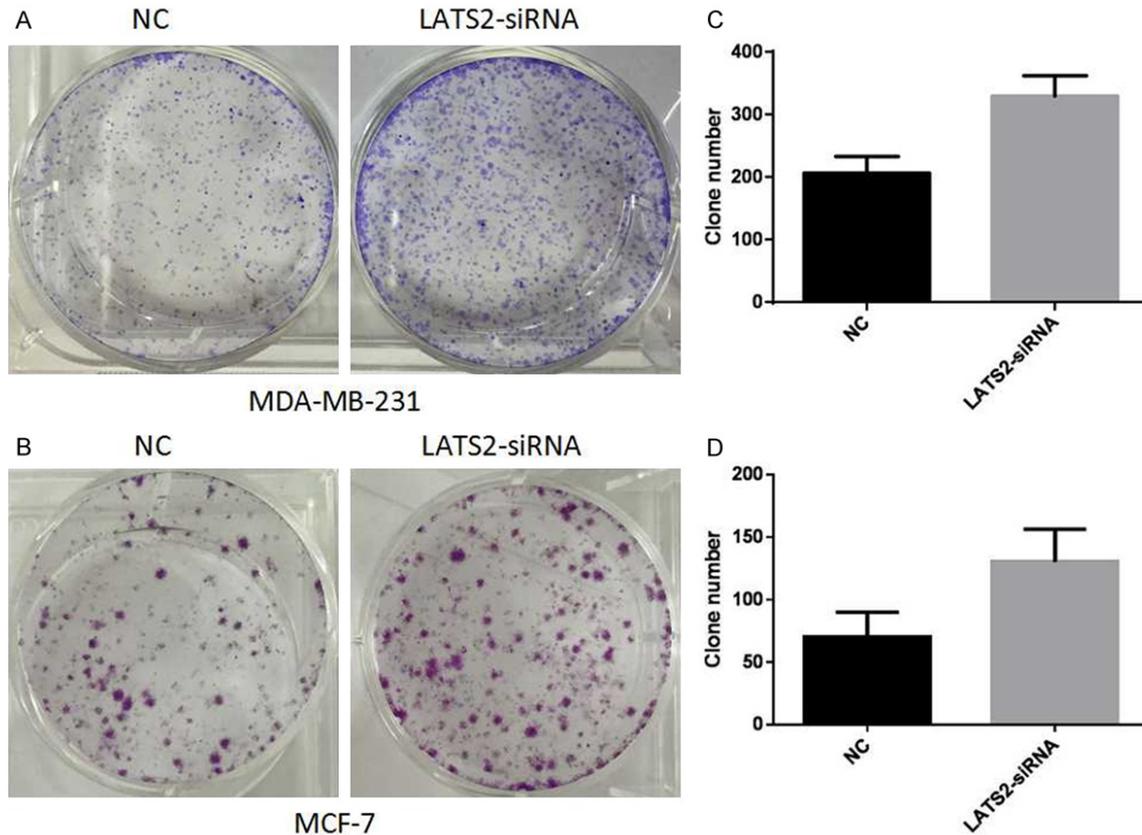
#### *Colony forming ability of breast cancer cells is promoted by the silencing of LATS2*

In order to detect the colony forming ability of breast cancer cells after silencing of LATS2, colony formation assays were conducted for three times in each breast cancer cell line. The size and the number of the colonies were measured in both LATS2-siRNA infected cell group and the non-silencing control siRNA infected group. As shown in **Figure 5**, the size and the number of the colonies in LATS2-siRNA group were significantly more obvious than that of non-silencing control group in MDA-MB-231 and MCF-7 cells ( $P < 0.05$ ). Our data indicated that silencing of LATS2 can promote colony forming ability of breast cancer cells.

