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MicroRNA-215-3p suppresses the growth and metastasis of cervical cancer cell via targeting SOX9

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Abstract. – OBJECTIVE: The aim of the current study was to investigate the potential roles of miR-215-3p in the progression of cervical cancer.

PATIENTS AND METHODS: The levels of miR-215-3p in both cervical cancer tissues and cell lines were detected using quantitative Real-time polymerase chain reaction (qRT-PCR) assay. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), colony formation, migration and invasion assays were applied to investigate the role of miR-215-3p on the growth and aggressiveness of cervical carcinoma SiHa cell. The expression of SRY-Box 9 (SOX9) was assessed by Western blotting assay. The Xenograft model and lung metastasis model were applied to reveal the impact of miR-215-3p on the growth and distant metastasis of cervical carcinoma cell *in vivo*. Moreover, miR-215-3p and a SOX9 siRNA were co-transfected into the SiHa cell to investigate the underlying mechanism of miR-215-3p-SOX9 on cervical cancer tumorigenesis.

RESULTS: We used genome-wide gene expression analysis using clinical cervical cancer samples to identify that miR-215-3p was down-regulated in cervical cancer. We then collected 31 pairs of cervical cancer and the corresponding non-cancerous tissues to determine miR-215-3p level and indicated that miR-215-3p was significantly down-expressed in cervical cancer. Furthermore, the functional analysis suggested that over-expression of miR-215-3p suppressed the aggressiveness of SiHa cell, whereas down-regulation led to the opposite results. We identified SOX9 as a direct target of miR-215-3p, and its level was negatively related to the level of miR-215-3p in cervical carcinoma tissue. Up-regulation of SOX9 reversed the suppressive impact of miR-215-3p on cervical carcinoma cell, and down-regulation of SOX9 reversed the promote effects of miR-215-3p

CONCLUSIONS: These findings showed the important role of the miR-215-3p/SOX9 axis in the progression of cervical carcinoma.

Key Words:
miR-215-3p, SOX9, Cervical cancer, Growth, Metastasis.

Introduction

Despite improvements in early diagnosis, surgical techniques, and chemotherapy, the local infiltration and metastasis account for the unfavorable prognosis of patients with cervical cancer[1-3]. Unfortunately, little is known concerning the reasons for the aggressiveness of and dismal prognosis for cervical cancer. Therefore, it is crucial to investigate the mechanisms that regulate cervical cancer metastasis in order to improve cervical cancer treatment[4-6]. During the metastatic process, the primary cancer cells undergo the following steps sequentially: migration, invasion, intravasation, extravasation and colonization at distant tissues[7]. To prevent cancer metastasis, it is essential to identify the mechanisms triggering the cervical cancer metastatic process.

MicroRNA (miRNA), which is a small non-coding RNA, regulates the gene expression through inhibiting mRNA translation[8,9]. A substantive amount of researches demonstrate that dysregulated miRNAs are associated with cancer cell proliferation, apoptosis, invasion, and chemo-sensitivity[10-12]. Sex-determining region Y-box 9 (SOX9) plays important roles in the organ development, embryogenesis, and maintenance of stem cells[13]. Previous investigations

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indicated that dysregulation of SOX9 participates into the progression of several types of cancers as an oncogene, including inhibits senescence, promotes cell proliferation and facilitates transformation[14]. Furthermore, the up-regulation of SOX9 has been proved to confer the properties of cancer stem cell (CSC) and correlates with epithelial-mesenchymal transition (EMT) *via* targeting the WNT/β-catenin pathway[15,16]. In addition, SOX9 has been observed been up-regulated in esophageal cancer and correlating with the unfavorable prognosis of patients with esophageal cancer[17,18]. Moreover, SOX9 regulates the self-renewal and tumorigenicity of hepatocellular carcinoma through promoting the symmetrical cell division of cancer stem cell[19]. Altogether, these investigations suggest that SOX9 is involved in the development of multiple tumor types, but its function in regulating cervical carcinoma development remains to be explained[20].

In this study, we demonstrated that miR-215-3p was down-expressed in cervical cancer and miR-215-3p acted as a suppressive miRNA in cervical cancer. The bioinformatic analysis and luciferase reporter gene assay identified that SOX9 was the potential target of miR-215-3p. Up-regulation of miR-215-3p inhibited the expression of SOX9 in cervical carcinoma SiHa cell and inhibited the growth, migration and invasion as well as the metastasis of cervical cancer cells. However, the down-regulation of miR-215a-3p led to the comple opposite results. In conclusion, our study provided the novel insights of the miR-215-3p-SOX9 in the progression of cervical carcinoma.

Materials and Methods

Cervical Cancer Tissues and Cells

31 pairs of cervical cancer tissues and adjacent normal tissues were obtained from the Fifth Affiliated Hospital of Sun Yat-sen University. Cervical cancer and corresponding normal tissues were frozen in liquid nitrogen. Tissue sections (4 μm thick) were non-specifically blocked with goat serum, followed by overnight incubation with anti-SOX9 (1:100; Beyotime Biotechnology, Nanjing, China) and then incubated with FITC-labeled secondary antibody (Beyotime Biotechnology) for 1.5 h. Finally, the sections were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Human cervical cancer cell lines (C-33A, SiHa, Ca-Ski, C-4-I), HEK293T and H8, a human normal cervical surface epithelial cell line used in the study were purchased from Cobioer Biotechnology Co., Ltd (Nanjing, Jiangsu, China). The cells were cultured in RPMI-1640 or DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and penicillin/streptomycin at 37°C in an atmosphere containing 5% CO_2 and 95% air.

Cell Transfection

MiR-215-3p mimics or miR-215-3p inhibitor (miR-215-3p^{inhi}) (Thermo Fisher Scientific, Waltham, MA, USA) was transfected into SiHa cell using Lipofectamine® 2000 (Thermo Fisher Scientific). The siRNA SOX9 (siSOX9) and the negative control (siCon) were obtained from GenePharma (Shanghai, China). The SOX9 overexpressing plasmid was constructed using pCDNA3.1(+) basic vector and was synthesized by GenePharma. Plasmids were transfected into SiHa cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) Assay

The growth of indicated the cells was analyzed using MTT (Thermo Fisher Scientific, Waltham, MA, USA) assay. 100 μl SiHa cells (2×10^3) were plated into 96-well plates. The medium was renewed after the cell was cultured for 24 h, 48 h, 72 h, and 96 h. MTT solution (100 μl) was added to 96 well plates and the cell was incubated for 4 h. The OD value was detected at 450 nm using Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA).

