

# MicroRNA-296 Targets Specificity Protein 1 to Suppress Cell Proliferation and Invasion in Cervical Cancer

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Cervical cancer is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths in women worldwide. MicroRNA-296 (miR-296) is aberrantly expressed in a variety of human cancer types. However, the expression levels, biological roles, and underlying molecular mechanisms of miR-296 in cervical cancer remain unclear. This study aimed to detect miR-296 expression in cervical cancer and evaluate its roles and underlying mechanisms in cervical cancer. This study demonstrated that miR-296 was significantly downregulated in cervical cancer tissues and cell lines. Restoring the expression of miR-296 inhibited the proliferation and invasion of cervical cancer cells. Moreover, miR-296 directly targeted the 3'-untranslated regions of specificity protein 1 (SP1) and decreased its endogenous expression at both the mRNA and protein levels. Similar to induced miR-296 expression, SP1 knockdown suppressed the proliferation and invasion of cervical cancer cells. Besides, resumption expression of SP1 rescued the tumor-suppressing roles of miR-296 in cervical cancer. These results indicated that miR-296 may act as a tumor suppressor in cervical cancer by directly targeting SP1. Therefore, SP1 may be developed as a therapeutic target for the treatment of patients with this malignancy.

**Key words: MicroRNA-296; Cervical cancer; Proliferation; Invasion; Specificity protein 1 (SP1)**

## INTRODUCTION

Cervical cancer is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths in women worldwide<sup>1</sup>. Approximately 530,000 new cases and 270,000 deaths due to cervical cancer are recorded worldwide annually<sup>1</sup>. Despite the remarkable advances in conventional treatments, including surgical resection, radiotherapy, and chemotherapy, the clinical outcome of patients with cervical cancer remains poor<sup>2</sup>. The overall 5-year survival rate for cervical cancer, particularly for patients at advanced stages, is less than 40%<sup>3</sup>. Therefore, the molecular mechanisms involved in cervical carcinogenesis and progression need to be elucidated, and novel therapeutic strategy for patients with this disease must be developed.

MicroRNAs (miRNAs) are a group of endogenous, single-stranded, noncoding and short RNA molecules that are 19–24 nucleotides in length<sup>4</sup>. They mainly function as negative regulators of gene expression through direct interaction with the 3'-untranslated regions (3'-UTRs) of their target genes in a base-pairing manner, resulting in mRNA destabilization and protein downregulation<sup>5</sup>. A certain

miRNA may regulate the expression of numerous target genes simultaneously; hence, miRNAs play important roles in various physiological and pathological processes, including cell proliferation, apoptosis, migration, invasion, metastasis, differentiation, and metabolism<sup>6</sup>. miRNAs are aberrantly expressed in various tumor types, such as cervical cancer<sup>7</sup>, gastric cancer<sup>8</sup>, colorectal cancer<sup>9</sup>, and bladder cancer<sup>10</sup>. miRNAs can act as either oncogenes by negatively regulating tumor suppressors or as tumor suppressors by inhibiting the expression of oncogenes<sup>11</sup>.

miR-296 is aberrantly expressed in a variety of human cancer types<sup>12–14</sup>. However, the expression levels, biological roles, and underlying molecular mechanisms of miR-296 in cervical cancer remain unclear. This study was aimed to detect miR-296 expression in cervical cancer and evaluate its roles and underlying mechanisms in cervical cancer.

## MATERIALS AND METHODS

### *Tumor Specimens*

This study was approved by the Ethics Committee of The Second Hospital of Jilin University. In addition,

written informed consent was obtained from all patients who participated in this research. A total of 31 paired cervical cancer tissues and matched adjacent normal cervical epithelial tissues were obtained from patients who were treated with surgical resection in The Second Hospital of Jilin University. None of the patients received radiotherapy or chemotherapy before surgery. All tissues were immediately frozen in liquid nitrogen and stored in  $-80^{\circ}\text{C}$  prior to RNA extraction.

#### *Cell Culture and Transfection*

Three cervical cancer cell lines (HeLa, SiHa, and Ca-Ski) and a human normal cervical epithelial cell line (Ect1/E6E7) were acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cell lines were cultured in a humidified atmosphere at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ .

miR-296 mimics, miRNA mimic negative control (miR-NC), small interfering RNA (siRNA) targeting specificity protein 1 (SP1) (SP1 siRNA), and negative control siRNA (NC siRNA) were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). SP1-overexpressing plasmid (pcDNA3.1-SP1) and empty plasmid (pcDNA3.1) were acquired from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cell transfection was carried out using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

#### *Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)*

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was conducted with PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) in accordance with the manufacturer's protocol. Afterward, the qPCR was performed using a SYBR Premix Ex Taq™ kit (Takara Biotechnology Co., Ltd.). Reverse transcription and qPCR were carried out on the Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). U6 snRNA and GAPDH were used as internal control for miR-296 and SP1 mRNA, respectively. Data were analyzed using the  $2^{-\Delta\Delta\text{Ct}}$  method.

