

## miR-181a-5p Promotes Proliferation and Invasion and Inhibits Apoptosis of Cervical Cancer Cells via Regulating Inositol Polyphosphate-5-Phosphatase A (INPP5A)

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Expression of miR-181a-5p associates with the proliferation and progression of cancer cells via its targets. This study was designed to investigate the effect of miR-181a-5p and its target inositol polyphosphate-5-phosphatase A (INPP5A) on the progression of cervical cancers. Upregulation of miR-181a-5p was revealed in the cervical cancer cell lines HeLa and SiHa in comparison with a normal cervical epithelium cell line End1/E6E7 ( $p < 0.001$ ). The inhibition and upregulation of miR-181a-5p in cervical cancer cell lines significantly reduced or increased cell proliferation and invasion capacity, accompanied with enhanced or reduced apoptosis ( $p < 0.05$ ). Moreover, INPP5A overexpression significantly inhibited cell proliferation and invasion capacity and enhanced cell apoptosis. The target relationship of miR-181a-5p to INPP5A was demonstrated by both the results of the Dual-Luciferase Reporter Assay and the fact that the miR-181a-5p mimic attenuated INPP5A's effect on cell proliferation, invasion, and apoptosis. To sum up, the overexpression of miR-181a-5p enhanced cell proliferation and invasion and inhibited apoptosis of cervical cancer cells by negatively targeting INPP5A. Therefore, inhibition of miR-181a-5p might benefit the inhibition of cervical cancer cell invasion.

**Key words:** Cervical cancer; miR-181a-5p; Proliferation; Inositol polyphosphate-5-phosphatase A (INPP5A)

### INTRODUCTION

Cervical cancer is a common female reproductive tract cancer with increasing occurrence and mortality<sup>1</sup>. Radiotherapy and surgery are still the major treatments for cervical cancer, although with relative high metastasis or recurrence because of the malignancy.

The dysregulation of microRNAs (miRNAs), gene-specific regulators, is common in cancers, including miR-181a-5p<sup>1</sup> and miR-196a<sup>2</sup> in cervical cancer, and miR-181a-5p<sup>3</sup> and miR-21<sup>4</sup> in breast cancer. The dysregulation of miR-181a-5p in various cancers has previously been reported. For example, miR-181a-5p was downregulated in hepatocellular carcinoma<sup>5</sup>, aggressive human breast cancer<sup>6</sup>, and lung cancer<sup>7</sup> and upregulated in gastric cancer<sup>8</sup>.

The expression of miR-181a-5p has been shown to control the proliferation and migration of hepatocellular carcinoma<sup>5</sup>, breast cancer<sup>6</sup>, and gastric cancers<sup>8,9</sup> via regulating the expression of its targets such as matrix metalloproteinase-14 (MMP14), c-Met, Ras association domain family member 6 (RASSF6), and their mediated signaling

pathways, including mitogen-activated protein kinase (MAPK), phosphate and tensin homolog/Akt/Forkhead box O1 (PTEN/Akt/FOXO1), vascular endothelial growth factor (VEGF) pathway, and wingless-related integration site (Wnt)/ $\beta$ -catenin signaling. In addition, the relative higher expression of miR-181a in cervical cancers<sup>10</sup>, and its promotion in cell metastasis of breast cancer<sup>11</sup> had been reported to be associated with its target or transcript factors.

The inositol polyphosphate-5-phosphatase A (INPP5A), encoding a 40-kDa membrane-associated type I protein inositol phosphatase, functions primarily as a signal-terminating enzyme associated with cell proliferation<sup>12</sup>. The loss of INPP5A had been reported to be linked to the development and progression of cancers and frequently described in head and neck mucosal squamous cell carcinoma<sup>12-14</sup>. However, there have been very few reports focusing on the effect of INPP5A in cervical cancers.

The present study aimed to investigate the effect of miR-181a-5p and its predicated target gene INPP5A on

the cell proliferation, invasion, and apoptosis of cervical cancer cells. Cervical cancer cell lines transfected with the miR-181a-5p mimic, inhibitor, and scramble sequences, as well as INPP5A expression vectors, were prepared for the exploration of cell functions. This study will provide us with direct information on the effect of miR-181a-5p and INPP5A expression on cervical cancer cells.

## MATERIALS AND METHODS

### *Cell Lines and Cell Culture Conditions*

Cervical cancer cell lines HeLa and SiHa (ATCC, Manassas, VA, USA), and End1/E6E7, a human normal cervical epithelium cell line (ATCC), were obtained and employed for the cellular experiments in this study. End1/E6E7 cells were maintained in keratinocyte serum-free medium (K-SFM; Gibco, New York, NY, USA) containing 0.1 ng/ml epithelial growth factor (EGF; Gibco), 0.05 mg/ml bovine pituitary extract (Gibco), and 1% streptomycin–penicillin (SP; Invitrogen, Carlsbad, CA, USA) as previously reported<sup>1</sup>. HeLa and SiHa cell lines were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS) and 1% SP at 37°C in 5% CO<sub>2</sub>.

