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miR-126-5p Restoration Promotes Cell Apoptosis in Cervical Cancer by Targeting Bcl2l2

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Cervical cancer is one of the most common cancers in females, with a high incidence and mortality around the world. However, the pathogenesis in cervical cancer is not completely known. In the present study, we investigated the role of miR-126-5p and Bcl2l2 in cervical cancer cells. First, miR-126-5p expression was aberrantly downregulated in human cervical cancer tumor tissues in comparison with normal tissues, as evaluated by RT-PCR. Consistently, the levels of miR-126-5p were also significantly reduced in cervical cancer cell lines when compared to normal cervical epithelial cells. Flow cytometric analysis showed that the rate of apoptosis of cervical cancer cells was significantly increased by miR-126-5p overexpression but inhibited by miR-126-5p inhibitor. A similar change pattern was observed in the expression of apoptosis-regulated protein caspase 3 in cervical cancer cells transfected with miR-126-5p mimic or inhibitor. By bioinformatic prediction with online databases and verification using luciferase reporter assay, we then identified that Bcl2l2 is a direct target of miR-126-5p in cervical cancer cells. The expression of Bcl2l2 was strongly downregulated by the miR-126-5p mimic but upregulated by the miR-126-5p inhibitor in cervical cancer cells, and Bcl2l2 expression was significantly increased in human cervical cancer tumor tissues, which was negatively correlated with miR-126-5p levels. Furthermore, we confirmed that the rate of apoptosis was significantly increased by Bcl2l2 silencing in cervical cancer cells, which was not affected by the miR-126-5p inhibitor. In addition, the increased apoptosis of cells by the miR-126-5p mimic was inhibited by Bcl2l2 overexpression. In summary, miR-126-5p plays an inhibitory role in human cervical cancer progression, regulating the apoptosis of cancer cells via directly targeting Bcl2l2. This might provide a potential therapeutic target for cervical cancer.

Key words: miR-126-5p; Cervical cancer; Apoptosis; Caspase 3; Bcl2l2

INTRODUCTION

Cervical cancer is one of the most common cancers among females, with a high incidence and mortality around the world. Invasive cervical cancer is the result of a multistep process in which the normal cervical epithelium is induced by oncogenic factors to transform into a cervical intraepithelial neoplasia (1). There are three predominant types: squamous carcinoma, adenocarcinoma, and adenosquamous carcinoma, according to the difference in histological characteristics. Most patients with cervical cancer correspond to the squamous carcinoma form, which can be classified into highly differentiated (grade I), moderately differentiated (grade II), and lowly differentiated squamous carcinoma (grade III). Of the cervical cancer cases, 15%–20% are adenocarcinoma, and 3%–5% are adenosquamous carcinoma (1). Although most patients receive appropriate surgery, radiotherapy, or chemotherapy, the efficiency of these therapies and their clinical outcomes vary significantly (2). Hence, further identification of pathogenesis in cervical cancer would be of considerable importance to improve the diagnosis and treatment of cervical cancer.

MicroRNAs (miRNAs) are a family of small noncoding single-stranded RNA molecules that play important roles as endogenous regulators in the regulation of gene expression (3). Most miRNAs can bind to different mRNAs to form a complicated regulatory network in the expression of genes that are implicated in multiple biological processes, including cell growth, differentiation, and death (4,5).

Dysregulation of miRNA expression has been reported in many cancers and has been identified to be involved in the pathogenesis of human cancers (6). In cervical cancer, aberrant expression of miRNAs has also been widely found and is associated with the progression of cervical cancer, including miR-199, miR-203, miR-145, miR-143,

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miR-21, miR-145, miR-127, miR-133, and others (7–12). In terms of miR-126, it is derived from a common precursor structure *egfl7* (epidermal growth factor-like domain 7) gene and has been reported to be downregulated in cervical cancer (13). Reduced expression of miR-126 is associated with poor prognosis and sensitivity to chemotherapeutic drug in patients with cervical cancer (14,15). However, the role of miR-126 in the progression of cervical cancer is still unexplored.