#### *Cell Counting Kit 8 (CCK-8) Assay*

The CCK-8 assay was utilized to assess cell proliferation in vitro. At 24 h posttransfection, cells were collected and plated into 96-well plates at a density of 2,000 cells per well. After incubation at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 0, 24, 48, and 72 h, the CCK-8 assay was performed. In brief,

10  $\mu\text{l}$  of CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added and incubated at  $37^{\circ}\text{C}$  for another 2 h. Finally, the absorbance was detected at a wavelength of 450 nm using a microplate reader. The experiments were done in triplicate and repeated three times.

#### *Cell Invasion Assay*

Transwell chambers (pore size, 8  $\mu\text{m}$ ; Costar; Corning Incorporated, Corning, NY, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used to evaluate cell invasive capacity in vitro. Transfected cells were collected at 48 h posttransfection, suspended in FBS-free DMEM medium, and seeded into the upper chamber with a density of  $1 \times 10^5$  cells/chamber. Subsequently, 500  $\mu\text{l}$  of DMEM containing 20% FBS was added into the lower chamber. After incubating at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 24 h, cells that did not pass through the membrane were removed using a cotton swab. The invasive cells were fixed in 100% methanol, stained with 0.1% crystal violet, and washed in PBS. Cells adhering to the bottom surface of the membrane were counted in five separate fields per membrane under an IX51 inverted microscope (Olympus Corporation, Tokyo, Japan). Each assay was repeated at least three times.

#### *Bioinformatics Analysis and Luciferase Reporter Assay*

TargetScan (version 7.1; <http://www.targetscan.org>) and microRNA.org (<http://www.microrna.org>) were used to predict the potential targets of miR-296.

Luciferase reporter plasmids, pmirGLO-SP1-3'-UTR wild type (Wt) and pmirGLO-SP1-3'-UTR mutant (Mut), were constructed by Shanghai GenePharma Co., Ltd. Cells were plated into 24-well plates at a density of 50%–60% confluence. After incubation overnight, miR-296 mimics or miR-NC was introduced into cells, together with pmirGLO-SP1-3'-UTR Wt or pmirGLO-SP1-3'-UTR Mut using Lipofectamine™ 2000 reagent. Transfected cells were harvested after transfection for 48 h. The luciferase activities were detected using the dual-luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The experiments were conducted in triplicate and repeated three times.

#### *Western Blotting Analysis*

Total protein of tissues or cells was isolated using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The concentration of total protein was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Equal amounts of protein were separated by 10% sodium

dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% nonfat dry milk in TBST, the membranes were incubated with primary antibodies against SP1 (sc-420) and GAPDH (sc-69778; both Santa Cruz Biotechnology, CA, USA) at 4°C overnight. Afterward, the membranes were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. GAPDH was used as a loading control.

#### Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis with Student's *t*-tests or one-way ANOVA. A value of  $p < 0.05$  was considered statistically significant.