### *Cell Transfection*

For inhibition and expression of miR-181a-5p, we transfected cervical cancer cell lines with the miR-181a-5p inhibitors, mimics, and scrambled oligonucleotide sequences (GenePharma, Shanghai, P.R. China) using Lipofectamine 2000 transfection reagent (Invitrogen). Cells at 40%–60% confluence were transferred to antibiotic-free medium and incubated for 24 h, followed by transfection with the miR-181a-5p inhibitors, mimics, and scrambled oligonucleotide sequences using Lipofectamine 2000 reagent. For the expression of INPP5A, we constructed the pCDNA3.1-INPP5A expression plasmids and transfected cervical cancer cell lines. Cells transfected with the empty vector pCDNA3.1 were considered as control.

### *Cell Proliferation Assay*

The proliferation analysis for HeLa, SiHa, and End1/E6E7 was performed using the bromodeoxyuridine (BrdU) cell proliferation assay (Millipore, Billerica, MA, USA)<sup>15</sup>. In brief, cells at a density of  $1 \times 10^4$  cells/well in 96-well plates were cultured for 48 h under the conditions described above for cell adhesion and confluence. Then the BrdU reagent (10 ng/ml) was added into the cell medium with a 12-h additional incubation time, and then the absorbance values at 450-nm wavelength were measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI).

### *In Vitro Invasion Assay*

The invasion of transfected cells was detected using 24-well Transwell chambers (Costar; Corning Incorporated, Corning, NY, USA)<sup>1</sup>. For cell invasion assay, Transwells were coated with 20  $\mu$ l of 1 mg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and  $2 \times 10^5$  transfected cells (24 h after transfection) were seeded into the upper chamber medium without FBS. The lower chamber medium was supplemented with 10% FBS and incubated for 48 h at 37°C under the above-described conditions. Forty-eight hours later, cells on the upper surface filters were removed, while the cells that adhered to the lower surface filters were fixed and prepared for crystal violet staining. The stained chambers were observed using a microscope.

### *Apoptotic Cell Analysis*

The percentage of apoptotic cells was determined with a flow cytometer using the Clontech Annexin V Apoptosis Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA)<sup>15</sup>. After transfection for 48 h, the cells were harvested, pelleted, and resuspended with 100  $\mu$ l of annexin-binding buffer consisting of annexin V and propidium iodide working solution for 15 min in the dark. Apoptotic cells were then analyzed immediately by subjecting them to a BD fluorescence-activated cell sorter (FACS) Calibur flow cytometer (BD Biosciences).

### *Dual-Luciferase Reporter Assay*

The target regions of miR-181a and INPP5A were predicted using TargetScan 7.1 (<http://www.targetscan.org>). Luciferase vectors (pmirGLO Dual-Luciferase miRNA Target Expression Vector; Promega, Madison, WI, USA) with wild-type and mutant INPP5A 3'-untranslated region (3'-UTR) target clones were cotransfected into the HeLa cells together with the miR-181a mimics and scrambled control for 48 h. The Luciferase Reporter Assay System (Promega) was used to analyze the relative *Renilla* luciferase activity.

### *Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)*

For qRT-PCR analysis of miR-181a and INPP5A mRNA, cellular total RNA was extracted from transfected cells (TRIzol lysis; Invitrogen), and the first-strand cDNA was then synthesized using a Moloney murine leukemia virus (M-MLV) Reverse Transcriptase Kit (Invitrogen). Primers for miR-181a and INPP5A mRNA detections were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, P.R. China). Expression levels of miR-181a and INPP5A mRNA were determined using a 7500 Fast System Real-Time PCR cycler (Applied Biosystems, ABI, Foster City, CA, USA) with a SYBR Green PCR Master Mix (Applied Biosystems). Glyceraldehyde

3-phosphate dehydrogenase (GAPDH) and U6 short nuclear RNA (shRNA) were used as the internal reference genes, and the relative expression levels of miR-181a and INPP5A mRNA were calculated using the  $2^{-\Delta\Delta Ct}$  method.

#### Western Blot Analysis

Cellular proteins were extracted from transfected cells, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Solarbio, Beijing, P.R. China), and were then electrotransferred onto to polyvinylidene fluoride (PVDF) membranes (Invitrogen). Membranes were then blocked with 5% skimmed milk for 1 h, probed with the specific primary antibodies against B-cell lymphoma 2 [Bcl-2; 1:1,000 dilution; Cell Signaling Technology (CST), Danvers, MA, USA], Bcl-2-associated X protein (Bax; 1:1,000 dilution; CST), INPP5A (1:2,000 dilution; CST), and GAPDH (1:2,000 dilution; Abcam PLC, Cambridge, UK) overnight at 4°C, and followed by additional incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Finally, the protein bands in the membranes were amplified by being subjected to the enhanced chemiluminescence (ECL) detection system (Beyotime, Shanghai, P.R. China), and the gray intensity of the Western blots was analyzed using a Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

#### Statistical Analysis

The data presented in this study were expressed as the mean  $\pm$  standard deviation (SD). All experiments

were conducted independently three times. The significant analysis was performed by GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Statistical differences between two groups were analyzed by *t*-test, and differences among more than two groups were analyzed using one-way ANOVA. Differences with a value of  $p < 0.05$  were considered as statistically significant.