Migration Assay

SiHa cell was cultured into 6 well plates for 24 h. Then, a scratch was generated using a sterile a 100 μL pipette tip. The non-adhesion cells were removed using PBS. The cell was cultured with medium without serum for 24 h[21].

Invasion Assay

The cell invasion ability was evaluated using 24-well transwell chamber (8 μm; Corning, Painted Post, NY, USA). Matrigel (BD Biosciences, San Diego, CA, USA) was pre-coated onto the upper chamber. 300 μl SiHa cells (2×10^4) were cultured into the upper chamber of a 24 well Millipore transwell chamber (Millipore, Braunschweig, Germany), and 600 μL medium

containing 20% FBS was added into the lower chamber. After 24 hours, the invaded cells were stained using crystal violet (Sigma, Shanghai, China) and were counted[22].

Luciferase Reporter Gene Assay

The wild-type (WT) or mutant type (MUT) 3'-UTR of SOX9 containing miR-215-3p binding sites was cloned into the psiCHECK™-2 vector (Promega, Madison, WI, USA). HEK293T cell was cotransfected with miR-215-3p and the SOX9-3'-UTR reporter plasmid using the Lipofectamine 3000 (Invitrogene, Carlsbad CA, USA). After 48 hours, the luciferase activities were detected using a luciferase reporter assay kit (Promega, Madison, WI, USA).

Quantitative real-time PCR (qRT-PCR) Assay

RNAs were extracted using TRIzol reagent (TakaraBio, Tokyo, Japan). The cDNA was synthesized using ABI 7500 PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences are as follows: SOX9, forward primer: 5'-AGCGAACGCACATCAAGAC-3', reverse primer: 5'-CTGTAGGCGATCTGTTGGGG-3'; GAPDH, forward primer: 5'-TGGATTTGGACGCATTGGTC-3', reverse primer: 5'-TTTGCACTGGTACGTGTTGATA-3'; miR-215-3p, forward primer: 5'-TGGATTTGGACGCATTGGTC-3', reverse primer: 5'-TTTGCACTGGTACGTGTTGATA-3'. The levels of miR-215-3p and SOX9 were normalized to U6 or GAPDH.

Immunoblotting Analysis

25 μg cell lysates were loaded on 8% SDS-PAGE and were transferred onto PVDF membranes. PVDF membranes were incubated with SOX9 antibody (Signalway Antibody, Nanjing, Jiangsu, China) and β-actin (Epitomics, Burlingame, CA, USA) followed by incubation with horseradish peroxidase-conjugated IgGs (Bioworld Biotechnology, Wuhan, Hubei, China). Target proteins were assessed using the ECL system (Millipore, Braunschweig, Germany) and visualized with the ChemiDoc XRS system (Bio-Rad, Shanghai, China).

In Vivo Animal Experiments

BALB/c nude mice were subcutaneously inoculated with tumor cells (1×10^6 per mouse). Nude mice were sacrificed 25 days later, and the tumors were removed. Tumor volume (Volume = 0.5 × tumor length × tumor width^2) was detected every 3 days using calipers. Tail-injected animals were sacrificed 4 weeks after injection, and lung tissues were removed. Lung tissue was fixed in 10% buffered formalin, immersed in an ascending series of alcohol washes, and paraffin embedded. The tissues were then sectioned (4-μm thick) and stained with hematoxylin and eosin (H&E). All procedures involving mice were conducted in accordance with the Fifth Affiliated Hospital of Sun Yat-sen University.

Statistical Analysis

SPSS 17.0 was used for all the statistical analyses. One-way ANOVA analysis of difference was used for comparisons among multiple groups, followed by Student's *post hoc* two-tailed *t*-test. Student's unpaired two-tailed tests were used for comparisons between two groups. The Pearson correlation was applied to analyze the relation between the miR-215-3p and SOX9. All values were represented as mean ± SD. $p<0.05$ was considered statistically significant.

Results

MiR-215-3p is Down-regulated in Cervical Carcinoma

To investigate the dysregulation of miRNA in cervical carcinoma, the GEO dataset, GSE81137 that containing cervical carcinoma tissues, as well as normal tissues, was selected to explore the expression patterns of miRNAs. As shown in Figure 1A, the heatmap that was generated using differential levels of miRNAs indicated that miR-215-3p was significantly down-expressed in cervical carcinoma. Next, we analyzed the levels of miR-215-3p in cervical carcinoma and control normal tissues using qRT-PCR analysis. As expected, miR-215-3p was markedly down-expressed in cervical cancer tissues (Figure 1B). Consistently, miR-215-3p was also significantly down-expressed in cervical cancer cells compared with normal cell (Figure 1C). Next, three bioinformatics prediction tools (TargetScan, miRanda and mirSVR) were used to reveal the potential target gene of miR-215-3p. As shown in Figure 1D, the 3'-UTR of SOX9 had sequences that bound to miR-215-3p. To future prove that SOX9 was the direct target gene of miR-215-3p, the luciferase assay using HEK293T cell that was cotransfected with wild type (WT)-SOX9 and miR-215-3p or cotransfected with miR-215-3p and mutant type (MUT)-SOX9 was conducted.