Bcl2 like 2 (Bcl2l2), also known as Bcl-w, is a member of the Bcl-2 protein family. This protein has been reported to promote cell survival and decrease apoptosis under cytotoxic conditions as antiapoptotic regulators by forming dimers (16,17). It has also been found to be aberrantly expressed in various cancer cells, contributing to carcinogenesis (18). In the present study, we investigated the role of miR-126-5p and Bcl2l2 in cervical cancer cells. miR-126-5p expression was decreased in cervical cancer and negatively regulated cell apoptosis by directly targeting Bcl2l2. Our results reveal a novel role of miR-126-5p dysregulation in cervical cancer pathogenesis, which might be a potential therapeutic target for cervical cancer.

MATERIALS AND METHODS

Patients and Tumor Samples

Frozen biopsy tumor specimens were collected from 14 patients with cervical cancer undergoing surgery at Taian City Central Hospital. Normal cervical tissues were obtained from 14 patients with benign gynecologic disease. This study was approved by the ethics committee of Taian City Central Hospital. Informed consent was obtained from all patients.

Cell Cultures

The human cervical cancer cell lines SiHa, ME-180, and HeLa cells as well as normal cervical epithelial cells were obtained from the American Type Culture Collection (ATCC). Cancer cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37°C in a 5% CO_2 atmosphere. Normal cervical epithelial cells were cultured in DMEM medium containing 10% FBS at 37°C.

Quantitative RT-PCR

TRIzol reagent (Sigma-Aldrich) was used to isolate the total RNA from cultured cells. The cDNA of miR-126-5p was prepared using MicroRNA First-Strand Synthesis and miRNA Quantitation kits (Takara, Dalian, China) according to the manufacturer's instructions. The CellAmp Direct RNA Prep kit for qPCR and a Protein Analysis kit (Takara) were used to test the expression of Bcl2l2. Ct values of U6 and GAPDH were used as the internal control to normalize the relative expression of miR-126-5p and Bcl2l2.

Western Blotting

Total proteins were isolated from cultured cells and separated by SDS-PAGE (Invitrogen), followed by electrophoretic transfer to PVDF membrane. Primary antibodies [rabbit anti-cleaved caspase 3, anti-Bcl2l2 1:1,000 dilution (Abcam); mouse anti-β-actin 1:3,000 dilution (ABclonal)] were incubated with blots at 4°C overnight. The membrane was then incubated with secondary antibodies for 1 h at room temperature. Bands were visualized by ECL (Amersham Pharmacia, NJ, USA).

Cell Transfection

miR-126-5p mimic, inhibitor, and negative control miRNA (NC) were purchased from RiboBio (Guangzhou, China). Cells were cultured to approximately 80% confluence and then transfected with 100 nM miR-126-5p mimic or inhibitor as well as NC miRNA using Lipofectamine 2000 (Invitrogen) twice for 48 h. Transfection of Bcl2l2 siRNA and negative oligonucleotides (NO) (RiboBio) was also performed using Lipofectamine 2000.

The open reading frame (ORF) of Bcl2l2 was amplified and cloned into the pAdTrack-CMV vector (Clontech). The recombinant adenovirus particles containing pAd-Bcl2l2 were obtained according to the manufacturer's protocol. Cells were infected with adenoviruses at 20 multiplicity of infection (moi) for 48 h without apparent cytotoxicity.

Cell Apoptosis

A cell apoptosis assay was performed using the Annexin-V-Fluorescein Isothiocyanate Kit (Immunotech, Marseille, France). In brief, following culture in serum-free DMEM for 16 h, cells were harvested with ice-cold PBS and resuspended with binding buffer. Cell suspensions (5×10^6 cells/well) were then stained with 0.5 μg/ml annexin V-fluorescein isothiocyanate and 0.6 μg/ml propidium iodide (PI) for 15 min, followed by analysis using a FACSCalibur™ (Becton Dickinson).