#### *LATS2-siRNA transfection accelerates breast cancer cells migration and invasion in vitro*

To research how silencing of LATS2 affects cellular migration and invasion, wound healing assays and transwell assays were performed in MDA-MB-231 and MCF-7 cells. Cells were transfected with LATS2-siRNA or NC. As shown in **Figure 6**, 24 h after drawing the "scratch" line on the monolayer MDA-MB-231 cells, the LATS2-siRNA group nearly filled in the gap, the NC group still showed a clear gap in the

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**Figure 5.** Colony formation assays showed Crystal Violet staining of the LATS2-siRNA transfected group and NC-transfected group. A and B. Silencing of LATS2 promoted colony formation ability of MDA-MB-231 and MCF-7 breast cancer cells. C and D. Clone number of MDA-MB-231 and MCF-7 cells.  $P < 0.05$ .

scratched region ( $P < 0.05$ ). The experiments conducted in MCF-7 cells also showed the similar trend. The results indicate that silencing of LATS2 in breast cancer cells accelerated cellular migration. The transwell invasion assay revealed that the number of MDA-MB-231 and MCF-7 cells penetrating the membrane significantly increased at 48 h after LATS2-siRNA transfection as compared to the NC group (**Figure 7**). Together these results showed that silencing of LATS2 by LATS2-siRNA can accelerate cellular migration and invasion in vitro.

### *LATS2-siRNA transfection regulates the cell cycle of breast cancer cells*

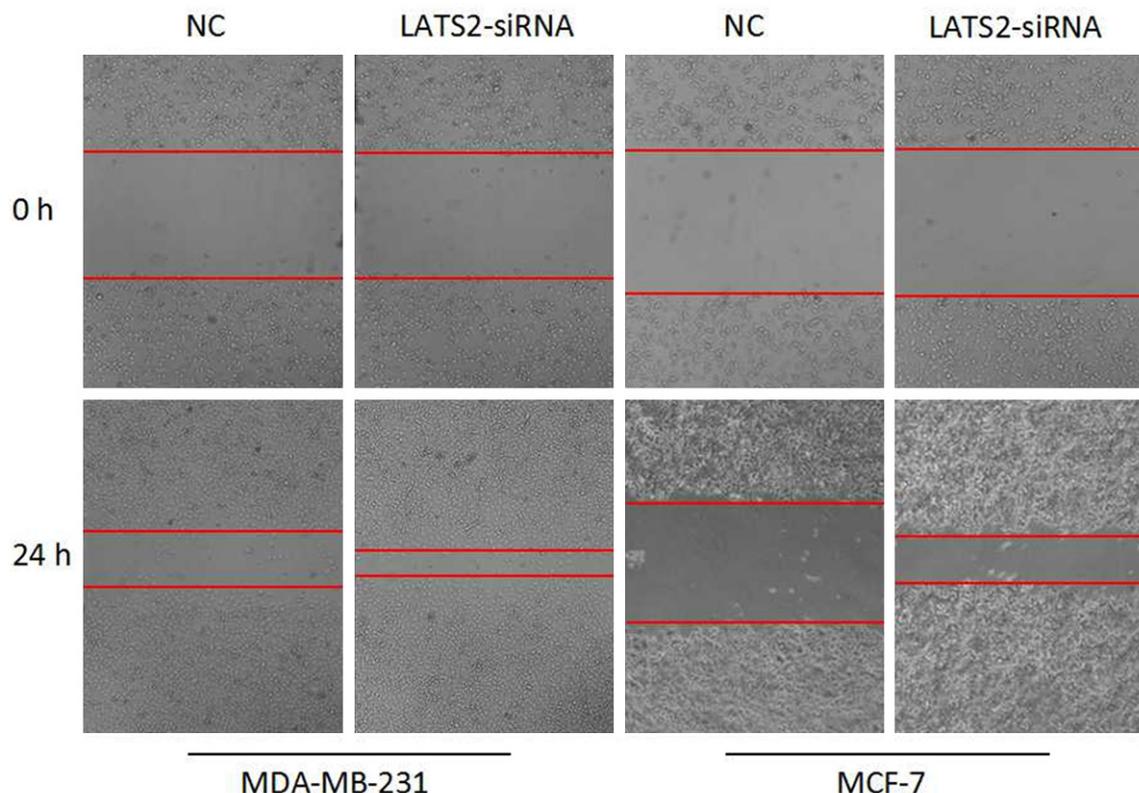
Twenty-four hours after the transfection of LATS2-siRNA or NC in MDA-MB-231 cells, flow cytometry analysis indicated that the percentage of G0/G1 phase cells ( $45.90 \pm 0.53\%$ ) dramatically decreased in the LATS2-siRNA group, when compared with that of the NC group ( $51.45 \pm 0.46\%$ ) ( $P < 0.05$ ). At the same time,

the proportion of S-phase cells increased in the LATS2-siRNA group ( $30.12 \pm 0.37\%$ ) compared with that of the NC group ( $27.97 \pm 0.23\%$ ) ( $P < 0.05$ ). The percentage of G2/M phase cells also elevated in the LATS2-siRNA group ( $23.98 \pm 0.26\%$ ) compared with that of the NC group ( $20.58 \pm 0.21\%$ ) ( $P < 0.05$ ). The experiments carrying out in MCF-7 cells also showed the similar trend (**Figure 8**). These findings revealed that silencing of LATS2 by LATS2-siRNA can lead to the upregulation of S-phase and G2/M phase cells.