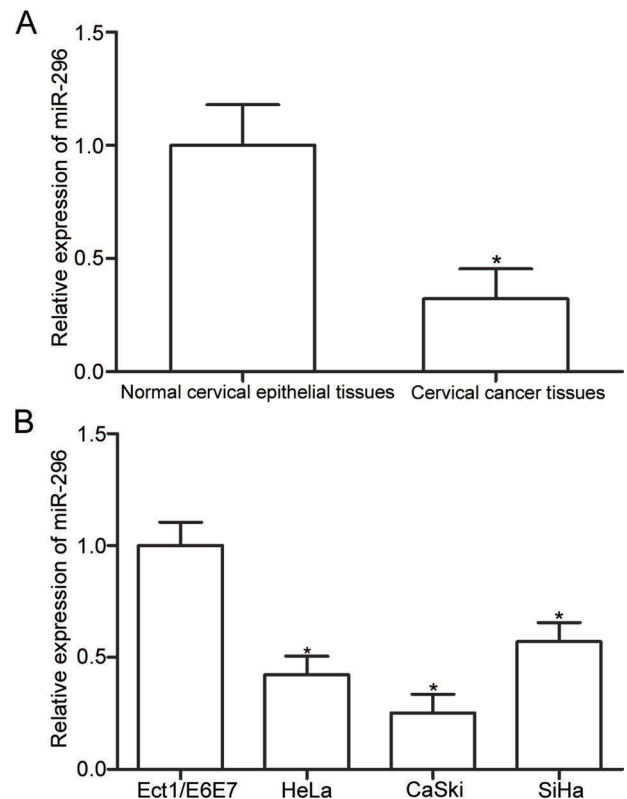
## RESULTS

### miR-296 Is Downregulated in Cervical Cancer Tissues and Cell Lines

RT-qPCR analysis was conducted to detect miR-296 expression in 31 paired cervical cancer tissues and matched adjacent normal cervical epithelial tissues. The results showed that miR-296 was downregulated in cervical cancer tissues compared with that in adjacent normal cervical epithelial tissues ( $p < 0.05$ ) (Fig. 1A). Furthermore, the expression levels of miR-296 in three cervical cancer cell lines (HeLa, SiHa, and Ca-Ski) and a human normal cervical epithelial cell line (Ect1/E6E7) were examined using RT-qPCR. The miR-296 expression level was significantly decreased in all cervical cancer cell lines compared with that in Ect1/E6E7 ( $p < 0.05$ ) (Fig. 1B). HeLa and SiHa cells expressed relatively low miR-296 expression and were used for further experiments. These results suggested that miR-296 may play important roles in the formation and progression of cervical cancer.

### miR-296 Overexpression Inhibits Cell Proliferation and Invasion in Cervical Cancer

miR-296 was overexpressed in HeLa and SiHa cells by transfection with miR-296 mimics to explore the roles of miR-296 in cervical cancer. RT-qPCR confirmed that miR-296 was markedly upregulated in HeLa and SiHa cells transfected with miR-296 mimics compared with that in cells transfected with miR-NC ( $p < 0.05$ ) (Fig. 2A). The effects of miR-296 overexpression on cervical cancer cell proliferation and invasion were analyzed with CCK-8 and cell invasion assays, respectively. As shown in Figure 2B and C, miR-296 upregulation attenuated the proliferation ( $p < 0.05$ ) (Fig. 2B) and invasion ( $p < 0.05$ )