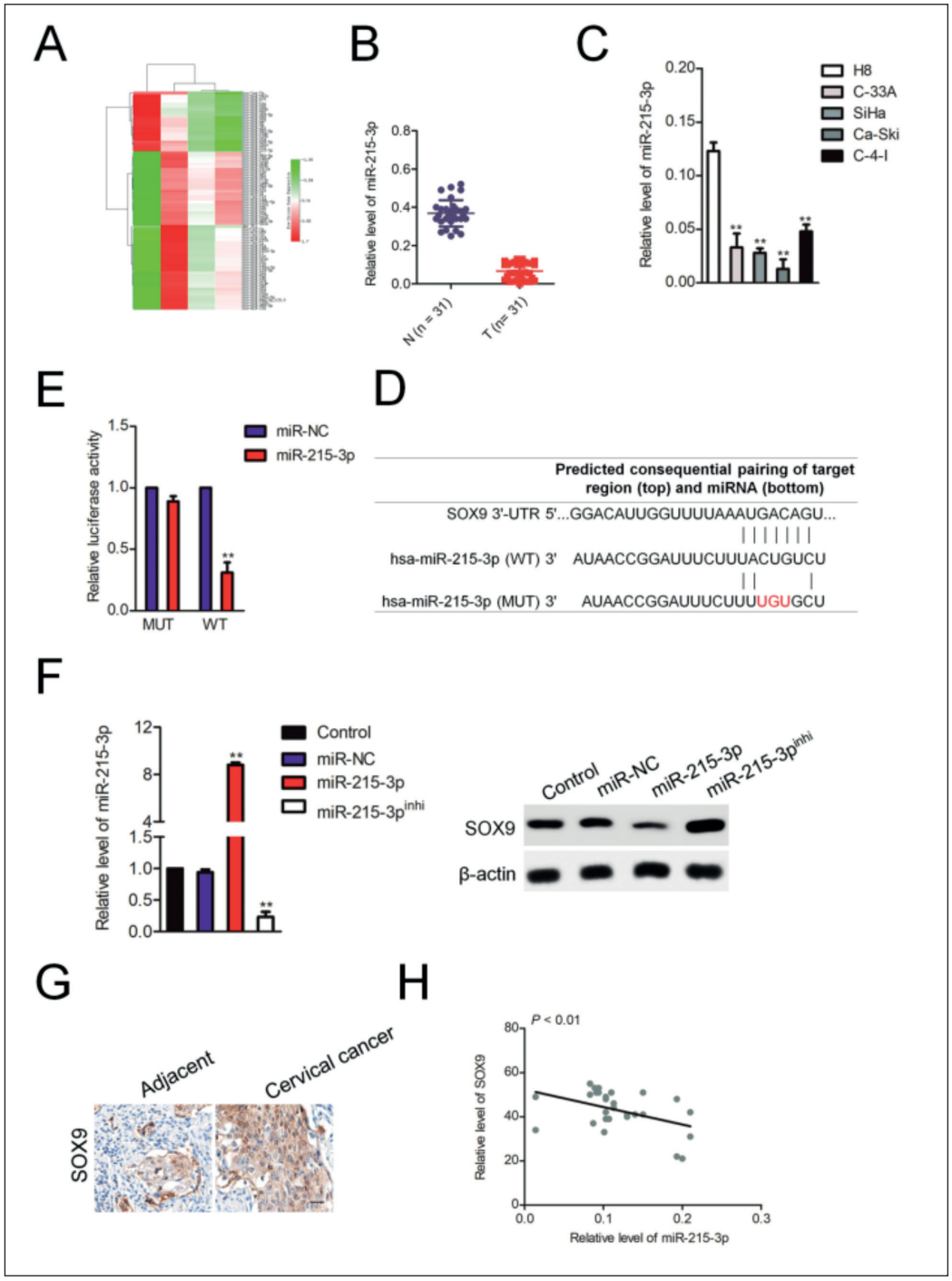

Figure 1. MiR-215-3p is down-regulated in cervical cancer samples and cells. **A,** Microarray analysis of miRNA expression in cervical cancer tissues from normal cervical tissues. **B,** The level of miR-215-3p in 31 adjacent normal control tissues (N) and 31 cervical cancer tissues (T) was determined by qRT-PCR. **C**, qRT-PCR analyzed the levels of miR-215-3p in cervical cancer cell lines. U6 was used as loading control. $^{**}p<0.01$ as compared to H8 cell. **D,** Schematic diagram of miR-215-3p binding sites in the SOX9 3′-UTR. Sequences were compared between the mature miR-215-3p and wild-type (WT) or mutant (MUT) putative target sites in the 3′-UTR of SOX9. **E,** HEK293T cells were co-transfected with the wild-type (WT) or mutant (MUT) SOX9 3′-UTR with miR-215-3p and the luciferase activity was examined. Firefly luciferase activity was measured and standardized by Renilla luciferase activity. $^{**}p<0.01$ as compared to miR-NC. **F,** SiHa cells were transfected with miR-215-3p and miR-215-3p inhibitor (miR-215-3p^{inhi}). SOX9 expression as determined by Western blotting assay. $^{**}p<0.01$ as compared to control. **G,** Immunohistochemical staining of SOX9 in normal human cervical cancer tissue and corresponding normal tissues. **H,** The correlation of miR-215-3p and SOX9 in tumor samples was analyzed by qRT-PCR assay.

The result indicated that miR-215-3p remarkably reduced the luciferase activity in HEK293T cell that was cotransfected with the WT 3'-UTR of SOX9 and miR-215-3p (Figure 1E). Then, the expression of SOX9 in SiHa cell transfected with miR-215-3p was analyzed using Western blotting assay. Up-regulation of miR-215-3p reduced the expression of SOX9 in SiHa cell (Figure 1F). Next, we examined the levels of SOX9 in cervical carcinoma tissues and corresponding normal tissues. The result revealed that SOX9 was over-expressed in cervical carcinoma in comparison with control of normal tissues (Figure 1G). Finally, the qRT-PCR analysis showed that the down-regulation miR-215-3p was negatively correlated with the overexpression of SOX9 in cervical carcinoma tissues (Figure 1H). All these findings suggested that miR-215-3p was down-expressed in cervical carcinoma.