Luciferase Reporter Assay

The luciferase reporter assay was performed as described previously (19). Briefly, the 3′-UTR of Bcl2l2 mRNA was cloned into the pGL3 luciferase vector (Promega, Madison, WI, USA) by PCR. The site-directed mutagenesis was introduced into the miR-126-5p binding site of Bcl2l2 3′-UTR using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). Recombinant pGL3 plasmid (200 ng) and miR-126-5p mimic (100 nM) were cotransfected into cultured cells (1×10^5 cells/well) in 96-well plates using Lipofectamine 2000. After 36 h of transfection, luciferase activity was analyzed using the Dual Luciferase Assay (Promega). *Renilla* activity was used as the internal control. Experiments were performed independently at least three times.

Statistical Analysis

All data are expressed as mean ± SEM. GraphPad Prism 6 (La Jolla, CA) was used to analyze the differences between groups. A value of $p < 0.05$ was considered as statistically significant.

RESULTS

miR-126-5p Is Aberrantly Downregulated in Human Cervical Cancer Tissues and Cells

To identify the possible role of miR-126-5p in human cervical cancer, we first investigated the expression of miR-126-5p in human cervical cancer tumor tissues from patients. As shown in Figure 1A, miR-126-5p expression was aberrantly downregulated in cervical cancer tumor tissues in comparison with normal tissues. We also measured the levels of miR-126-5p expression in the human cervical cancer cell lines SiHa, ME-180, and HeLa cells. Results showed that the levels of miR-126-5p were significantly reduced in all cervical cancer cells when compared to normal cervical epithelial cells (Fig. 1B). These results demonstrate that the dysregulation of miR-126-5p expression may contribute to tumorigenesis in human cervical cancer.

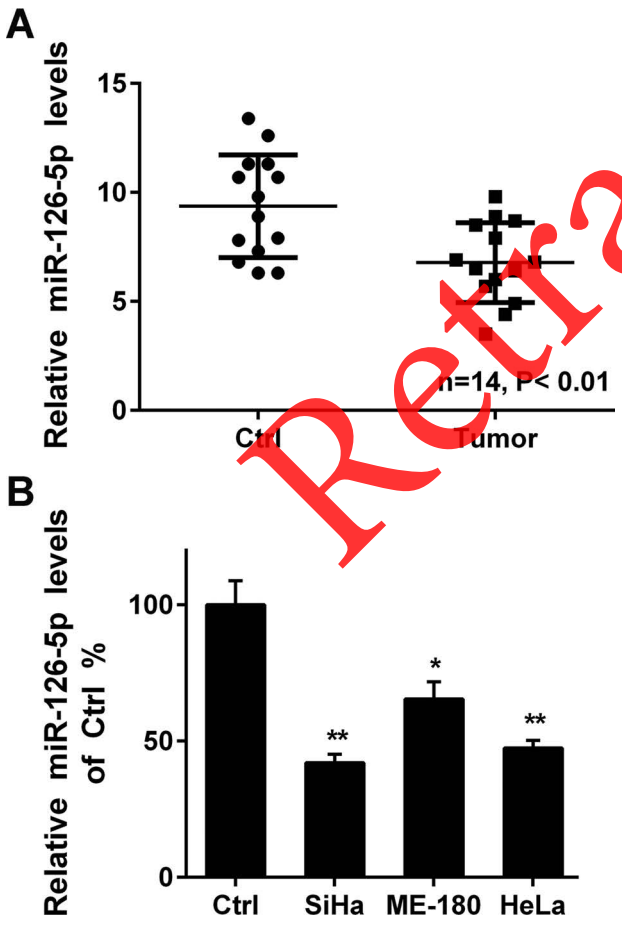

Figure 1. The expression of miR-126-5p in human cervical cancer. (A) The expression levels of miR-126-5p in human cervical cancer tumor tissue and normal tissues (Ctrl) were examined using RT-PCR. (B) The expression levels of miR-126-5p in human cervical cancer cell lines SiHa, ME-180, and HeLa cells as well as the normal cervical epithelial cells (Ctrl) were examined using RT-PCR. $^{**}p < 0.01$; $^{*}p < 0.05$ versus Ctrl.