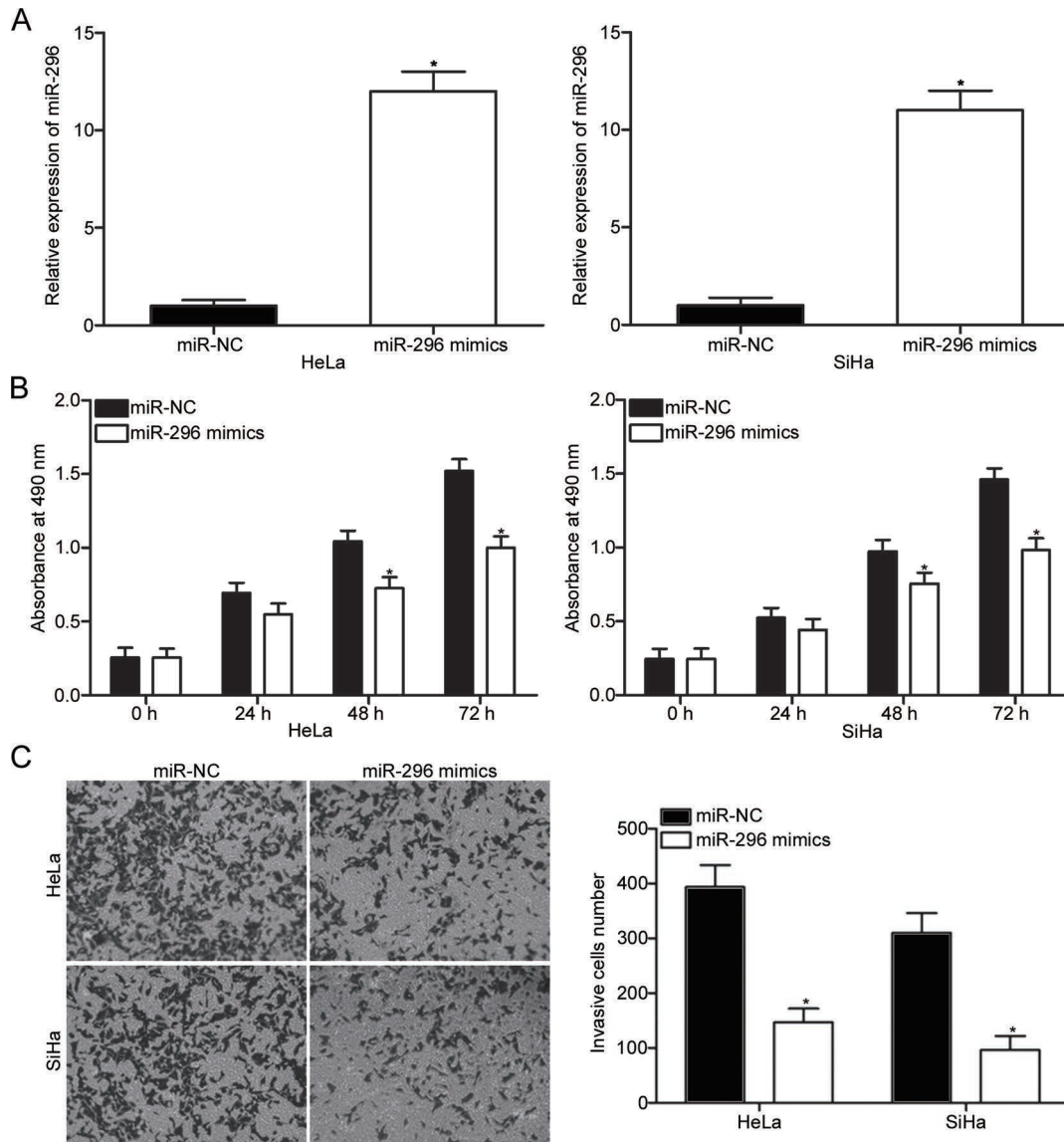


**Figure 1.** Low expression level of microRNA-296 (miR-296) in cervical cancer tissues and cell lines. (A) The expression level of miR-296 was analyzed in 31 paired cervical cancer tissues and matched adjacent normal cervical epithelial tissues by reverse transcription quantitative polymerase chain reaction (RT-qPCR). \* $p < 0.05$  compared with normal cervical epithelial tissues. (B) Relative miR-296 expression was determined in three cervical cancer cell lines (HeLa, SiHa, and Ca-Ski) and a human normal cervical epithelial cell line (Ect1/E6E7). \* $p < 0.05$  compared with Ect1/E6E7.

(Fig. 2C) of HeLa and SiHa cells. These results suggested that miR-296 may play tumor-suppressing roles in cervical cancer growth and metastasis.

### SP1 Is a Direct Target of miR-296 in Cervical Cancer

To elucidate the mechanism on how miR-296 affected cervical cancer cell proliferation and invasion, bioinformatics analysis was carried out to predict the potential targets of miR-296. SP1, which was highly expressed in cervical cancer and contributed to cervical cancer occurrence and development, was selected for further confirmation (Fig. 3A). Luciferase reporter assay was conducted to confirm whether the 3'-UTR of SP1 could be directly targeted by miR-296. HeLa and SiHa cells were cotransfected with miR-296 mimics or miR-NC and pmirGLO-SP1-3'-UTR Wt or pmirGLO-SP1-3'-UTR Mut. These results revealed that luciferase activities were evidently



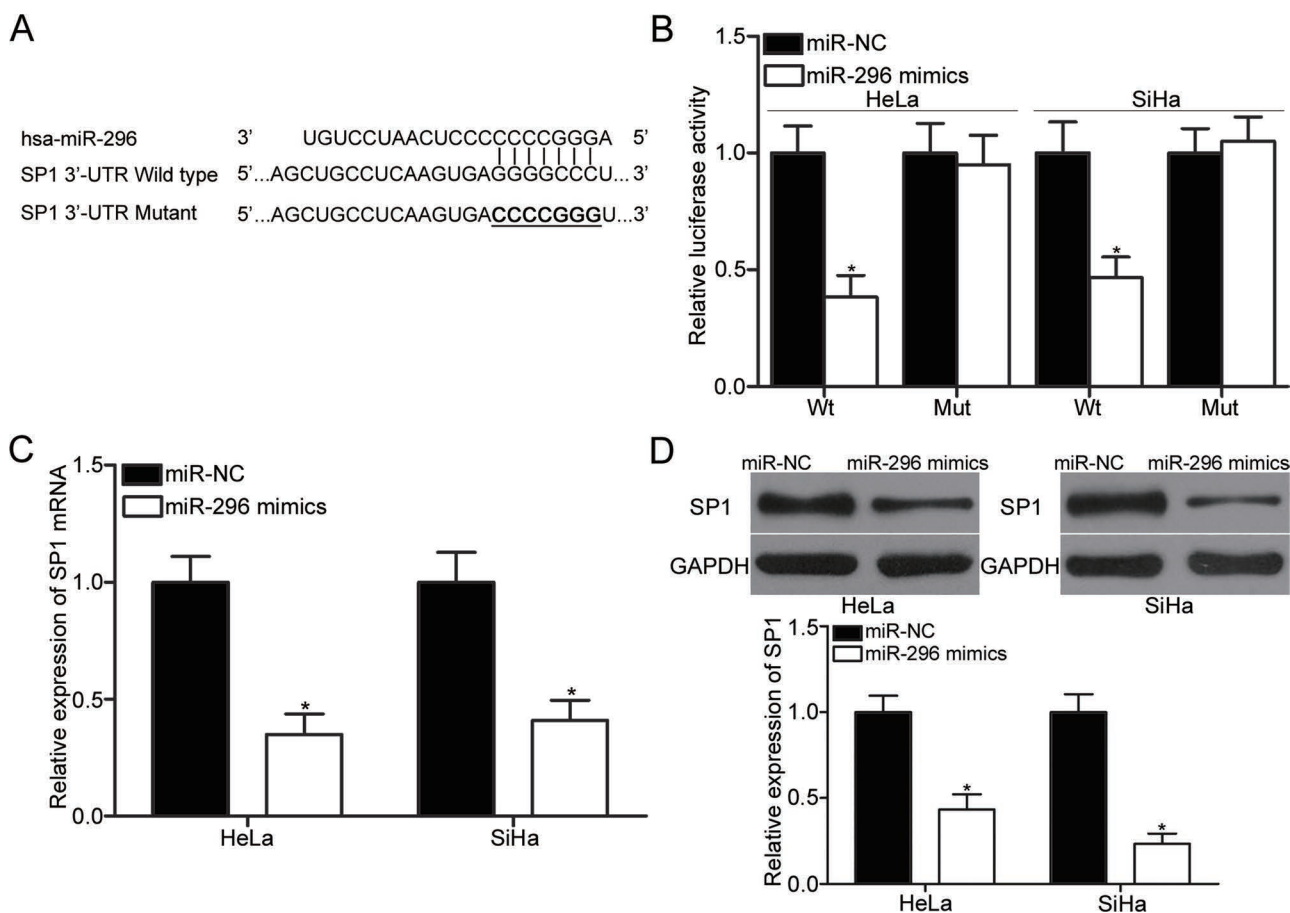
**Figure 2.** Restored miR-296 expression inhibits cell proliferation and invasion in cervical cancer. (A) At 48 h after transfection with miR-296 mimics or miR-NC in HeLa and SiHa cells, RT-qPCR analysis was utilized to detect the miR-296 expression. \* $p < 0.05$  compared with miR-NC. Cell counting kit 8 (CCK-8) (B) and cell invasion (C) assays were performed to examine the cell proliferation and invasion in HeLa and SiHa cells transfected with miR-296 mimics or miR-NC, respectively. \* $p < 0.05$  compared with miR-NC.