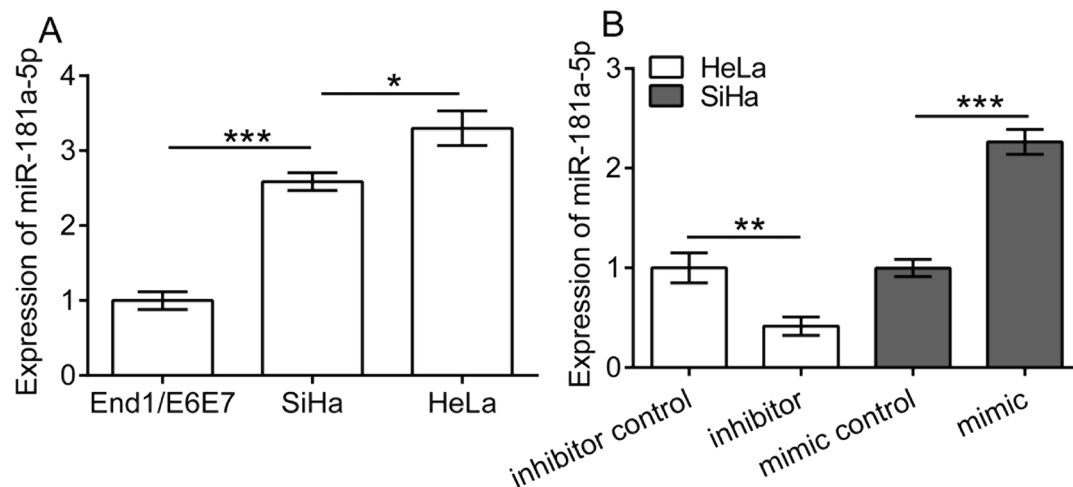
## RESULTS

### miR-181a-5p Decreases in Cervical Cancer Cells

To investigate the expression of miR-181a-5p in cervical cancer cells, we detected the expression of miR-181a-5p in the two cervical cancer cell lines HeLa and SiHa and the human normal cervical epithelium cell line End1/E6E7 using qRT-PCR. The results showed that the expression of miR-181a-5p was significantly upregulated in HeLa and SiHa cells compared to End1/E6E7 ( $p < 0.01$ ) (Fig. 1A). Moreover, a relatively higher expression of miR-181a-5p was observed in HeLa cells in comparison with SiHa cells ( $p < 0.05$ ). Accordingly, we transfected SiHa cells with the miR-181a-5p mimic and scramble sequences (mimic control), and HeLa cells with the miR-181a-5p inhibitor and scramble sequences (inhibitor control), respectively. The results showed that mimic and inhibitor transfection significantly upregulated and downregulated the expression of miR-181a-5p in SiHa cells ( $p < 0.001$ ) and HeLa cells ( $p < 0.01$ ), respectively (Fig. 1B).

### miR-181a-5p Promotes Proliferation and Invasion of Cervical Cancer Cells

Using the BrdU assay, we detected the proliferation of transfected cells and found that the miR-181a-5p inhibitor



**Figure 1.** Expression of miR-181a-5p in cervical cancer cells. (A) The expression of miR-181a-5p in cervical cancer cell line HeLa and SiHa and in the normal cervical epithelial cell line End1/E6E7. (B) HeLa cells were transfected with the miR-181a-5p inhibitor and scramble sequences (inhibitor control), and SiHa cells were transfected with the miR-181a-5p mimic and scramble sequences (mimic control). \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

transfection into HeLa cells significantly decreased the proliferation of HeLa cells, and the miR-181a-5p mimic transfection into SiHa cells significantly increased the proliferation of SiHa cells compared with control ( $p < 0.05$ ) (Fig. 2A). Moreover, the cell invasion assay showed that the miR-181a-5p inhibitor transfection decreased the invasion ability of HeLa cells, and the miR-181a-5p mimic transfection significantly increased the invasion ability of SiHa cells, compared with the control, respectively ( $p < 0.01$ ) (Fig. 2B). These results demonstrated that the miR-181a-5p inhibitor inhibited the cell proliferation and invasion of HeLa cells, and the miR-181a-5p mimic promoted the cell proliferation and invasion of SiHa cells, revealing that miR-181a-5p promoted the cell proliferation and invasion of cervical cancer cells.

