

miR-146a regulates the function of Th17 cell differentiation to modulate cervical cancer cell growth and apoptosis through NF- κ B signaling by targeting TRAF6

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Abstract. The aim of the present study was to investigate whether miRNA-146a regulated the function of Th17 cell differentiation to modulate cervical cancer cell growth and apoptosis. miR-146a expression was increased in human cervical cancer. Both overall survival (OS) and disease-free survival (DFS) of low miR-146a expression were higher than those of high miR-146a expression. Additionally, IL-17a expression was lower in patients with high miR-146a expression compared to that of patients with lower miR-146a expression. In a co-culture of cervical cancer and CD4⁺ T cells, downregulation of miR-146a inhibited cell growth and induced apoptosis of cervical cancer cells, while overexpression of miR-146a promoted cell growth and reduced apoptosis of cervical cancer cells. Downregulation of miR-146a induced TRAF6 and NF- κ B protein expression, increased IL-6, IL-17A and IL-21 levels, and enhanced p-STAT3 protein expression. The inhibition of TRAF6 attenuated the effects of anti-miR-146a on the function of Th17 cell differentiation to modulate cervical cancer cell growth and apoptosis. Collectively, miR-146a regulated the function of Th17 cell differentiation to modulate cervical cancer cell growth and apoptosis through NF- κ B signaling by targeting TRAF6. miR-146a may function as an oncogene in cervical cancer via Th17 cell differentiation by targeting TRAF6.

Introduction

Cervical cancer is one of the most common gynecological malignancies. Its morbidity is ranked in the third place among global female malignancies (1). Furthermore, it is ranked in the second place in developing countries in terms of both morbidity and mortality, only second to breast cancer. In recent years, the morbidity of cervical cancer is constantly increasing and exhibits a younger trend along with shortened course of disease (2). Cervical cancer screening has been extensively popularized (2). However, a large number of patients are diagnosed with invasive cervical cancer. It has currently been ascertained that the genesis of cervical cancer is related to the persistent infection of high risk human papilloma virus (HPV) (3). According to statistics, HPV infection has resulted in 99.7% of cervical cancer cases (3).

Currently, persistent HPV infection has been revealed to be the initial step of cervical cancer genesis. The genesis and development of cervical cancer is the result of a joint action of multiple factors. Existing studies indicate that, epigenetic modification plays a key role in tumor genesis and development (4). MicroRNAs (miRNAs) are important factors involved in that process. The role of miRNAs in cervical cancer has been determined with increasingly profound basic research on miRNAs (5). Research has revealed that numerous miRNAs are involved in regulating multiple biological processes of cervical cancer, such as proliferation, apoptosis, invasion and metastasis (5). In addition, it is closely correlated with the susceptibility and clinical prognosis of cervical cancer. miRNAs can regulate the expression of both oncogenes and tumor-suppressor genes (5), thus promoting or inhibiting cancer genesis (6).

Th17 cells are a subgroup of helper T cells that has attracted wide attention in recent years (7). Its roles in inflammatory response and autoimmune disease have been determined (7). In recent years, the role of Th17 cells in tumors has also been studied. In gastric cancer, Th17 cells were determined to be markedly increased in peripheral blood (8), which was more obvious in stage III-IV gastric cancer patients than in early-stage patients. This suggests that Th17 cells are related to

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tumor development and are negatively correlated with patient survival (9). IL-17a is the primary effector secreted by Th17 cells that has a potent pro-inflammatory effect. Research has revealed that its role in tumors is closely related to the enhanced formation of tumor microvessels (9).