Up-regulation of MiR-215-3p Inhibits the Aggressiveness of Cervical Carcinoma Cell

To future explore the impact of miR-215-3p in the progression of cervical carcinoma cell, SiHa cells were transfected with miR-215-3p or miR-215-3p inhibitor (miR-215-3p^{inhi}). As shown in Figure 1F, miR-215-3p mimic transfection increased the level of miR-215-3p whereas miR-215-3p inhibitor (miR-215-3p^{inhi}) reduced the level of miR-215-3p in SiHa cell. Then, the proliferation of SiHa cell was detected at 24 h, 48 h, 72 h or 96 h using the MTT assay. We found that up-regulation of miR-215-3p inhibited the growth of SiHa cell whereas miR-215-3p^{inhi} increased cell proliferation (Figure 2A). Consistently, the colony formation assay indicated that miR-215-3p inhibited the colony formation of cervical carcinoma cell *in vitro* whereas the down-regulation of miR215-

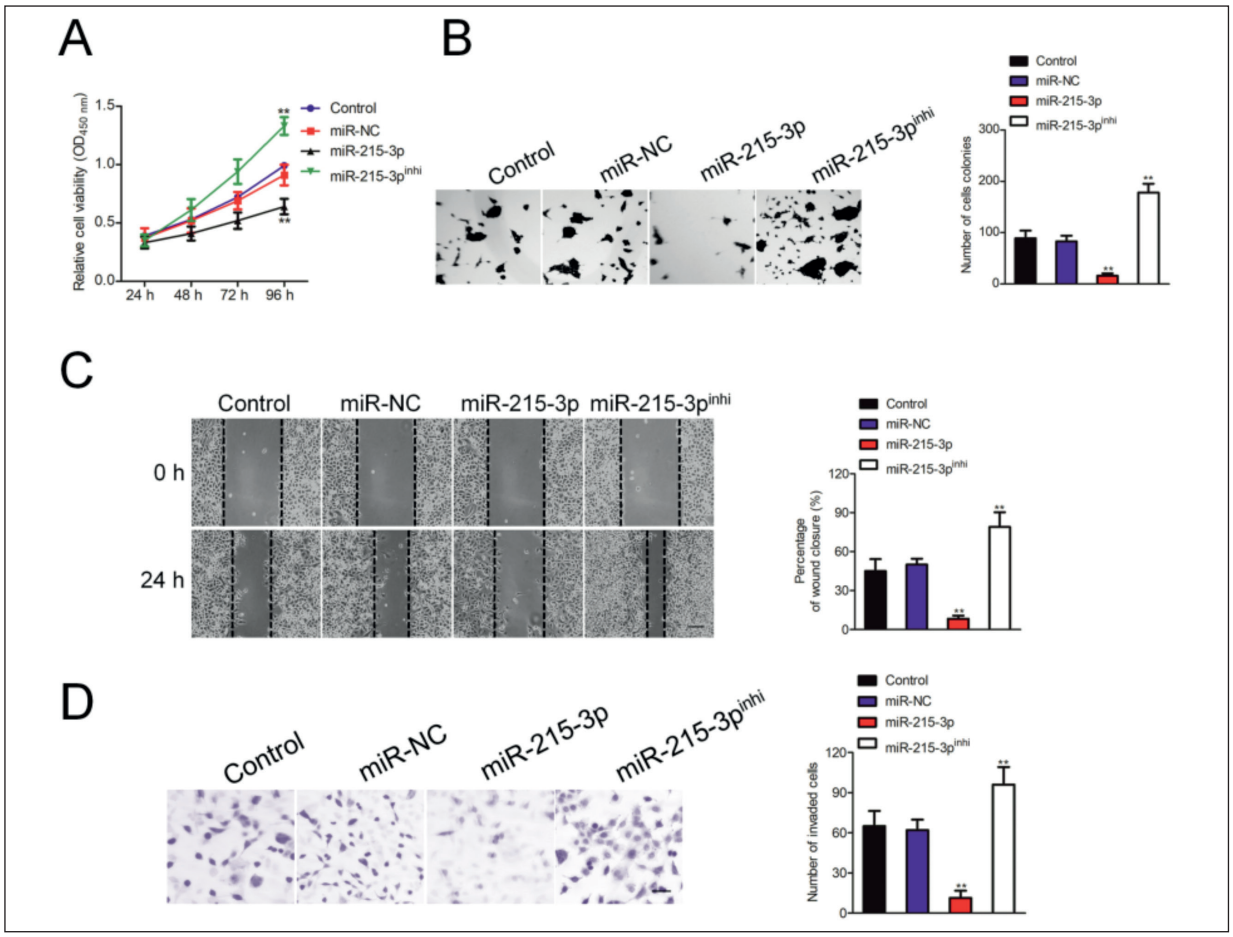

Figure 2. Effect of miR-215-3p in growth, migration and invasion in SiHa cells. **A,** Cells were transfected with miR-215-3p mimic or miR-215-3p^{inhi}. Cell proliferation rates were determined by the MTT assay. **B,** Colony formation assays of SiHa cells. Representative images for each group were shown. **C,** SiHa cells were transfected with miR-215-3p mimic or miR-215-3p^{inhi} and the ability of migration in cervical cancer cells was determined by wound closure assay. Scale bar: 200 μm. **D,** The invasion of SiHa cells was determined by transwell assay. $^{**}p<0.01$ as compared to control cell. Scale bar: 200 μm.

5p resulted in opposite outcomes (Figure 2B). Next, we evaluated the role of miR-215-3p on the migration and invasion abilities of SiHa cells. As shown in Figure 2C-2D, the results from wound healing and transwell invasion assay indicated that the over-regulation of miR-215-3p inhibited cell mobility whereas miR-215-3p inhibitor accelerated the invasion and migration of SiHa cell.

Down-regulation of SOX9 Inhibits the Growth and Metastasis of Cervical Cancer Cell

To reveal the role of SOX9 in cervical carcinoma, SOX9 was knocked-down using the specific siRNA against SOX9 (siSOX9) and the expression of SOX9 was assessed using immunoblotting assay. We found that the level of SOX9 was significantly reduced in siSOX9 transfected SiHa cells (Figure 3A). The proliferation and colony formation of SiHa cells was significantly inhibited by siSOX9 *in vitro* (Figure 3B-3C). Consistently, the migration and invasion abilities of SiHa cell were remarkably decreased by siSOX9 (Figure 3D-3E). An experimental lung metastasis model was constructed to future explore the function of SOX9 in the metastasis of cervical cancer cells *in vivo*. As shown in Figure 3F, down-expression of SOX9 inhibited the lung metastasis of SiHa cell. We also analyzed the impact of SOX9 on the tumor growth of SiHa cells *in vivo*. Parental or SiHa cell that was transfected with siSOX9 was injected subcutaneously into nude mice. As shown in Figure 3G, down-expression of SOX9 significantly suppressed the size and weight of