Overexpression of miR-126-5p Promotes Cell Apoptosis in Cervical Cancer Cells

We further employed specific mimic and inhibitor of miR-126-5p to elucidate the potential effect of low miR-126-5p levels on human cervical cancer cells. The SiHa and HeLa cells were used in the subsequent experiment. Efficiency of the specific mimic and inhibitor was confirmed using RT-PCR assay. Compared with the control, miR-126-5p expression was significantly increased by the mimic and reduced by the inhibitor in both cervical cancer cells (Fig. 2A).

Flow cytometric analysis was then performed to measure the apoptosis of cervical cancer cells. Results showed that the rate of apoptosis of both SiHa and HeLa cells was significantly increased by miR-126-5p overexpression compared with the control (Fig. 2B). On the contrary, the miR-126-5p inhibitor specially decreased the levels of apoptosis in cervical cancer cells (Fig. 2B). We further tested the expression of apoptosis-regulated protein in cervical cancer cells by Western blotting assay. As seen in Figure 2C, cleaved caspase 3 levels were enhanced by miR-126-5p overexpression and reduced when miR-126-5p was suppressed. These data suggest that miR-126-5p has a role in the regulation of cervical cancer apoptosis.

Bcl2l2 Is the Direct Target of miR-126-5p

By searching the mirbase and miRDB databases, we predicted the possible target of miR-126-5p. There was a potential binding site of miR-126-5p found in the 3′-UTR of Bcl2l2 mRNA, as shown in Figure 3A. To identify this prediction, we performed a luciferase reporter analysis, in which the wild and mutant 3′-UTR of Bcl2l2 mRNA was cloned into a luciferase vector (Fig. 3A). Results showed that the luciferase activity of the Bcl2l2-3′-UTR-wt reporter was reduced in SiHa and HeLa cells transfected with the miR-126-5p mimic (Fig. 3B). However, there was no inhibitory effect observed in the luciferase activity of Bcl2l2-3′-UTR-mut reporter induced by the miR-126-5p mimic (Fig. 3B).

Then the expression of Bcl2l2 was detected to be significantly downregulated by the miR-126-5p mimic but upregulated by the miR-126-5p inhibitor in SiHa and HeLa cells (Fig. 3C). We further tested the expression of Bcl2l2 in human cervical cancer tumor tissues. Consistently, compared to the normal tissues, Bcl2l2 expression was