decreased by miR-296 overexpression in pmirGLO-SP1-3'-UTR Wt in HeLa and SiHa cells ( $p < 0.05$ ) (Fig. 3B). In addition, the inhibitory effect of miR-296 overexpression was abolished in pmirGLO-SP1-3'-UTR Mut. RT-qPCR and Western blot analysis were performed to explore whether miR-296 exerted regulatory effects on endogenous SP1 expression in cervical cancer. The data showed that restored miR-296 expression reduced the SP1 expression in HeLa and SiHa cells at both the mRNA ( $p < 0.05$ ) (Fig. 3C) and protein ( $p < 0.05$ ) (Fig. 3D) levels. Collectively, SP1 is a direct target gene of miR-296 in cervical cancer.

#### SP1 Is Involved in miR-296-Mediated Proliferation and Invasion of Cervical Cancer Cells

To investigate whether SP1 contributed to miR-296-mediated cell proliferation and invasion of cervical cancer, SP1 siRNA was transfected into HeLa and SiHa cells to knock down its expression. Western blot analysis demonstrated that SP1 was significantly downregulated in HeLa and SiHa cells after transfection with SP1 siRNA compared with that in cells transfected with NC siRNA ( $p < 0.05$ ) (Fig. 4A). Subsequently, CCK-8 and cell invasion assays were utilized to evaluate the effects of SP1





**Figure 3.** Specificity protein 1 (SP1) is a direct target of miR-296 in cervical cancer. (A) The putative miR-296 binding sites in the 3'-untranslated region (3'-UTR) of SP1 were predicted using bioinformatics analysis. (B) HeLa and SiHa cells were cotransfected with miR-296 mimics or miR-NC and the wild type or mutant of the SP1 3'-UTR luciferase reporter plasmid. Luciferase activities were measured at 48 h posttransfection using a dual-luciferase assay system. \* $p < 0.05$  compared with miR-NC. The SP1 mRNA (C) and protein (D) expression levels in HeLa and SiHa cells transfected with miR-296 mimics or miR-NC were detected using RT-qPCR and Western blot analysis, respectively. \* $p < 0.05$  compared with miR-NC.

knockdown on cervical cancer cell proliferation and invasion, respectively. The data revealed that SP1 downregulation suppressed the proliferation ( $p < 0.05$ ) (Fig. 4B) and invasion ( $p < 0.05$ ) (Fig. 4C) of HeLa and SiHa cells; this effect was similar to that induced by miR-296 overexpression. These results suggested that miR-296 may act as a tumor suppressor in cervical cancer by downregulating SP1.