### *LATS2-siRNA transfection inhibits the apoptosis of breast cancer cells*

To examine whether knockdown of LATS2 effects the apoptosis of MDA-MB-231 and MCF-7 cells, cells were transfected with 100 nmol/l of LATS2-siRNA for 24 h. Flow cytometry revealed a lower percentage of early and late apoptotic breast cancer cells in the LATS2-siRNA group (MDA-MB-231:  $1.9 \pm 0.05\%$ ; MCF-7:

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**Figure 6.** Cell scratch assay was used to detect the migration ability of MDA-MB-231 and MCF-7 cells. Silencing of LATS2 in breast cancer cells accelerated cellular migration *in vitro*.  $P < 0.05$ .

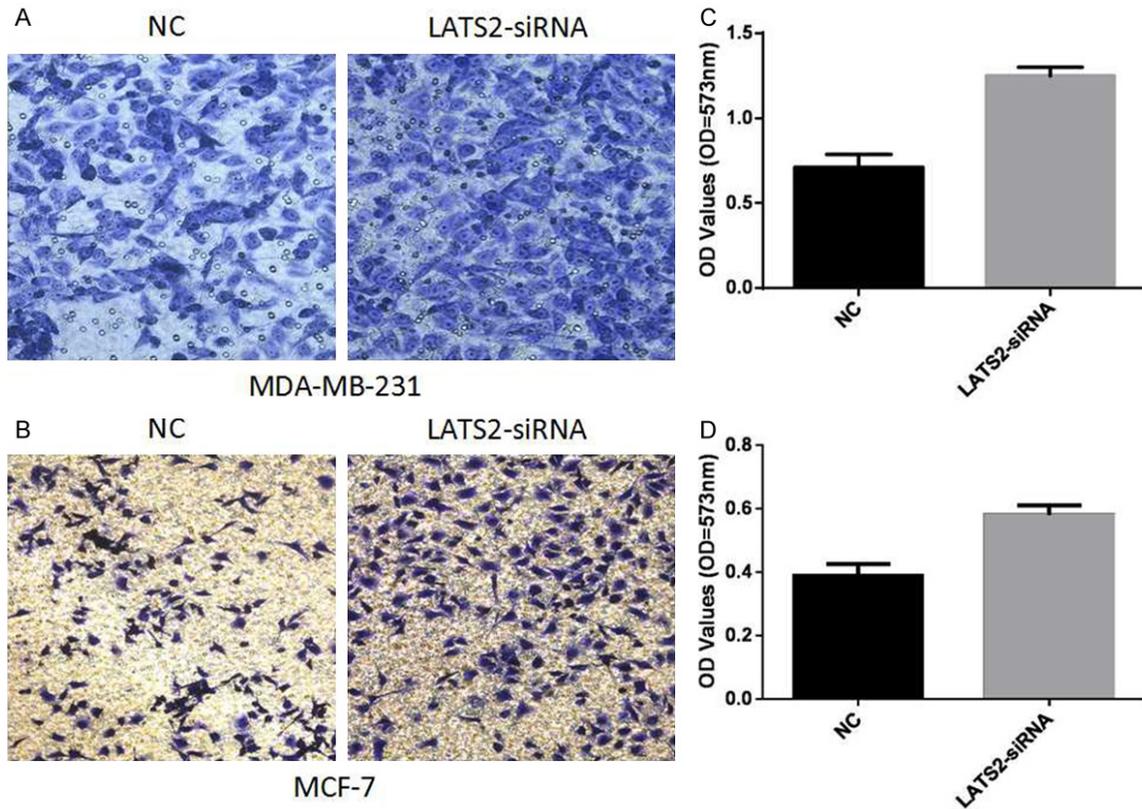
1.3±0.06%) compared with the NC group (MDA-MB-231: 4.4±0.09%; MCF-7: 2.9±0.08%) ( $P < 0.05$ ; **Figure 9**). These data indicated that downregulation of LATS2 expression induced apoptosis.