Tumor necrosis factor receptor-associated factors (TRAFs) are important adaptor molecules. They play a key role in multiple signaling pathways. There are 7 members (TRAF1-7) in the TRAF family. TRAF1 and TRAF2 were the two first discovered members. They serve as the signal transduction proteins of tumor necrosis factor receptor II (TNFR2). Subsequently, the other 5 family members (TRAF3-7) were successively discovered. TRAF6-activated downstream signals mainly include NF- κ B and AP-1. These two are involved in the transcription and expression of multiple genes *in vivo* (including inflammation- and anti-apoptosis-related genes). Therefore, TRAF6 also possesses extensive biological functions. It not only participates in regulating natural immune response, but it is also closely related to the maintenance of T-cell peripheral immune tolerance. In addition, it can take part in the maturation and differentiation of an osteoclast *in vitro*. Recent research revealed that TRAF6 also plays a critical role in tumor genesis, development, invasion and metastasis. The aim of the present study was to investigate whether miRNA-146a regulates the function of Th17 cell differentiation to modulate cervical cancer cell growth and apoptosis.

Materials and methods

Patients with cervical cancer. The present hospital-based case-control study consisted of 68 female cervical cancer patients (range, 57-72 years) and 24 female cancer-free controls (range, 55-66 years). All patients and cancer-free controls were recruited from The Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, Guangdong), between January 2011 and January 2016. The cancer-free control subjects showed no evidence of a genetic relationship with the cases. All cervical cancer patients were undergoing surgery treatment. The cancer tissue samples were saved at -80°C to measure the expression of miRNA-146a. This study was approved by the Ethics Review Board of The Third Affiliated Hospital of Sun Yat-sen University and all patients provided written informed consent.

RNA isolation and quantitative RT-PCR (qPCR). Total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) from tumor tissue samples or cell samples. RNA in exosomes was extracted using the TOPscript Reverse Transcriptase (Enzymomics, Inc., Daejeon, Korea). qPCR was performed using the 7500 Fast Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) by HiFast Probe Lo-ROX and HiFast SYBR Lo-ROX Master Mix (PCR Biosystems, London, UK). The qPCR cycle was set to an initial 95°C for 10 min, 40 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Relative quantification analysis was performed using the comparative quantification cycle (Cq) method ($2^{-\Delta\Delta Cq}$) (10).

Isolation of tumor-infiltrated T cells. Cancer and peri-tumor tissues samples were cut into small pieces and digested in

RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.), 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml Collagenase type IV and 100 μ g/ml DNase. Dissociated cells were filtered using a 75- μ m cell strainer and mononuclear cells were washed and incubated with RPMI-1640 with 10% FBS. T cells were purified with anti-CD3 magnetic Dynabeads (Thermo Fisher Scientific, Inc.).

Exosome extraction from human serum. Blood samples were centrifuged at 1,000 x g for 10 min at 4°C and serum samples were collected. Exosome samples were isolated using a Total Exosome Isolation kit (Thermo Fisher Scientific, Inc.) and incubated with exosome isolation reagent for 30 min at 4°C. Then, the pellets were centrifuged at 1,000 x g for 10 min at 4°C and resuspended with phosphate-buffered saline (PBS).

In vitro culture of CD4⁺ T cells. Lentivirus with miRNA-146a, anti-miRNA-146a and control negative mimics were purchased from Shanghai GeneChem Co., Ltd., (Shanghai, China). Splenic cells were harvested from mice and then subjected to magnetic-activated cell sorting (MACS) using a Mouse Naive CD4⁺ T Cell Isolation kit (Miltenyi Biotec, Inc., Cambridge, MA, USA) according to the manufacturer's instructions. The STAT3 decoy ODN sequences were: 5-CATTTCCCGTAAATC-3 and 5-GATTTACGGGAAATG-3.

Cell lines and miRNA mimic transfection. Human cervical cancer cell line HeLa was purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained at 37°C in an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) with 10% FBS (American Amereseo, Solon, OH, USA). HeLa cells were transduced with lentivirus with miRNA-146a, anti-miRNA-146a, STAT3 decoy ODN, si-TRAF6 and control negative mimics. Transfected HeLa cells and CD4⁺ T cells were co-cultured with anti-CD3 (5 mg/ml) and anti-CD28 (2 mg/ml).