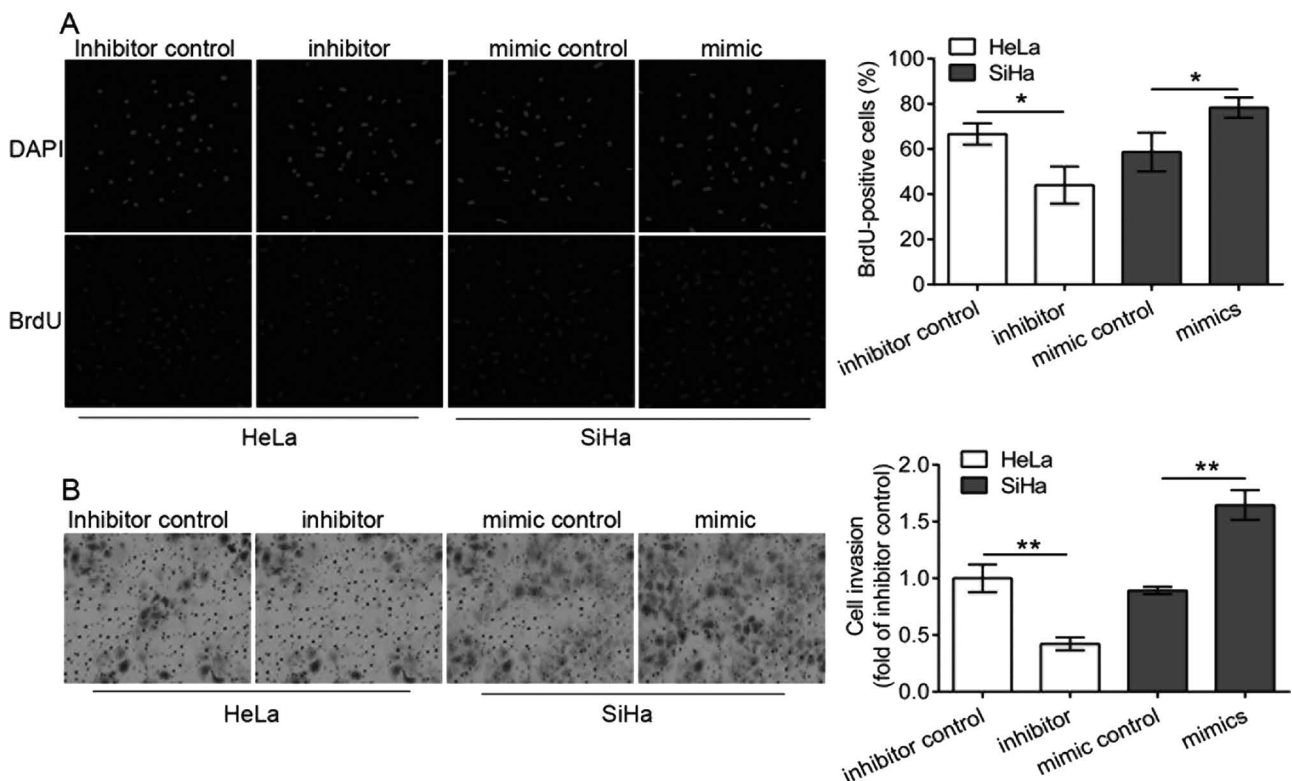
#### miR-181a-5p Inhibits Apoptosis of Cervical Cancer Cells

Using a flow cytometer, we detected the apoptotic percentage of transfected cells. We demonstrated that the miR-181a-5p inhibitor transfection significantly enhanced the apoptotic percentage of HeLa cells ( $p < 0.01$ ) (Fig. 3A),

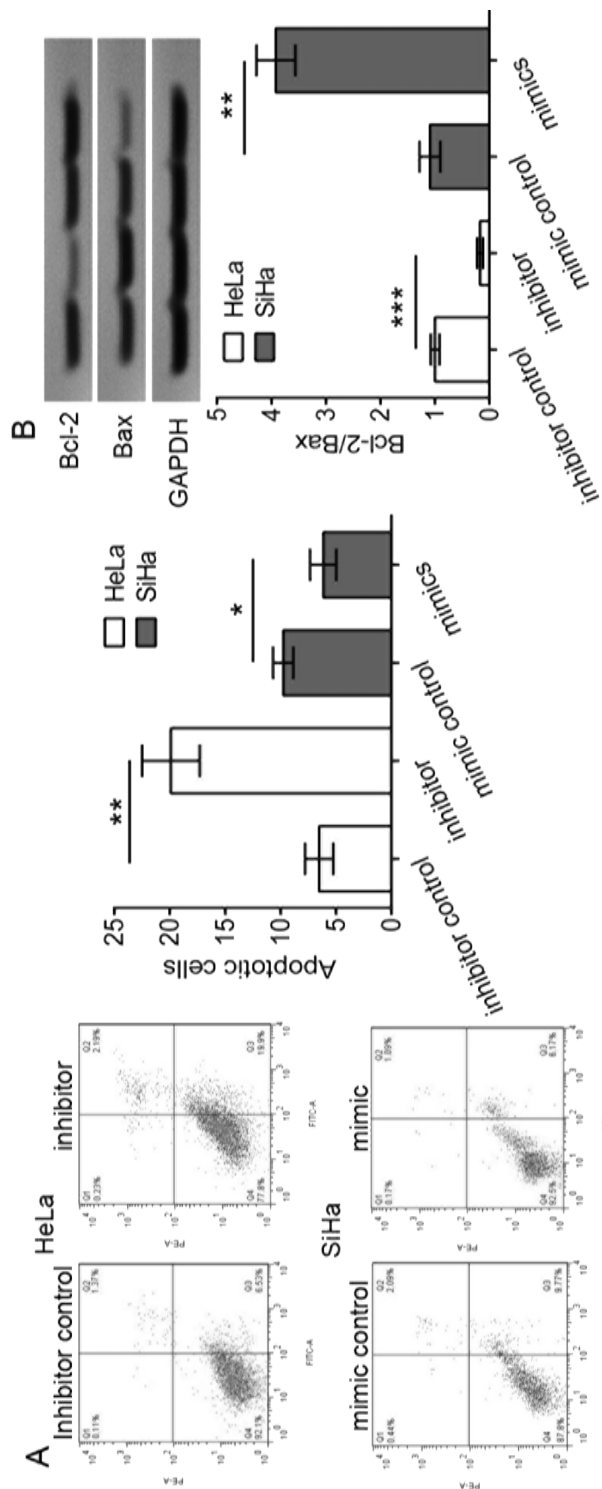
and the miR-181a-5p mimic transfection significantly decreased the apoptotic percentage of SiHa cells compared with control ( $p < 0.05$ ), respectively. In addition, the expression of cell apoptosis-related proteins Bcl-2 and Bax was significantly inhibited and enhanced in the miR-181a-5p inhibitor-transfected HeLa cells, respectively, thus a significant reduction in the Bcl-2/Bax ratio ( $p < 0.001$ ) (Fig. 3B). In contrast, the expression of Bcl-2 and Bax was significantly enhanced and inhibited in the miR-181a-5p mimic-transfected SiHa cells, respectively, thus a significant increase in the Bcl-2/Bax ratio ( $p < 0.01$ ) (Fig. 3B). These results suggested that the miR-181a-5p inhibitor promoted the cell apoptosis of HeLa cells, and the miR-181a-5p mimic inhibited the cell apoptosis of SiHa cells, demonstrating that miR-181a-5p inhibited cell apoptosis of cervical cancer cells.