### Discussion

Genetic mutations and epigenetic modifications have been demonstrated to be involved in the tumorigenesis and development of cancer [11]. The selective targeting of cancer cells would reduce the adverse side effects of conventional chemotherapies by inhibiting tumor growth and invasion while sparing the surrounding normal cells. Therefore, it is critical to search therapeutic molecules that specially target cancer cells [12]. In these decades, RNA-based gene therapy has considered as a potential approach to target various human diseases effectively and safely [13]. Our study aimed to identification of an antineoplastic target in breast cancer cells and investigation of the effects of silencing the respective gene on breast cancer cell function. Our experiments demonstrated that siRNA mediated silencing of

LATS2 gene has a significant influence on the survival of breast cancer cells. The proliferation rate, colony forming ability, migration and invasion ability was promoted while the apoptosis rate was induced by the absence of LATS2. It is clear that LATS2 has played a significant role in the proliferation and progression of breast cancer cells.

LATS2, a member of the LATS tumor suppressor family, is located in human chromosome 13q11-12 [14]. Loss of heterozygosity at the LATS2 locus is demonstrated in ovarian cancer initially [15]. Recent studies have verified that LATS2 regulates diverse cellular processes, such as proliferation, angiogenesis, apoptosis, migration and invasion [16, 17]. One paper has reported that LATS2 plays a significant role in the regulation of cell cycle on post-transcriptional level, and promotes the development and progression of colorectal cancer [18]. LATS2 also plays a crucial role in mediating the Hippo signaling pathway and deregulation of this signaling pathway has been found in several human cancers [19-22]. YAP, the most critical effector of the Hippo pathway, is regulated



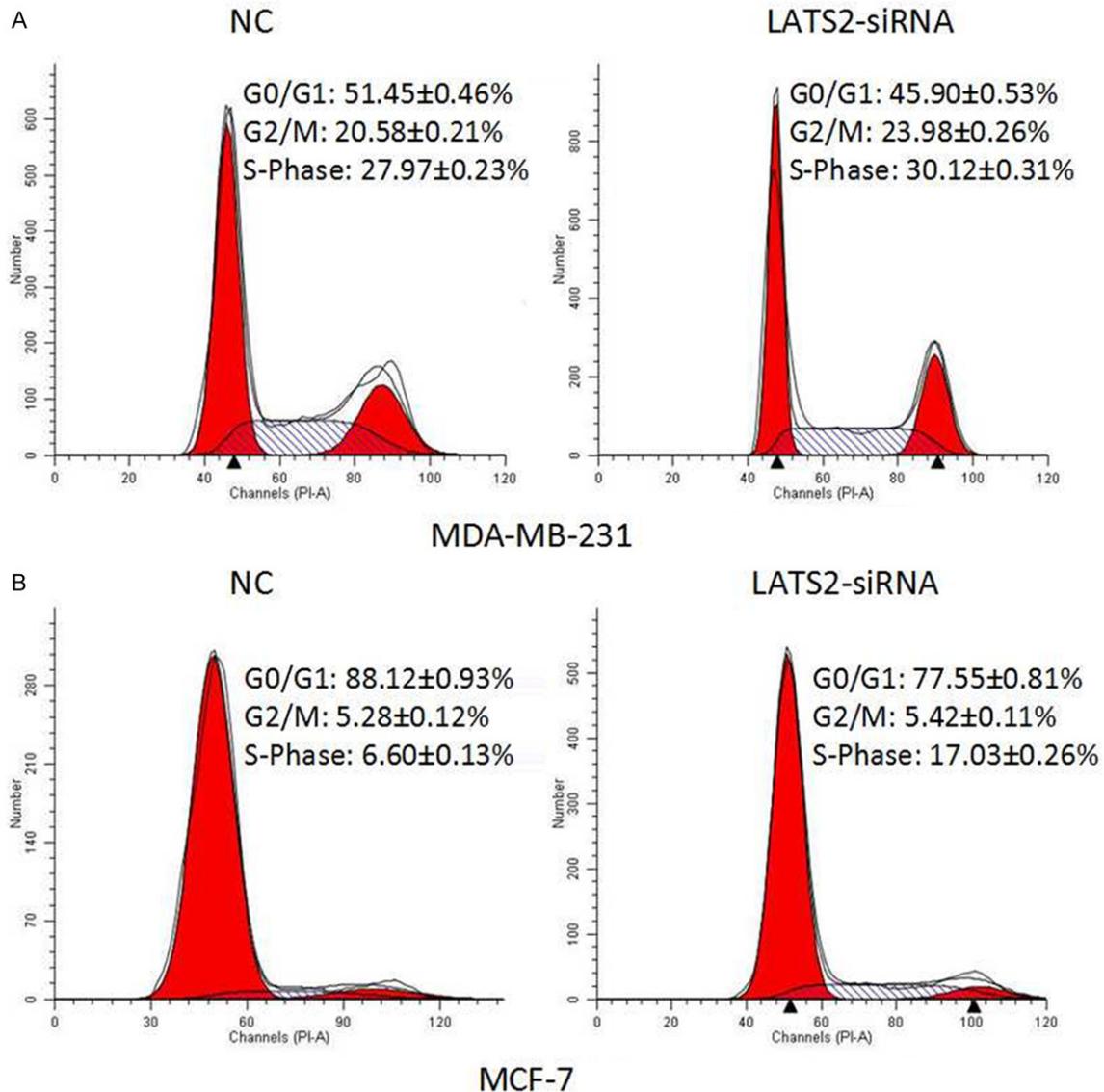
**Figure 7.** Transwell invasion assay was performed to evaluate the invasion ability of MDA-MB-231 and MCF-7 cells. A and B. Representative pictures of invasion on an inverted microscope with ×200 magnification. C and D. Invasion rates were determined by solubilization of crystal violet and spectrophotometric reading at OD 573 nm. Data represent means ± SD;  $P < 0.05$ .