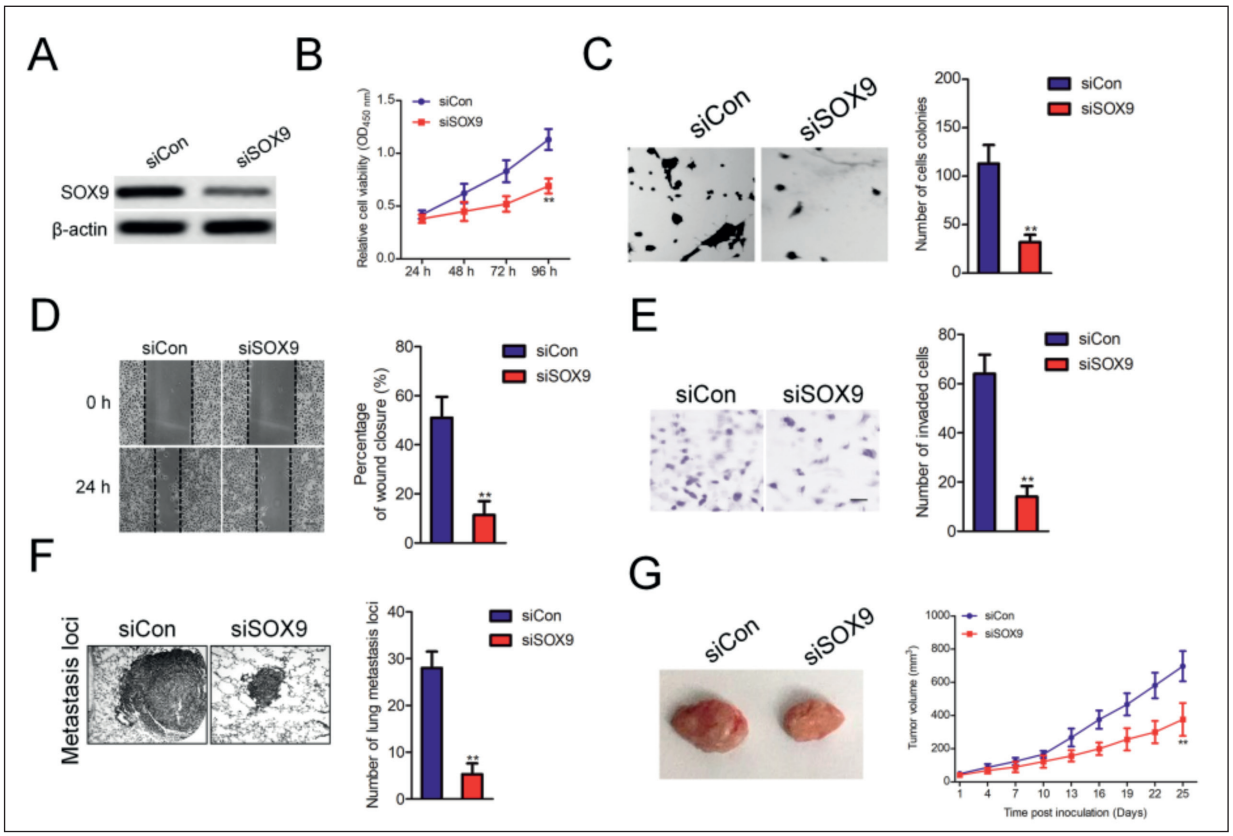

Figure 3. Loss of expression of SOX9 inhibits cervical cancer cells growth and metastasis. **A,** SiHa cells were transfected with either the negative control siRNA (siCon) or siSOX9. The expression levels of SOX9 were detected by Western blotting assay. **B,** After transfected with siCon or siSOX9, the cell proliferation rates were determined by the MTT assay. **C,** Colony formation assays of SiHa cells. **D,** SiHa cells were transfected with siSOX9 and the ability of migration was determined by wound scratch assay. Scale bar: 200 μm. **E,** Cervical cancer cells with silent expression of SOX9 exhibit less invasive abilities in Transwell invasion assay. The invaded cells were stained with crystal violet and counted. Scale bar: 200 μm. **F,** siSOX9 cells or control cells were injected into nude mice *via* lateral vein. Representative pictures of lungs from mice were taken after four weeks. Numbers of lung metastasis were quantified. **G,** Knocked-down of SOX9 inhibited the growth of cervical cancer cells-engrafted tumors. Representative tumors were photographed at 25 days after mice inoculation with siSOX9 or control cells. $^{**}p<0.01$ as compared to siCon.

tumor tissue from mice that were injected SOX-9 down-expressing cell. These results indicated that down-regulation of SOX9 inhibited the tumor growth and lung metastasis of SiHa cell.

Over-expression of SOX9 Rescues the Effects of MiR-215-3p in Cervical Cancer Cell

To investigate the potential influence of SOX9 in the growth, migration, and invasion of cervical cancer cells regulated by miR-215-3p, we assessed whether the suppressive impact of miR-215-3p on SiHa cell could rescue by over-expression of SOX9. Firstly, SiHa cells were cotransfected with miR-215-3p and SOX9 plasmid and the expression of SOX9 was assessed using qRT-PCR assay (Figure 4A). Then, the MTT and colony formation experiment suggested that the up-regulation of SOX9 rescued the suppressive effect of miR-215-3p on the proliferation and colony formation of SiHa cells *in vitro* (Figure 4B-4C). Consistently, the migration and invasive abilities of SiHa which were inhibited by miR-215-3p transfection were significantly rescued by the up-regulation of SOX9 (Figure 4D-4E).

Down-regulation of SOX9 Reverses the Impact of MiR-215-3plinhibitor on Cervical Cancer Cell

Next, we future showed the suppressive influence of miR-215-3p on the growth and aggressiveness of SiHa cell dependent on SOX9. SiHa cells were cotransfected with miR-215-3p^{inhi} and/or SOX9 siRNA (siSOX9) and the mRNA level of SOX9 was measured using qRT-PCR analysis (Figure 5A). Then, the MTT and colony formation tests were applied to the analysis, the growth and colony formation of SiHa cells *in vitro*. As shown in Figure 5B-5C, down-regulation of SOX9 reversed the effects of miR-215-3p^{inhi} on the proliferation and colony formation of SiHa cell. Consistently, the migration and invasion abilities of SiHa cell that was cotransfected with siSOX9 and miR-215-3p^{inhi} were restored compared to the cell that was transfected with miR-215-3p^{inhi} alone (Figure 5D-5E). All these findings suggested that miR-215-3p suppressed the growth and aggressiveness of SiHa cell through targeting SOX9.