#### miR-181a-5p Suppresses INPP5A Expression in Cervical Cancer Cells

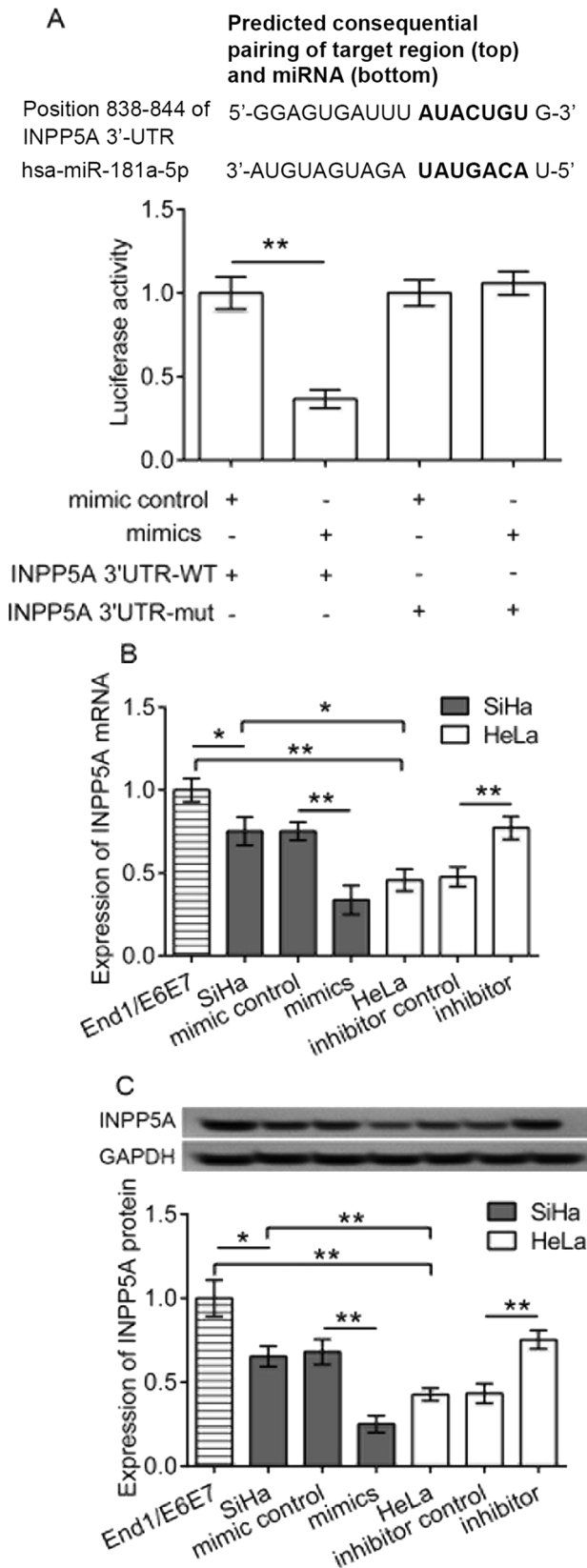
Using the TargetScanHuman software, we predicted that INPP5A was a target of miR-181a-5p. We then validated the target relationship of miR-181a-5p to INPP5A



**Figure 2.** Proliferation and invasion of transfected cervical cancer cells. Cell proliferation ability was determined using the bromodeoxyuridine (BrdU) assay (A), and the cell invasion ability assay was detected using the 24-well Transwell chambers. Nuclei are counterstained with 4,6-diamidimino-2-phenylindole (DAPI). (B) Cell numbers at five randomly selected fields were averaged. HeLa cells were transfected with the miR-181a-5p inhibitor and scramble sequences (inhibitor control), and SiHa cells were transfected with the miR-181a-5p mimic and scramble sequences (mimic control), respectively. \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 3.** Apoptosis of transfected cervical cancer cells. HeLa cells were transfected with the miR-181a-5p inhibitor and scramble sequences (inhibitor control), and SiHa cells were transfected with the miR-181a-5p mimic and scramble sequences (mimic control), respectively. (A) The cell apoptosis percentage was detected using a flow cytometer, and apoptotic cells that were annexin V<sup>+</sup> and propidium iodide<sup>-</sup> (PI<sup>-</sup>) were counted, and the percentage was calculated. (B) Cell apoptosis-related proteins including B-cell lymphoma 2 (Bcl-2) and BCL-2-associated X protein (Bax) were detected using Western blot analysis. \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.