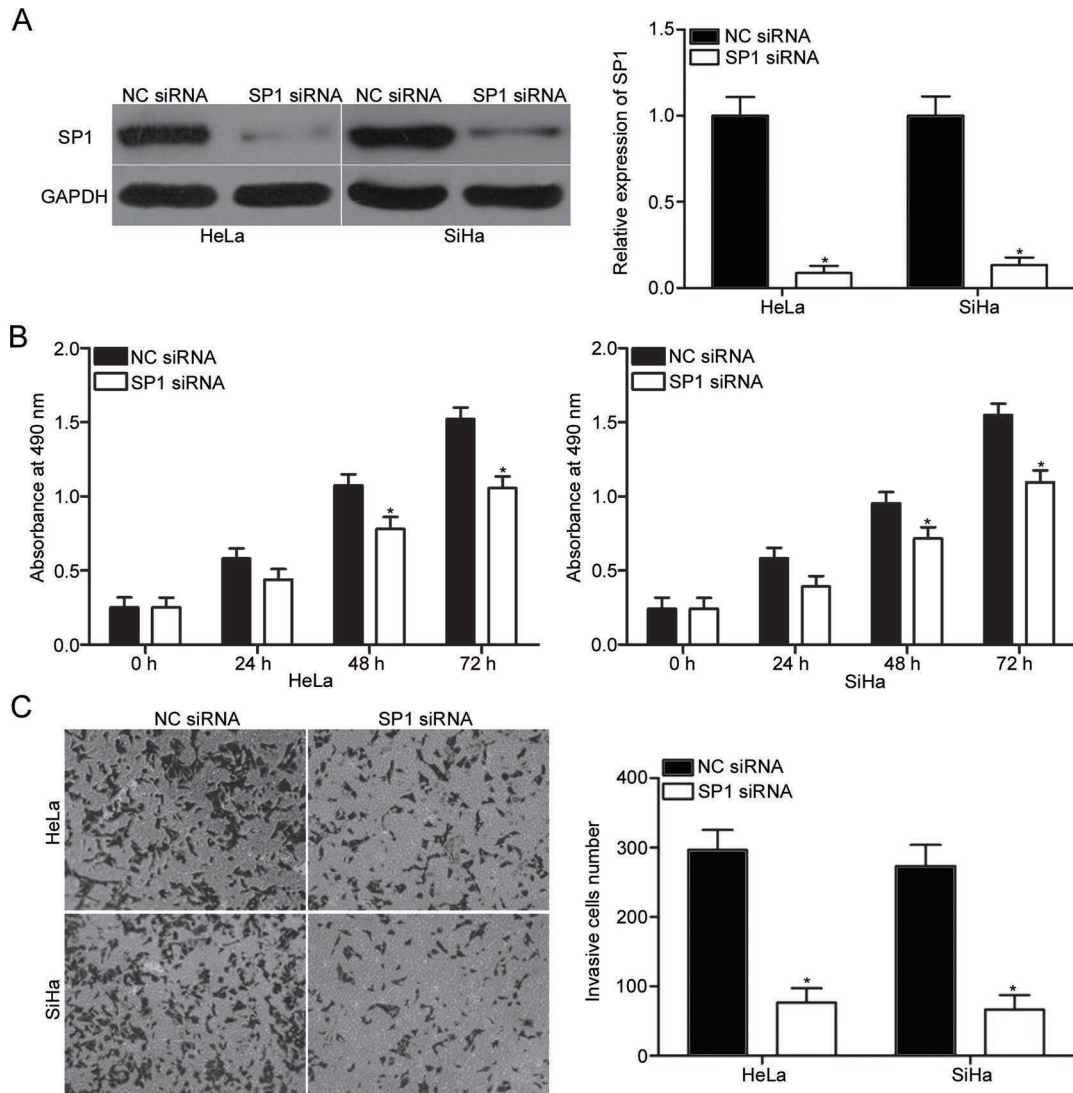
#### *Restored SP1 Expression Rescues the Tumor-Suppressing Roles of miR-296 in Cervical Cancer*

Rescue experiments were performed to further confirm whether SP1 is responsible for the functional roles of miR-296 in cervical cancer. HeLa and SiHa cells were transfected with miR-296 mimics together with pcDNA3.1 or pcDNA3.1-SP1. Following transfection, Western blotting analysis revealed that the downregulation of SP1 induced by miR-296 mimics was recovered

in HeLa and SiHa cells after cotransfection with pcDNA3.1-SP1 ( $p < 0.05$ ) (Fig. 5A). Subsequent functional assays demonstrated that upregulation of SP1 rescued the tumor-suppressing effects of miR-296 on cell proliferation ( $p < 0.05$ ) (Fig. 5B) and invasion ( $p < 0.05$ ) (Fig. 5C) in cervical cancer. These results further suggested that miR-296 may serve as a tumor suppressor in cervical cancer, at least in part, by regulation of SP1.