Up-regulation of MiR-215-3p Inhibits the Progression of Cervical Cancer Cell in Vivo

Finally, we examine the role of miR-215-3p on the growth and distant metastasis of SiHa cell *in vivo*. The nude mice were subcutaneously inoculated with a cervical cancer SiHa cell that was transfected with miR-NC or miR-215-3p. As shown in Figure 6A, the tumor that was injected growth in mice with miR-215-3p-overexpressing cell was significantly inhibited compared to the control group. Furthermore, miR-215-3p was up-regulated and SOX9 was down-regulated in the tumor tissues from the mice inoculated with miR-215-3p-overexpressing cervical cancer cells when compared to the miR-NC group (Figure 6B). Subsequently, we analyzed the expression of SOX9 in the tumor tissue using immunohistochemistry (ICH) assay. As shown in Figure 6C, the expression of SOX9 was significantly reduced in tumor tissue that was formed by miR-215-3p-overexpressing SiHa cell compared to that in tumor tissue that was formed by the control cell. To reveal the impact of miR-215-3p on the metastasis of SiHa cell *in vivo*, cervical cancer SiHa cell transfected with miR-215-3p or miR-NC were injected into nude mice *via* tail vein. After four weeks, the number of metastatic nodes in the lung tissue from nude mice that was injected with miR-215-3p transfected cell were remarkably reduced compared to that in the mice that was injected with control cell (Figure 6D). These results suggested that up-regulation of miR-215-3p inhibited the growth and lung metastasis of SiHa cell *in vivo*.

Discussion

Previous investigations have demonstrated that miRNAs are involved in the growth and metastasis of various types of cancers[23]. MiRNAs act as either tumorigenic or tumor-suppressing genes[24,25]. Metastasis is characteristic of cervical cancer and a crucial step in cervical cancer progression. Although, a substantial amount of evidence indicates that miRNAs contribute to tumor development, the function of miR-215-3p in cervical cancer metastasis still remains undefined. Our study identified that miR-215-3p acted as a suppressor gene in cervical carcinoma and regulated the progression of cervical carcinoma cells.

In this study, we verified that miR-215-3p was remarkably down-expressed in cervical carcinoma. Furthermore, we demonstrated that the up-regulation of miR-215-3p markedly inhibited the cervical carcinoma cell growth and metastatic phenotypes. Moreover, the down-regulation of miR-215-3p significantly promoted cervical