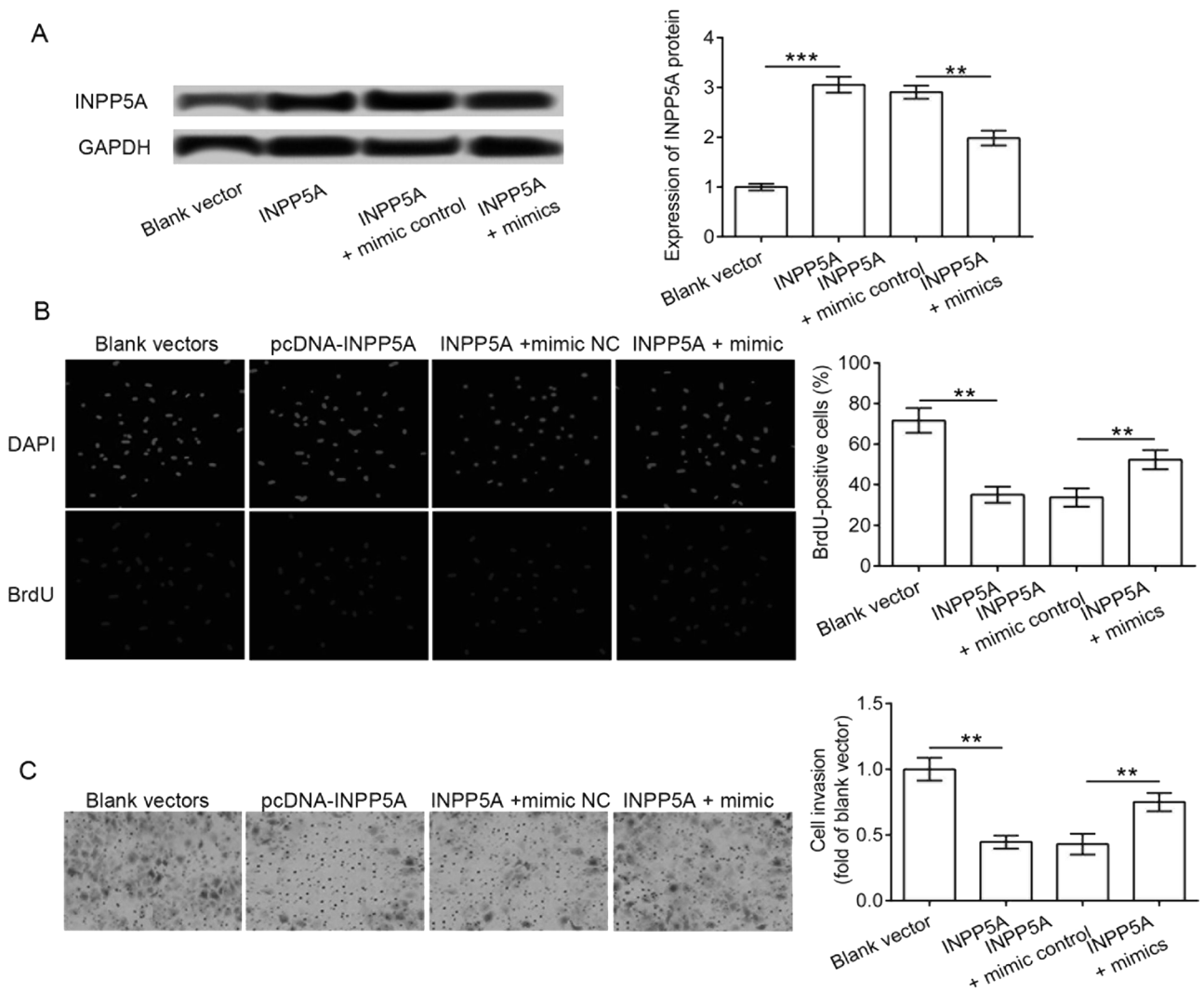
using the Dual-Luciferase Reporter Assay in HeLa cells. We found that HeLa cells cotransfected with the miR-181a-5p mimic, and INPP5A-3'UTR-WT luciferase vectors showed a significant reduction in *Renilla* luciferase activity in comparison with those of the cells transfected with mimic control or INPP5A-3'UTR-mut luciferase vectors ( $p < 0.05$ ) (Fig. 4A), demonstrating the direct targeting relationship of miR-181a-5p to INPP5A. Accordingly, we detected the expression of INPP5A mRNA and protein in transfected cells, and the results revealed that INPP5A expression was significantly downregulated in the two kinds of cervical cell lines (SiHa and HeLa) compared to that in the control of normal End1/E6E7 cells ( $p < 0.05$ , SiHa vs. control;  $p < 0.05$ , HeLa vs. control) (Fig. 4B and C). However, when cells were transfected with the miR-181a-5p mimic, the expression of INPP5A in the two kinds of cervical cell lines were significantly decreased compared with cells transfected with the mimic control (for SiHa cells,  $p < 0.01$ , mimics vs. mimic control; for HeLa cells,  $p < 0.01$ , mimics vs. mimic control) (Fig. 4B and C). Also, the same regulatory relationship was also observed in the inhibitor-transfected groups. These data suggested a negative relationship between miR-181a-5p and INPP5A expression. Taken together, the data demonstrated that miR-181a-5p negatively regulated the expression of INPP5A in HeLa and SiHa cells.