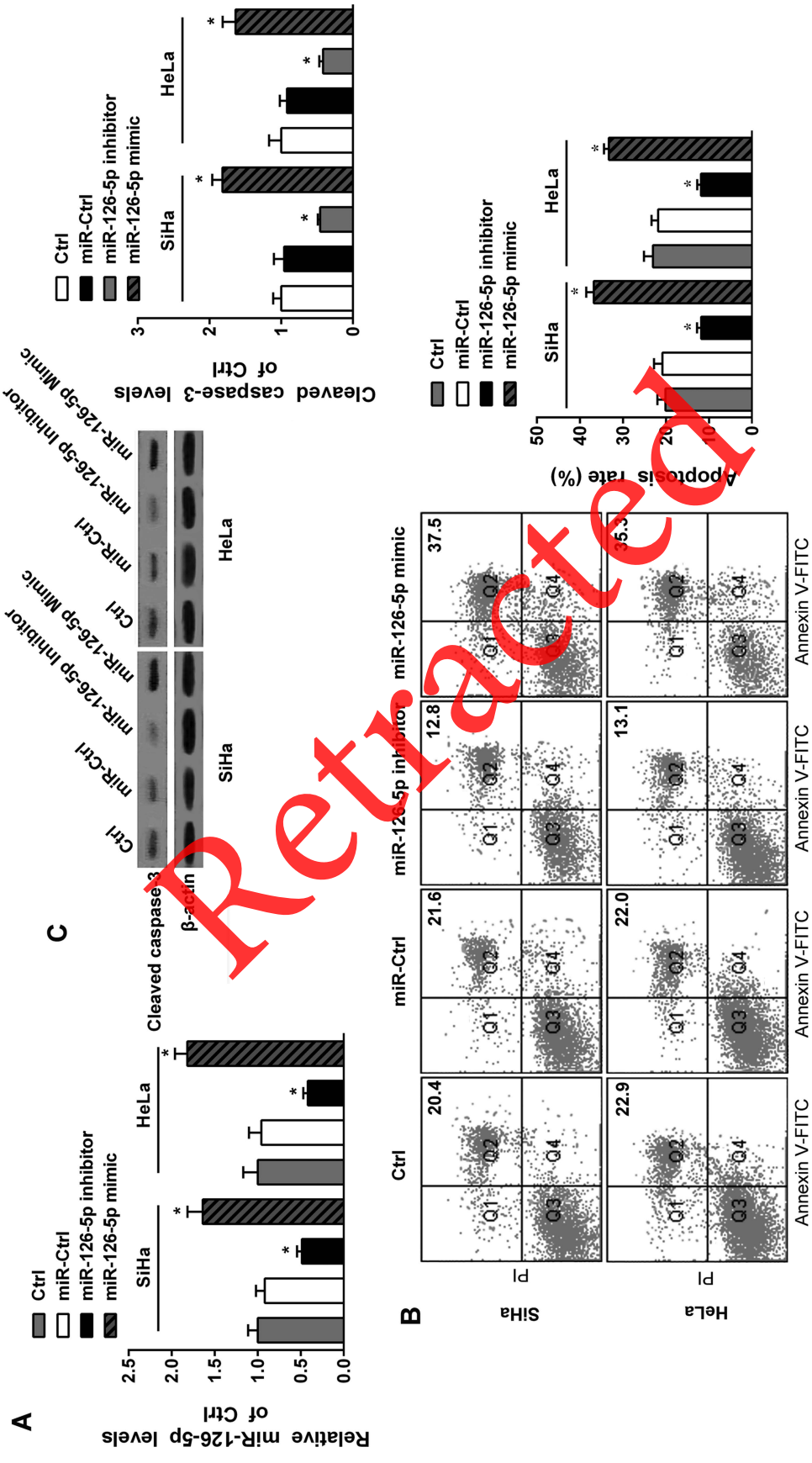

Figure 2. Effects of miR-126-5p on cervical cancer cell apoptosis. SiHa and HeLa cells were cultured in medium (Ctrl), followed by transfection with miR-126-5p mimic, inhibitor, or negative control miRNA (miR-Ctrl). (A) The expression levels of miR-126-5p were determined using RT-PCR in SiHa and HeLa cells transfected with the miR-126-5p mimic, inhibitor, or negative control miRNA (miR-Ctrl). (B) Cell apoptosis was measured using flow cytometric analysis. (C) The levels of cleaved caspase 3 were tested using Western blotting. *$p < 0.05$ versus Ctrl.