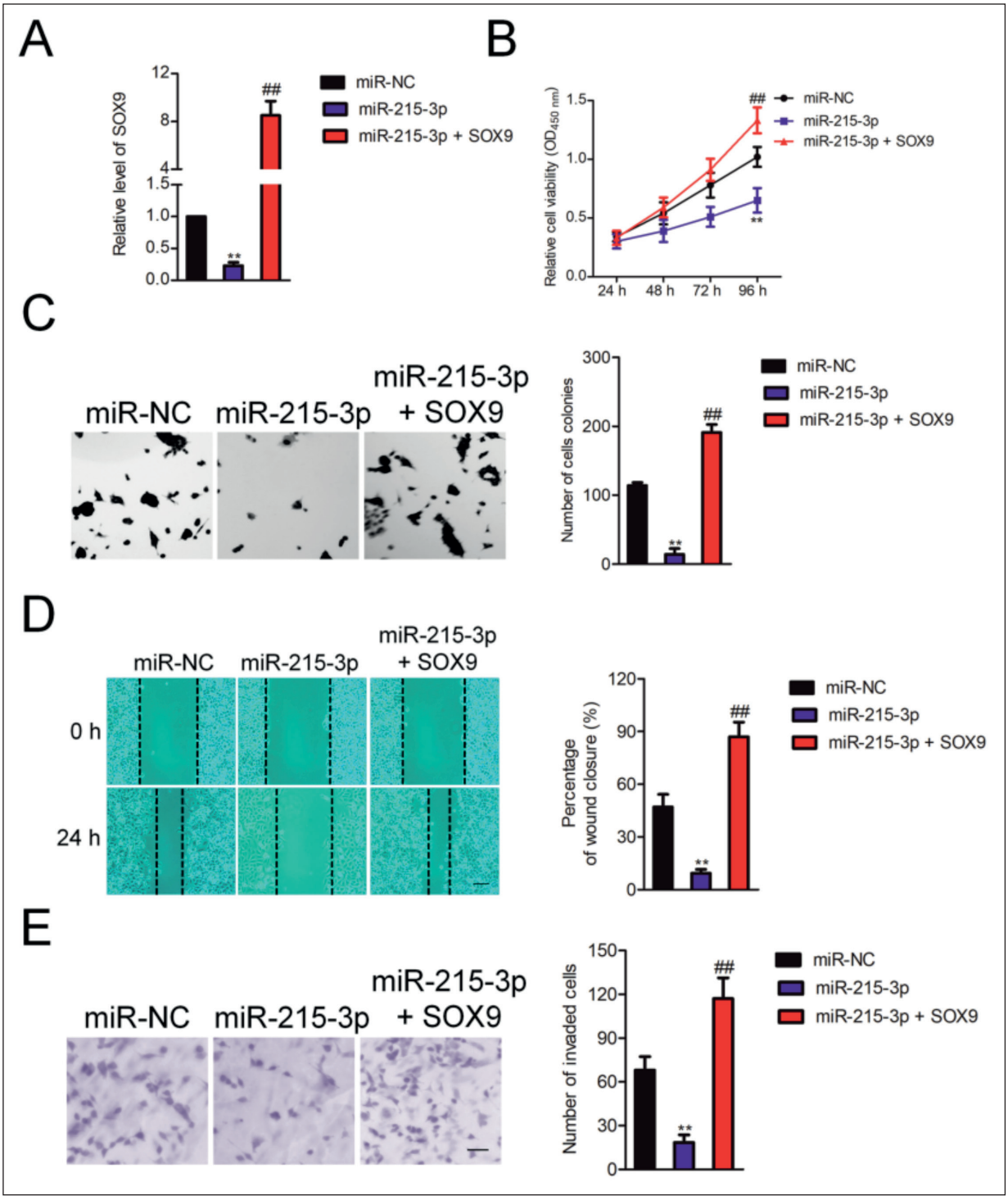

Figure 4. Up-regulation of SOX9 rescues the effects of miR-215-3p. **A,** SiHa cells were transfected with miR-215-3p mimic, or co-transfected with SOX9 over-expression plasmid and miR-215-3p mimic. The mRNA level of SOX9 was detected by qRT-PCR assay. **B,** Cells were transfected with miR-215-3p mimic alone, or co-transfected with SOX9 and miR-215-3p mimic, and then were seeded into 96 well plates. After 24 h, 48 h, 72 h and 96 h, the MTT assay was performed to analysis cell proliferation. **C**. Colony formation assay of SiHa cells. **D,** SiHa cells co-transfected with SOX9 over-expression plasmid and miR-215-3p were subjected to wound healing assay and images were taken at 0 and 24 h (left panel). The percentage of wound closure was quantified (right panel). **E,** Transwell invasion assay was performed after transfection of SiHa cells with SOX9 plasmid and miR-215-3p mimic. The invaded cells were stained with crystal violet and counted. $^{**}p<0.01$ as compared to control, $^{\#\#}p<0.01$ as compared to miR-215-3p.

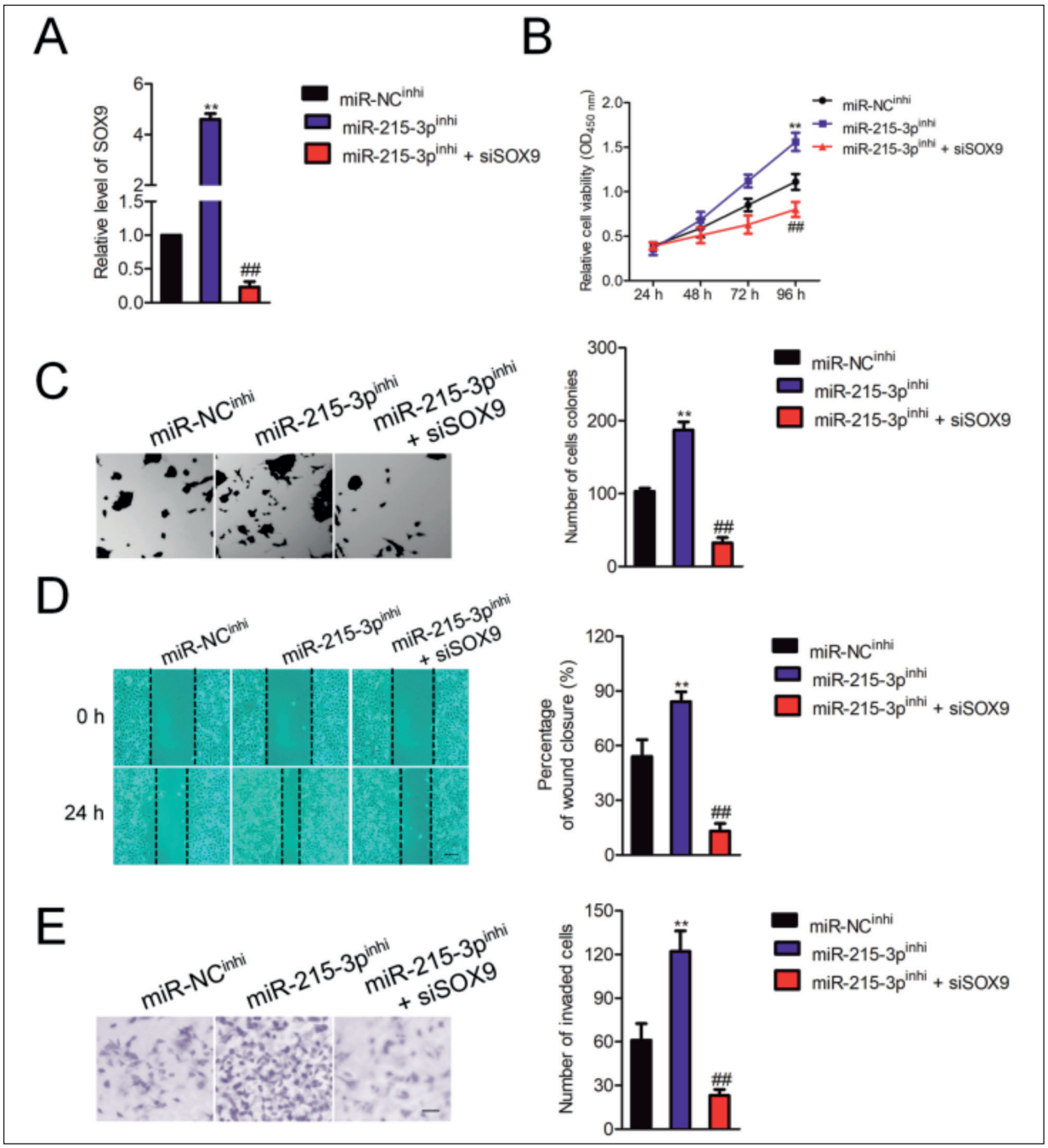

Figure 5. SOX9 knocked-down reversed the effects of miR-215-3p inhibition in cervical cancer. **A,** SiHa cells were transfected with miR-215-3p^{inhi}, co-transfected with siSOX9 and miR-215-3p^{inhi}. The expression of SOX9 was determined by qRT-PCR analysis. **B,** SiHa cells were transfected with miR-215-3p^{inhi}, or co-transfected with siSOX9 and miR-215-3p^{inhi}, and then were seeded into 96 well plates. After 24 h, 48 h, 72 h and 96 h, the MTT assay was performed to analysis cell proliferation. **C,** Colony formation assays of SiHa cells. **D,** SiHa cells co-transfected with siSOX9 and anti-miR-215-3p were subjected to wound closure assay. The percentage of wound closure was quantified (right panel). Scale bar: 200 μm. **E,** Transwell invasion assay was performed after transfection of SiHa cells with siSOX9 and miR-215-3p^{inhi}. The invaded cells were stained with crystal violet and counted. Scale bar: 200 μm. $^{**}p<0.01$ as compared to control and $^{\#\#}p<0.01$ as compared to miR-215-3p^{inhi}.