by LATS2. Specifically, phosphorylated and activated LATS2 can phosphorylate transcription coactivator YAP at S127 and S89, leading to the YAP cytoplasm retention by 14-3-3 or degradation [23]. As effective inhibitors of gene expression like siRNA, several miRNAs have been found to inhibit LATS2 expression in different types of cancer cells. For example, miR-181b regulates ovarian cancer cell growth and invasion by targeting LATS2 [24]. MiR-93 can promote tumor angiogenesis and metastasis by suppressing LATS2 in human breast cancer cells [25]. MiR-372 disrupts cell cycle in gastric cancer cells through direct regulation of LATS2 [26]. It is reported that knockdown of endogenous LATS2 by LATS2-siRNA promotes cell proliferation and inhibits cell apoptosis in human colorectal cancer cells [18]. But the specific role of LATS2 and the Hippo pathway in breast cancer has not been well described.

To investigate the effects of the activity of LATS2 on breast cancer, we conducted a series of experiments. Firstly, qRT-PCR and western

blot analysis was used to detect the expression of LATS2 in human breast cancer cell lines MDA-MB-231, MCF-7, MDA-MB-436, HCC1937 and non-malignant breast epithelial cell line MCF-10A. Our results indicated that LATS2 expression was markedly lower at the mRNA and protein level in four collected breast cancer cell lines, and the expression level in MDA-MB-231 was the lowest compared to other cell types. As two of the most representative breast cancer cell lines, MDA-MB-231 and MCF-7 cells were selected in our following experiments. In our present study, the effects of LATS2-siRNA on the biological behavior of breast cancer cells, including cell proliferation, colony formation abilities, migration and invasion abilities, were investigated *in vitro*. Our results indicated that LATS2-siRNA can significantly reduce the mRNA and protein levels of LATS2 in MDA-MB-231 and MCF-7 cells. As a downstream gene in the Hippo pathway, YAP protein expression was upregulated. The transfection of LATS2-siRNA promoted the proliferation, migration and invasion abilities of the transfected

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**Figure 8.** LATS2 affected cell cycle distribution. A and B. Cell cycle distribution was analyzed by flow cytometry 24 h after transfection. Silencing of LATS2 by LATS2-siRNA can lead to the upregulation of S-phase and G2/M phase cells. Data represent means  $\pm$  SD;  $P < 0.05$ .

cells. Cell cycle and apoptosis assays were also conducted in the transfected cells. The results showed that silencing of LATS2 by LATS2-siRNA can lead to the upregulation of S-phase and G2/M phase cells. Meanwhile, the apoptosis rate was markedly decreased after LATS2-siRNA transfection. Hence it is clear that LATS2 has played a critical role in the proliferation and progression of breast cancer cells, and that this effect was mediated, at least partly, by regulating the Hippo pathway.