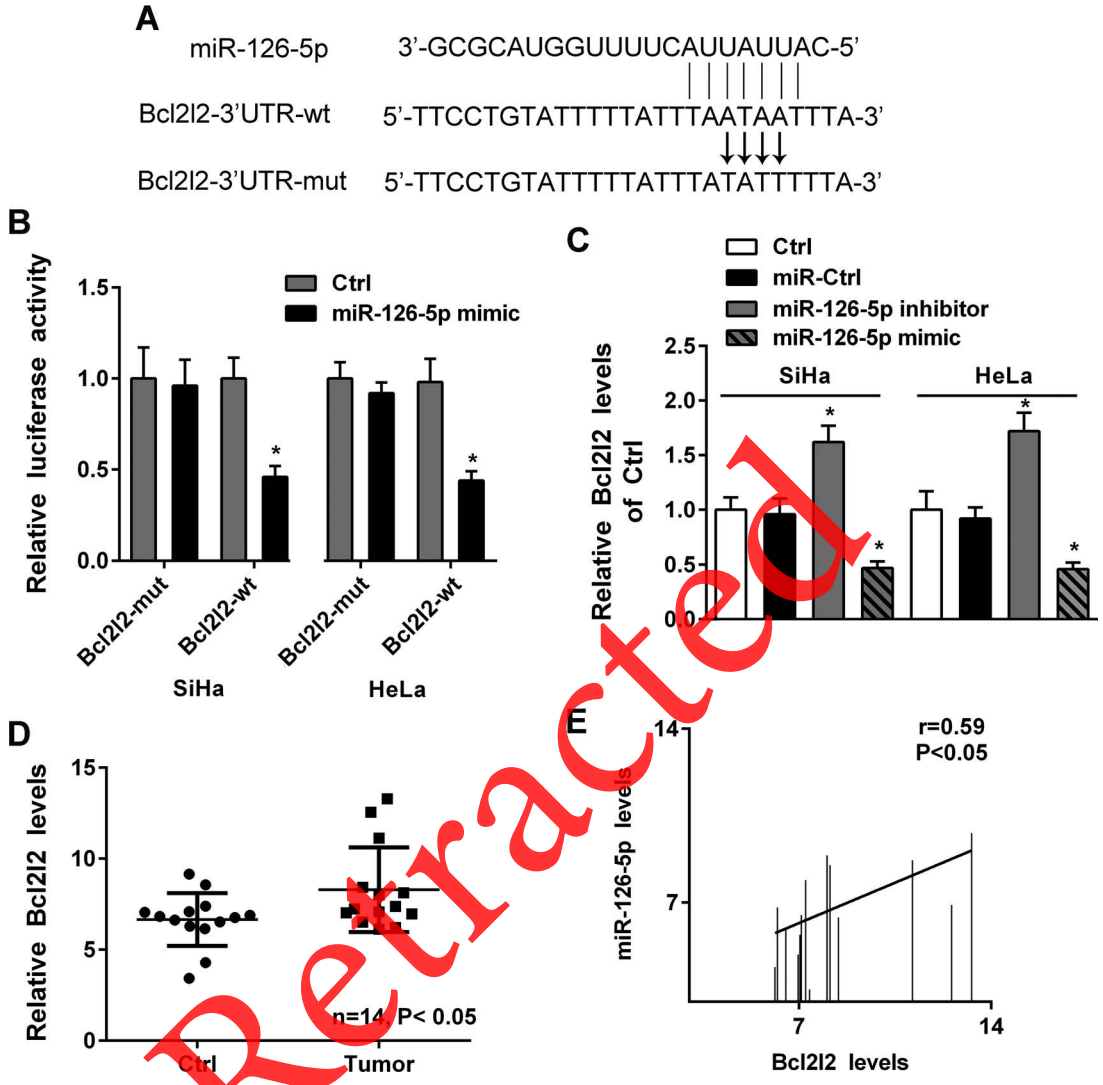

Figure 3. miR-126-5p directly targets Bcl2l2. (A) The prediction of potential binding site of miR-126-5p in the 3′-UTR of Bcl2l2 mRNA. (B) Luciferase activity was tested in SiHa and HeLa cells transfected with Bcl2l2-3′-UTR-wt (Bcl2l2-wt) or Bcl2l2-3′-UTR-mut (Bcl2l2-mut) as well as miR-126-5p mimic. (C) The expression of Bcl2l2 in SiHa and HeLa cells was measured using RT-PCR. (D) Expression of Bcl2l2 in human cervical cancer tumor tissue and normal tissues (Ctrl) was measured using RT-PCR. (E) The correlation between Bcl2l2 and miR-126-5p expression in cervical cancer tumor tissues. *$p<0.05$ versus Ctrl.

significantly increased in cervical cancer tumor tissues, which was negatively correlated with miR-126-5p levels (Fig. 3D and E). These data indicate that Bcl2l2 is a direct target of miR-126-5p in cervical cancer cells.

Bcl2l2 Inhibits miR-126-5p-Mediated Cell Apoptosis

Previous studies have suggested that Bcl2l2 confers an antiapoptotic effect in various human cancers (20). To clarify whether Bcl2l2 is involved in the miR-126-5p-mediated cervical cancer cell apoptosis, we further performed a series of experiments in SiHa and HeLa cells. First, we employed Bcl2l2 siRNA and adenovirus transduction to inhibit or overexpress Bcl2l2, respectively. As seen in Figure 4A, the efficiency of the Bcl2l2 siRNA and adenovirus was confirmed using RT-PCR assay. Compared with the control, Bcl2l2 expression was significantly increased by the adenovirus transfection and reduced by the specific siRNA in both cervical cancer cells. In addition, the rate of apoptosis was significantly increased by Bcl2l2 silencing in both SiHa and HeLa cells, which was not affected by the miR-126-5p inhibitor (Fig. 4B). Besides, the increased apoptosis of cells by the miR-126-5p mimic was inhibited by Bcl2l2 overexpression (Fig. 4C), indicating that miR-126-5p modulates the apoptosis of cervical cancer cells by directly targeting Bcl2l2 expression.