carcinoma cell growth, migration as well as invasion *in vitro*. Meanwhile, over-expression of miR-215-3p inhibited the tumorigenicity of cervical carcinoma cell *in vivo*. Additionally, metastatic nodules in the lung from mice that was inoculated with miR-215-3p-transfected cells were markedly

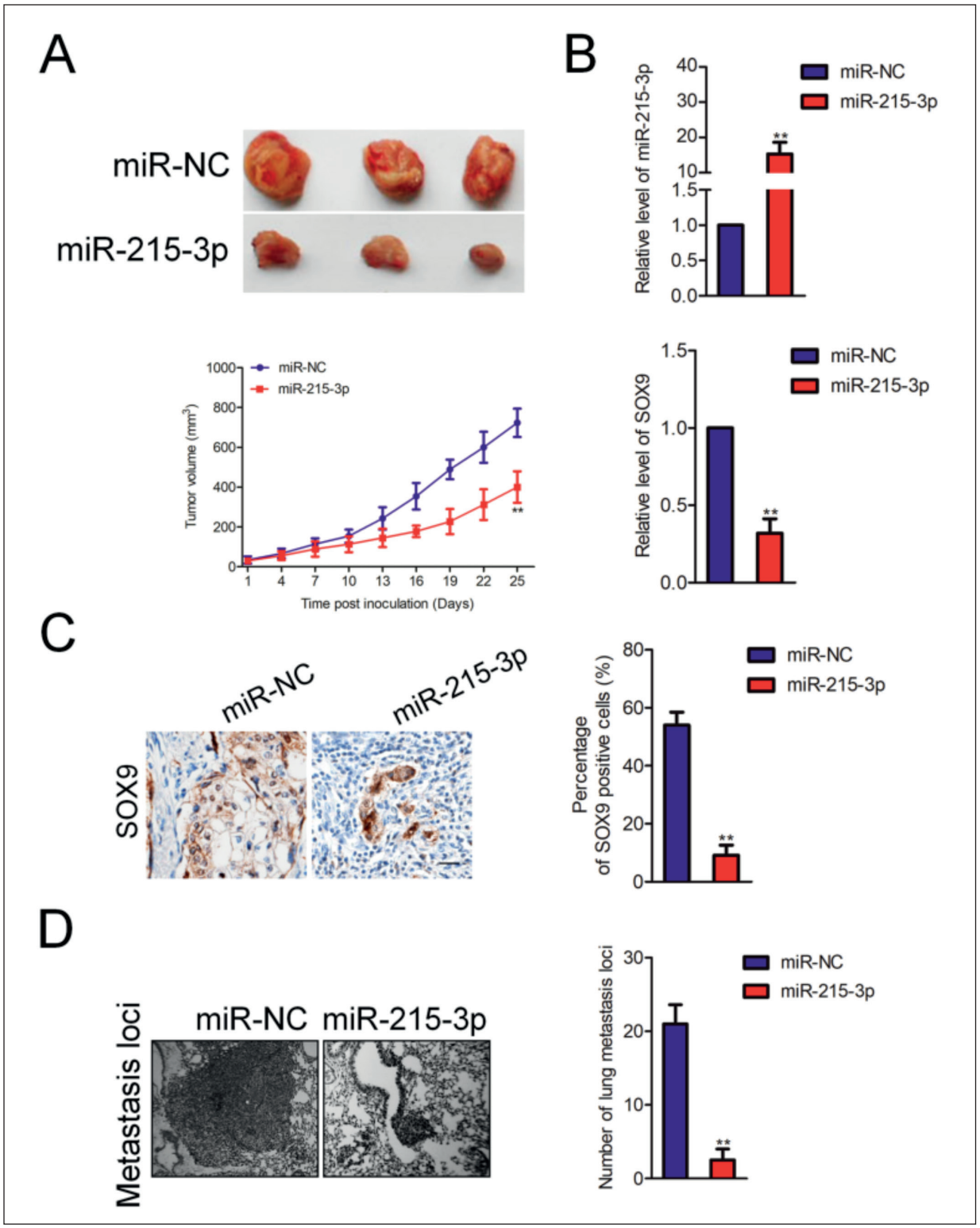

Figure 6. Up-regulation of miR-215-3p inhibits cervical cancer cells growth and metastasis *in vivo*. **A,** miR-215-3p-stably over-expressing or parental cervical cancer cells were injected into nude mice by subcutaneous. Tumor volumes were measured and growth curves were generated. **B,** The expression of miR-215-3p and SOX9 was determined by qRT-PCR assay in the tumor tissues. **C,** The expression of SOX9 in tumor tissue was detected by immunohistochemistry assay, respectively. **D,** The numbers of lung metastatic nodes of lungs from mice induced by miR-215-3p or miR-NC transfetced cervical cancer cells were displayed. Histopathology of metastasis was determined with H&E staining. $^{**}p<0.01$ compared to control group.

fewer than those in control. Mechanistically, we determined that SOX9 was the target gene of miR-215-3p, and its level was negatively associated with the level of miR-215-3p in cervical cancer.

As non-coding RNAs, miRNAs execute functions by targeting several protein-coding genes[26]. There are more and more studies have indicated that SOX9 is over-regulated in various cancers, including colorectal cancer, prostate cancer and hepatocellular carcinoma[13,15,19]. In cervical cancer, SOX9 inhibits the growth of cervical tumor cells *via* trans-activating cyclin-dependent protein kinase inhibitor (CDKI)[27]. Consistent with the previous investigations, we studied the function of SOX9 in cervical cancer and proved that its knockdown significantly repressed cervical cancer cell growth, migration, and invasion.

Finally, we revealed that over-expression of SOX9 rescued the impacts of miR-215-3p in cervical cancer cell and down-regulation of SOX9 reversed the impact of miR-215-3p inhibitor on cervical cancer, which indicated that miR-215-3p inhibited the growth as well as metastasis of cervical carcinoma cell dependent on SOX9.

Conclusions

We demonstrated that miR-215-3p inhibits the growth, invasion and metastasis of cervical cancer cell *via* regulating SOX9. These results reveal the vital function of the miR-215-3p/SOX9 axis in regulating cervical cancer cell growth and aggressiveness.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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