Cell viability and Transwell assays and the apoptosis rate by flow cytometer. Following transduction, cells (10⁵ cell/well) were seeded into a 96-well plate and 20 μ l of MTT was added to the cells. The cells were incubated for 4 h at 37°C and then, the medium was removed. Dimethyl sulphoxide (DMSO) solution was added to the cells for 20 min at 37°C. The absorbance was assessed using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 492 nm.

For the Transwell assay, following transduction, cells (1x10⁶ cells/ml) in serum-free medium containing 5% FBS were plated in the top chamber of each Transwell insert (Corning Costar, Corning, NY, USA) and incubated at 37°C for 48 h. Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab. Then, the cells that remained in the upper chambers were removed and the cells that had migrated into the bottom chambers and attached to the lower surface of the membrane were fixed and stained with dye solution containing 20% methanol violet and 0.1% crystal violet. The cells were then counted using an optical inverted microscope (magnification, x200; Nikon Corp., Tokyo, Japan).

For the apoptosis rate, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min. The cells were

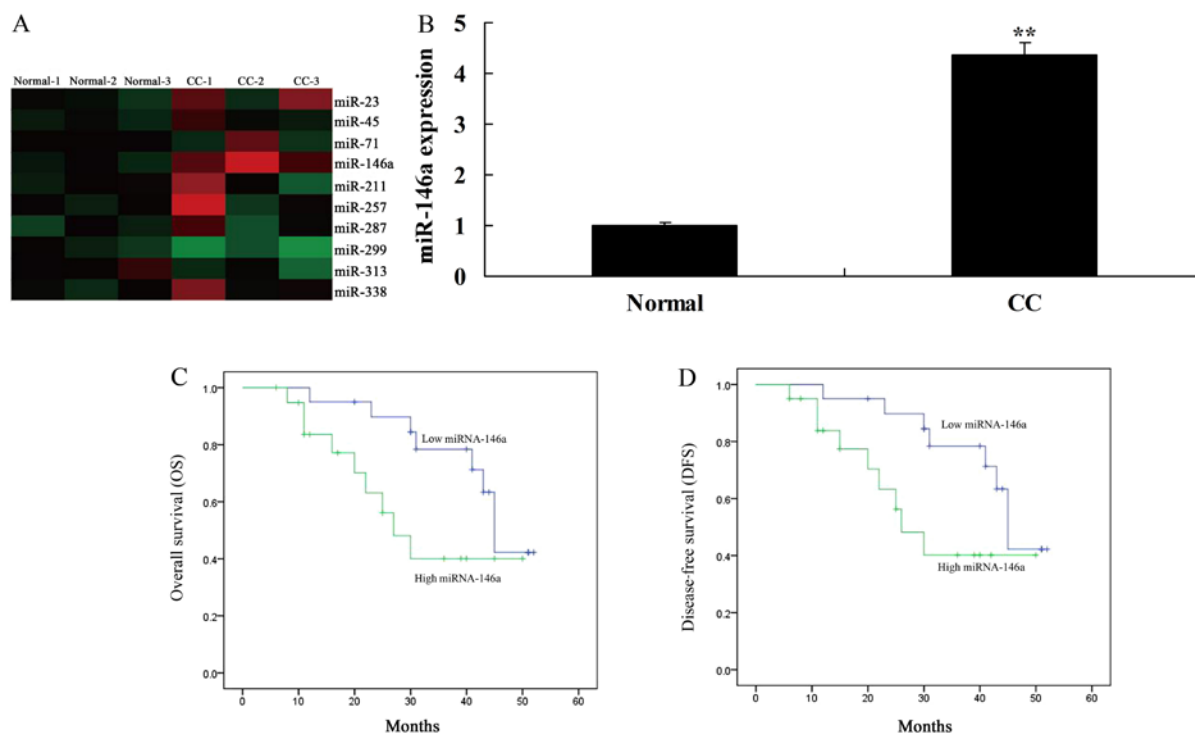


Figure 1. miRNA-146a expression in human cervical cancer. (A) Heat map for miRNA expression, and (B) qPCR for miRNA-146a expression in human cervical cancer tissues. (C) OS and (D) DFS of cervical cancer patients. OS, overall survival; DFS, disease-free survival; normal, normal control group; CC, cervical cancer tissue sample group. ** $P < 0.01$ compared with the normal control group.