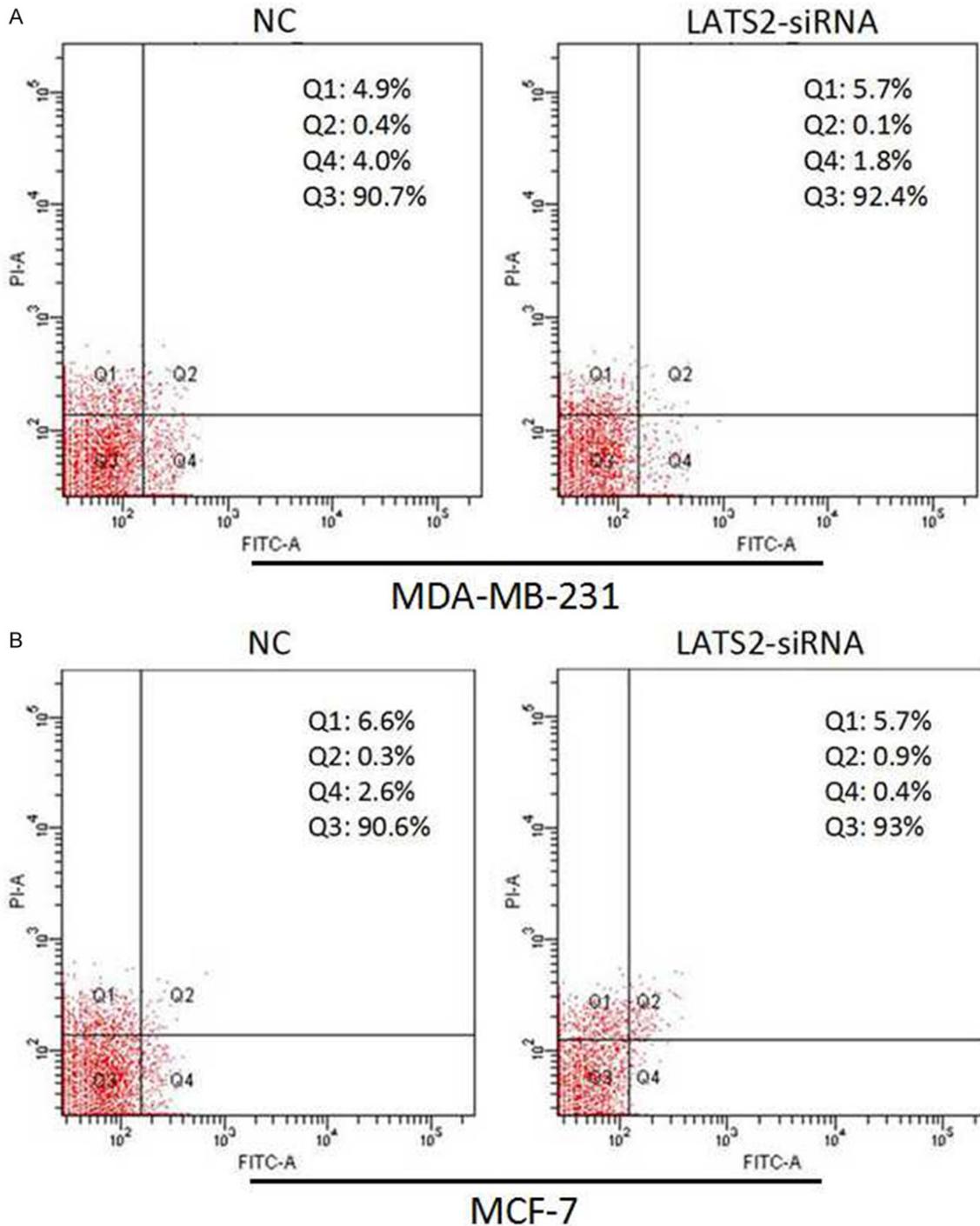
In summary, our results showed that LATS2 was frequently downregulated in breast cancer

cell lines. This study identifies LATS2 as a critical gene in the breast cancer cell survival and growth, suggesting that LATS2 may be a potential new target for the treatment of breast cancer.

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**Figure 9.** Effects of LATS2 in breast cancer cell apoptosis. A and B. Inhibition of LATS2 promoted the ability of cells to undergo apoptosis.  $P < 0.05$ .

### Disclosure of conflict of interest

None.

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