## DISCUSSION

miRNAs play key roles in cervical cancer occurrence, development, clinical outcome, and treatment response. Therefore, miRNAs are considered potential targets for the treatments of patients with this disease. In the present study, miR-296 expression was significantly downregulated in cervical cancer tissues and cell lines. The resumption expression of miR-296 inhibited the proliferation

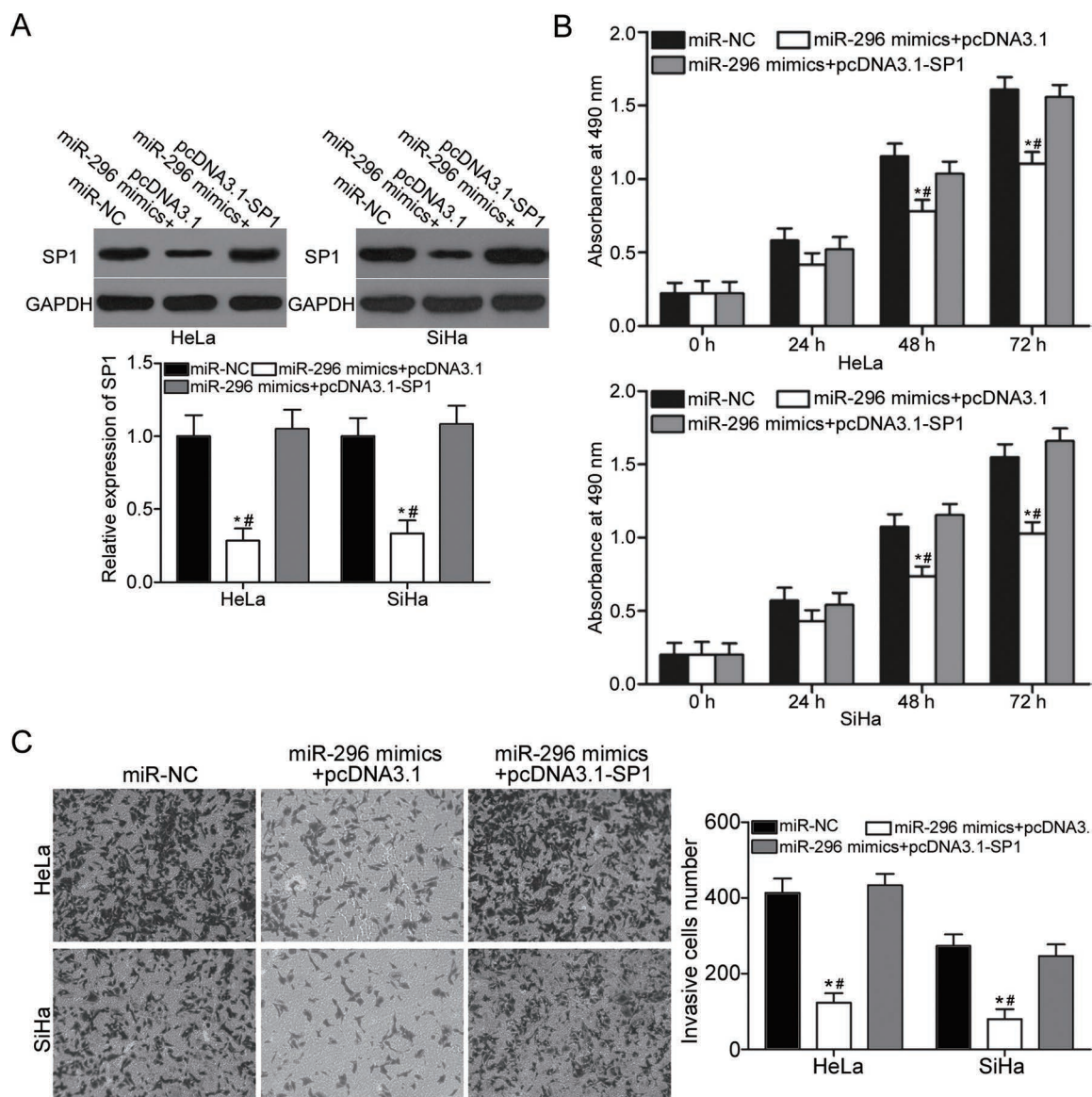


**Figure 4.** SP1 downregulation reduces cervical cancer cell proliferation and invasion. (A) Western blot analysis of SP1 protein expression in HeLa and SiHa cells following transfection with SP1 siRNA or NC siRNA. \* $p < 0.05$  compared with NC siRNA. The effects of SP1 knockdown on cervical cancer cell proliferation (B) and invasion (C) were evaluated using CCK-8 and cell invasion assays, respectively. \* $p < 0.05$  compared with NC siRNA.