#### miR-181a-5p Promotes Proliferation and Invasion of SiHa Cells via INPP5A

In order to investigate whether the effect of miR-181a-5p on cervical cancer cells was mediated by INPP5A, we transfected the SiHa cells with both miR-181a-5p mimics and pcDNA3.1-INPP5A vectors. The results showed that the enhanced expression of INPP5A protein in SiHa cells was significantly reduced by the miR-181a-5p mimic addition ( $p < 0.001$ ) (Fig. 5A). Moreover, pcDNA3.1-INPP5A administration significantly reduced the cell proliferation and invasion abilities of SiHa cells compared to the control ( $p < 0.01$ ) (Fig. 5B and C). In contrast, the addition of miR-181a-5p significantly attenuated the effect of pcDNA3.1-INPP5A vectors on cell proliferation and invasion. Cells cotransfected with

#### FACING COLUMN

**Figure 4.** Validation of inositol polyphosphate-5-phosphatase A (INPP5A) as a target of miR-181a-5p. (A) The Dual-Luciferase Reporter Assay in HeLa cells after transfection of mimics and/or INPP5A wild-type (WT) or mutant (mut) 3'-untranslated region (3'-UTR). The mRNA (B) and protein (C) expression of INPP5A in transfected cervical cancer cell lines HeLa and SiHa and normal cervical epithelial End1/E6E7 cells as determined by polymerase chain reaction (PCR) and Western blotting, respectively. \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 5.** Effect of INPP5A and miR-181a-5p on cell proliferation and invasion. SiHa cells were cotransfected with both miR-181a-5p mimics and pcDNA3.1-INPP5A vectors. (A) Expression of INPP5A protein in transfected cells as determined by Western blotting. (B) Cell proliferation assay using BrdU incorporation. (C) Cell invasion analysis using Transwell cultures. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

pcDNA3.1-INPP5A vectors and miR-181a-5p showed a significantly higher proliferation and invasion abilities in comparison with the mimic control ( $p < 0.01$ ). Taken together, we concluded that the miR-181a-5p mimic promoted the proliferation and invasion abilities of SiHa cells partially by modulating INPP5A.

#### *miR-181a-5p Inhibits Apoptosis of SiHa Cells via INPP5A*

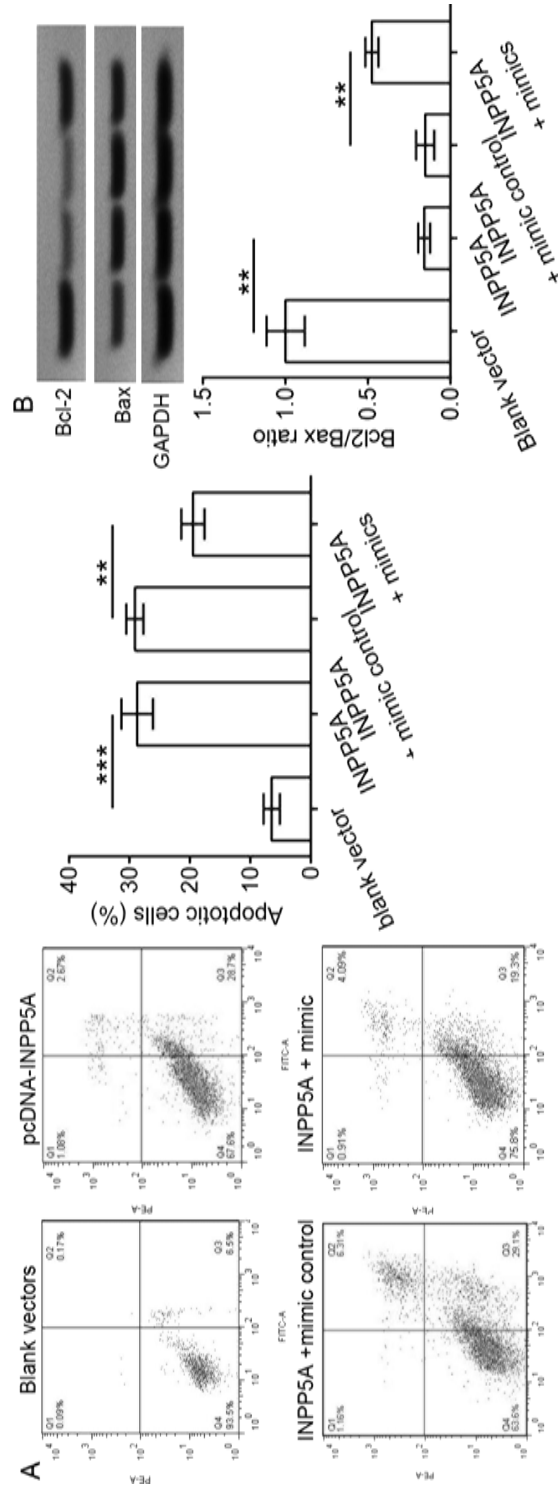
The apoptosis analysis results showed that the pcDNA3.1-INPP5A administration to SiHa cells significantly enhanced cell apoptotic percentage compared to control ( $p < 0.001$ ) (Fig. 6A). In contrast, the addition of miR-181a-5p significantly attenuated the effect of pcDNA3.1-INPP5A vectors on cell apoptosis ( $p < 0.01$ )