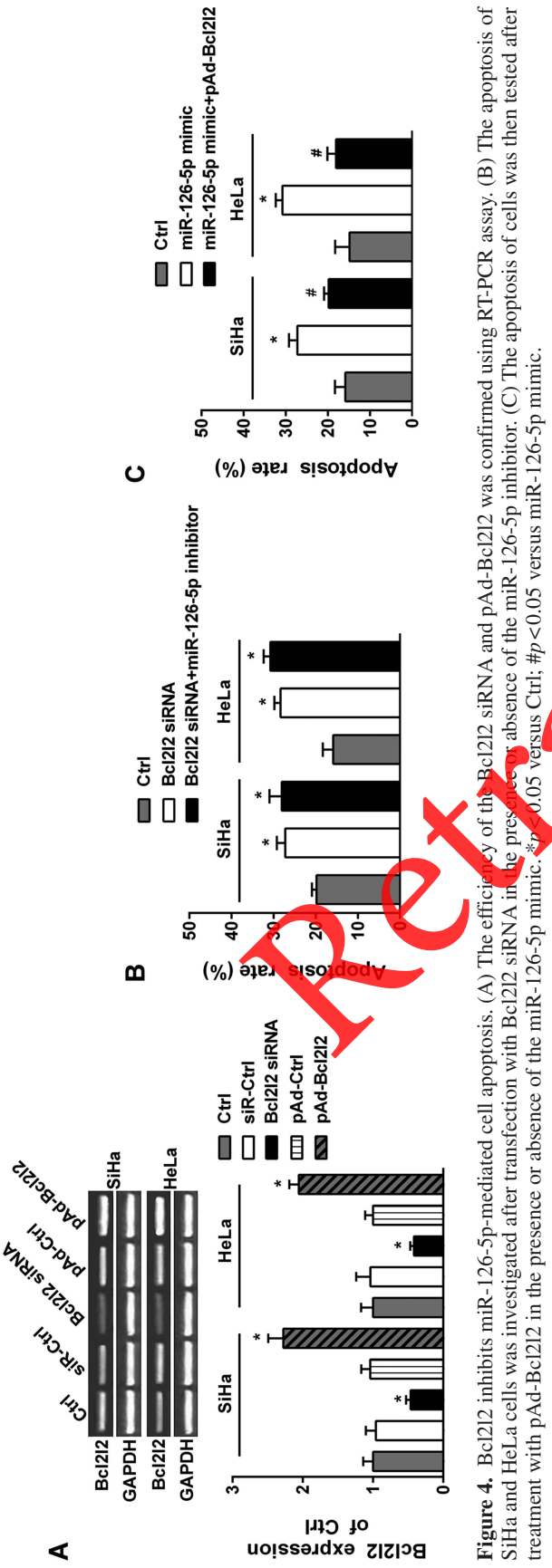

Figure 4. Bcl2l2 inhibits miR-126-5p-mediated cell apoptosis. (A) The efficiency of the Bcl2l2 siRNA and pAd-Bcl2l2 was confirmed using RT-PCR assay. (B) The apoptosis of SiHa and HeLa cells was investigated after transfection with Bcl2l2 siRNA in the presence or absence of the miR-126-5p inhibitor. (C) The apoptosis of cells was then tested after treatment with pAd-Bcl2l2 in the presence or absence of the miR-126-5p mimic. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus miR-126-5p mimic.