and invasion of cervical cancer cells. Additionally, SP1 was demonstrated as a novel direct target of miR-296 in cervical cancer. The SP1 downregulation suppressed the proliferation and invasion of cervical cancer cells; this result was similar to that induced by miR-296 overexpression. Restored SP1 expression rescued the tumor-suppressing roles of miR-296 in cervical cancer. These results suggested that miR-296 could be developed as a potential therapeutic agent in the treatment of patients with cervical cancer.

miR-296 is aberrantly expressed, and it plays important roles in multiple types of human cancer. For example, miR-296 is downregulated in pancreatic cancer. miR-296 upregulation attenuates cell proliferation, migration, and

invasion in pancreatic cancer<sup>15</sup>. The expression level of miR-296 is low in colorectal cancer and is evidently associated with adverse clinical parameters and poor prognosis. Functional experiments indicated that miR-296 overexpression suppresses the metastasis and epithelial-mesenchymal transition of colorectal cancer<sup>12</sup>. miR-296 underexpression has been observed in non-small-cell lung cancer tissues and cell lines. The restoration expression of miR-296 inhibits cell proliferation in non-small cell lung cancer in vitro<sup>13</sup>. miR-296 is also a tumor suppressor in prostate cancer<sup>14,16</sup>, breast cancer<sup>17</sup>, and ovarian cancer<sup>18</sup>. Nevertheless, in gastric cancer, miR-296 is upregulated in tumor tissues and cell lines. miR-296 upregulation promotes cell proliferation in gastric cancer<sup>19</sup>. Hong



**Figure 5.** Upregulation of SP1 rescues the tumor-suppressing effects of miR-296 overexpression on cervical cancer cell proliferation and invasion. HeLa and SiHa cells were transfected with miR-NC, miR-296 mimics+pcDNA3.1, or miR-296 mimics+pcDNA3.1-SP1. (A) Western blotting analysis was performed to detect SP1 expression of indicated cells. \* $p < 0.05$  compared with miR-NC. # $p < 0.05$  compared with miR-296 mimics+pcDNA3.1-SP1. CCK-8 and cell invasion assays were used to examine cell proliferation (B) and invasion (C) in indicated cells, respectively. \* $p < 0.05$  compared with miR-NC. # $p < 0.05$  compared with miR-296 mimics+pcDNA3.1-SP1.

et al. found that miR-296 is highly expressed in esophageal squamous cell carcinoma. miR-296 underexpression decreases the cell growth of esophageal cancer both in vitro and in vivo<sup>20</sup>. These conflicting findings indicated that the expression pattern and biological roles of miR-296 are varied and tissue dependent in human cancer. These studies also suggested that miR-296 may be investigated as a novel and efficient therapeutic target for their treatment.

Several direct targets of miR-296, including AKT2<sup>15</sup> in pancreatic cancer, S100A4<sup>12</sup> in colorectal cancer, Pin1<sup>14</sup>

and HMGAI<sup>16</sup> in prostate cancer, Scribble<sup>17</sup> in breast cancer, PLK1<sup>13</sup> in non-small cell lung cancer, and CDX1<sup>19</sup> in gastric cancer, were validated. In the present study, SP1 was identified as a direct target of miR-296 in cervical cancer. SP1, mapped to 12q13.1, is a sequence-specific DNA-binding protein, and it encodes a protein of 785 amino acids<sup>21</sup>. SP1 is highly expressed in many types of human cancer, such as lung cancer<sup>22</sup>, colorectal cancer<sup>23</sup>, hepatocellular carcinoma<sup>24</sup>, glioma<sup>25</sup>, and prostate cancer<sup>26</sup>. SP1 plays a major role in many biological

functions associated with tumorigenesis and tumor development, such as cell proliferation, apoptosis, migration, metastasis, and epithelial–mesenchymal transition<sup>27,28</sup>. In cervical cancer, the expression level of SP1 is higher in tumor tissues than in normal cervical epithelial tissues<sup>29</sup>. Functional experiments revealed that SP1 contributes to cervical cancer initiation and progression through regulation of cell proliferation, apoptosis, radiosensitivity, and metastasis<sup>30–33</sup>. Combined with these findings, the miR-296/SP1 pathway may be investigated as a therapeutic target for inhibition of rapid growth and metastasis in cervical cancer.

In summary, to the best of our knowledge, this study is the first to provide evidence that miR-296 was down-regulated in cervical cancer tissues and cell lines. Ectopic miR-296 expression decreased cell proliferation and invasion through direct targeting of SP1. Further research exploring the anticancer role of miR-296 in cervical cancer may contribute to the development of novel therapeutic strategies for cervical cancer patients.

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