(Fig. 6B). We confirmed that the miR-181a-5p mimic inhibited cell apoptosis partially by targeting INPP5A.

## DISCUSSION

We demonstrated that the expression of miR-181a-5p was downregulated in cervical cancer cells, and miR-181a-5p mimic transfection promoted proliferation and invasion and inhibited cell apoptosis of the cervical cancer SiHa and HeLa cells. Moreover, the miR-181a-5p effect on cell function was mediated by a target INPP5A.

Both the down- or upregulation of miR-181a-5p in various cancers and its effect on cell proliferation and migration have been reported<sup>5-8</sup>. In this study, we demonstrated that the expression of miR-181a-5p was significantly upregulated in both cervical cancer cell lines SiHa



**Figure 6.** Effect of INPP5A and miR-181a-5p on cell apoptosis. SiHa cells were cotransfected with both miR-181a-5p mimics and pcDNA3.1-INPP5A vectors. (A) Cell apoptotic percentage depicted by flow cytometry and descriptively in transfected SiHa cells. (B) Cell apoptosis-related proteins including Bcl-2 and Bax were detected using Western blot analysis. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



and HeLa compared to the normal control cervical epithelium cell line End1/E6E7. Moreover, the transfection of the miR-181a-5p inhibitor or mimic into cervical cancer cells prominently reduced or enhanced cell proliferation and invasion, respectively, accompanied with enhanced or reduced cell apoptosis. We confirmed that the expression of miR-181a-5p appeared to contribute to the progression of cervical cancers.

BCL2 family members encode antiapoptotic proteins including Bcl-2 or proapoptotic proteins including Bax, which play essential roles in cell death, apoptosis, migration, and invasion<sup>16-19</sup>. The Bcl-2/Bax ratio is considered as a predictive sign for cell apoptosis<sup>20,21</sup>. The decreasing Bcl-2/Bax ratio or increasing Bcl-2/Bax ratio promotes or inhibits cell apoptosis, respectively<sup>21</sup>. Previous reports showed that a decreasing Bcl-2/Bax ratio was associated with upregulation of caspase 3<sup>22-24</sup>. In accordance with the previous report<sup>1</sup>, we confirmed that the downregulation of miR-181a-5p in cervical cancer cells resulted in the decrease in Bcl-2/Bax ratio and the increase in cell apoptotic percentage in this study.

Further studies demonstrated that the expression of INPP5A was regulated by the administration of the miR-181a-5p mimic or inhibitor and suggested INPP5A was a target of miR-181a-5p. Previous studies have shown that miR-181a-5p modulates cancer cell proliferation and migration via targeting its targets including Kirsten RAS (Kras)<sup>7</sup> and c-Met<sup>5</sup>. In the present study, we demonstrated that INPP5A was a direct target of miR-181a-5p, via which miR-181a-5p promoted cell proliferation and invasion of cervical cancer cells.

The loss of INPP5A had been frequently reported in head and neck mucosal squamous cell carcinoma<sup>12-14</sup>. INPP5A is reported to be a negative regulator of inositol signaling<sup>13</sup>. In addition, the expression of INPP5A has been described as being associated with the differentiation increase and proliferative decrease in normal human keratinocytes<sup>12</sup>. In this study, we showed that the expression of INPP5A was associated with the proliferation, invasion, and apoptosis of cervical cancer cells. Interestingly, the expression of INPP5A or downregulation of miR-181a-5p in cervical cancer cells increased the Bcl-2/Bax ratio and decreased the cell apoptotic percentage. This demonstrated that INPP5A as well as miR-181a-5p might take crucial roles in the proliferation, invasion, and apoptosis of cervical cancer cells.

In summary, we revealed that the overexpression of miR-181a-5p took crucial roles in enhancing cell proliferation and invasion and in inhibiting apoptosis of cervical cancer cells by negatively targeting INPP5A. Inhibition of miR-181a-5p, or overexpression of INPP5A, decreased cell proliferation and invasion and promoted the apoptosis of cervical cancer cells. These results demonstrated that miR-181a-5p or INPP5A might be explored as a therapeutic strategy for cervical cancer treatment.

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