DISCUSSION

As key endogenous regulators of gene expression, miRNAs have important roles in various physiological and pathological processes of development and diseases (21). Dysregulation of miRNA expression has been identified to be involved in multiple progression of human diseases, including cancers (22). In cervical cancer, aberrant expression of miRNAs has also been reported (23,24). miR-126 is derived from the common precursor structure *egfl7* gene and is conserved among different species. The loss of miR-126 has been reported in many human cancers, such as colon, lung, prostate, and breast cancers (25,26). Aberrant expression of miR-126 exhibits a tumor oncogenic function during tumorigenesis. Liu et al. reported that miR-126 expression is downregulated in lung cancer cells in vitro and in vivo. miR-126 restoration could decrease VEGF expression and repress the growth of lung cancer cells (26). In another study, the reduction of miR-126 was found to be associated with angiogenesis in tumorigenesis. The loss of miR-126 results in the free proangiogenic adrenomedullin, contributing to the enhanced vascular growth and invasive carcinoma transition (27). Additionally, the downregulation of miR-126 caused by methylation was proven to be implicated in glioma progression (28). In cervical cancer, repression of miR-126 expression was also identified to exert a role in the angiogenesis of cervical cancer and is associated with poor prognosis and sensitivity to chemotherapeutic drug in patients with cervical cancer (15,29,30). In the present study, we further determined a novel role for miR-126-5p, the complement of miR-126, in cervical cancer. miR-126-5p expression is strongly downregulated in human cervical cancer tumor tissues as well as in cultured human cervical cancer cell lines. Further study found that the dysregulation of miR-126-5p is involved in the inhibition of apoptosis in cervical cancer cells, indicating the role of miR-126-5p in human cervical cancer tumorigenesis.

Bcl2l2 comprise a family of apoptosis-regulated proteins (Bcl-2 family). Previous studies report that the Bcl2l2 protein is aberrantly expressed in various cancer cells, including non-small cell lung, colon, and gastric cancers (18). Altered expression of Bcl2l2 can promote cell survival and decrease apoptosis in response to cytotoxic conditions as antiapoptotic regulators by forming dimers, contributing to carcinogenesis. By bioinformatic prediction with online databases and verification using luciferase reporter assay, we identified that Bcl2l2 mRNA is a direct target of miR-126-5p in cervical cancer cells, with a binding site on the 3′-UTR. The expression level of Bcl2l2 was strongly increased in human cervical cancer tumor tissues and presented a negative correlation with miR-126-5p levels, suggesting the involvement of miR-126-5p and Bcl2l2 in human cervical cancer tumorigenesis.

Another approach we took to elucidate the role of miR-126-5p is to detail the potential mechanisms involved in miR-126-5p-mediated apoptosis. It has been known that caspase 3 activity is essential for apoptosis induced by serum starvation. The Bcl-2 protein family has regulatory roles in the modulation of caspase-dependent apoptosis (16,31). Bcl-2 can protect cells against apoptosis by preventing the release of mitochondrial cytochrome c and caspase 3-dependent proteolytic cascade (20,32). On the contrary, a shift in the Bax/Bcl-2 ratio may activate caspase 3, contributing to apoptosis initiation (33). Herein, we also found that ectopic expression of miR-126-5p increased the expressions of caspase 3 in human cervical cancer cells, and the enhanced apoptosis of cells by miR-126-5p was reversed by Bcl2l2 overexpression. Together, we provide evidence that miR-126-5p may promote apoptosis in human cervical cancer cells by targeting Bcl2l2.

Our study is not without limitation. Bcl2l2 is just one of the targets regulated by miR-126-5p. Exploring the interactions between the dysfunction of miRNAs and the functional categories of target genes may be requisite to clearly elucidate the role of dysregulated miRNAs in cervical cancer. Additionally, the regulation of miRNA expression and the interaction between miRNAs and target genes are very complicated. In human cervical cancer, there are various differentially expressed miRNAs that are involved in cancer-related signal transduction pathways. Therefore, interactive studies on different miRNAs will be an important part of our next study to identify the potential mechanism involved in the pathological process of cervical cancer.

In conclusion, we demonstrated that miR-126-5p plays an inhibitory role in human cervical cancer progression, regulating the apoptosis of cancer cells via directly targeting Bcl2l2. This might provide a potential therapeutic target for cervical cancer.

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