then stained with 5 μ l of-FITC and 5 μ l of propidium iodide (PI) for 15 min in the dark. Flow cytometry was performed on a BD AccuriC6 (BD Biosciences, Franklin Lakes, NJ, USA) and data was analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Western blot analysis. Total cellular protein was extracted using RIPA assay Pierce; Thermo Fisher Scientific, Inc.) and protein concentration was determined using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing Tween-20 (TBST) for 1 h at 37°C and probed with TRAF6 (1:1,000; cat. no. sc-7221), NF- κ B (1:1,000; cat. no. sc-71675) and Bax (1:1,000; cat. no. sc-6236), p-Stat3 (1:500; cat. no. sc-8001-R) and GAPDH (1:5,000; cat. no. sc-51631; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. Then, the membranes were washed with TBST for 15 min and probed with horseradish peroxidase-labeled goat-anti-rabbit IgG (1:5,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at 37°C and visualization was performed by Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA).

Statistical analysis. Data are presented as the mean \pm SEM. Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used to perform statistical comparisons. A value of $P < 0.05$ was considered to indicate a statistically significant result.

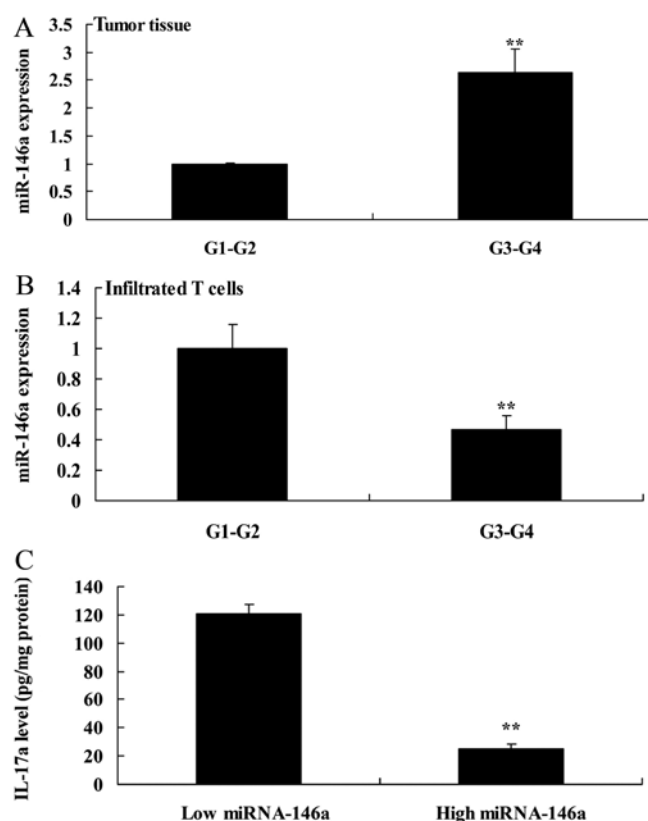


Figure 2. miRNA-146a expression in tumor tissues and infiltrated T cells. miRNA-146a expression in (A) tumor tissues, (B) infiltrated T cells, and (C) IL-17a levels. G1-G2, G1-G2 grade cervical cancer patients; G3-G4, G3-G4 grade cervical cancer patients; low miRNA-146a, cervical cancer patients with low miRNA-146a expression; high miRNA-146a, cervical cancer patients with high miRNA-146a expression. ** $P < 0.01$ compared with G1-G2 grade or the low miRNA-146a group.

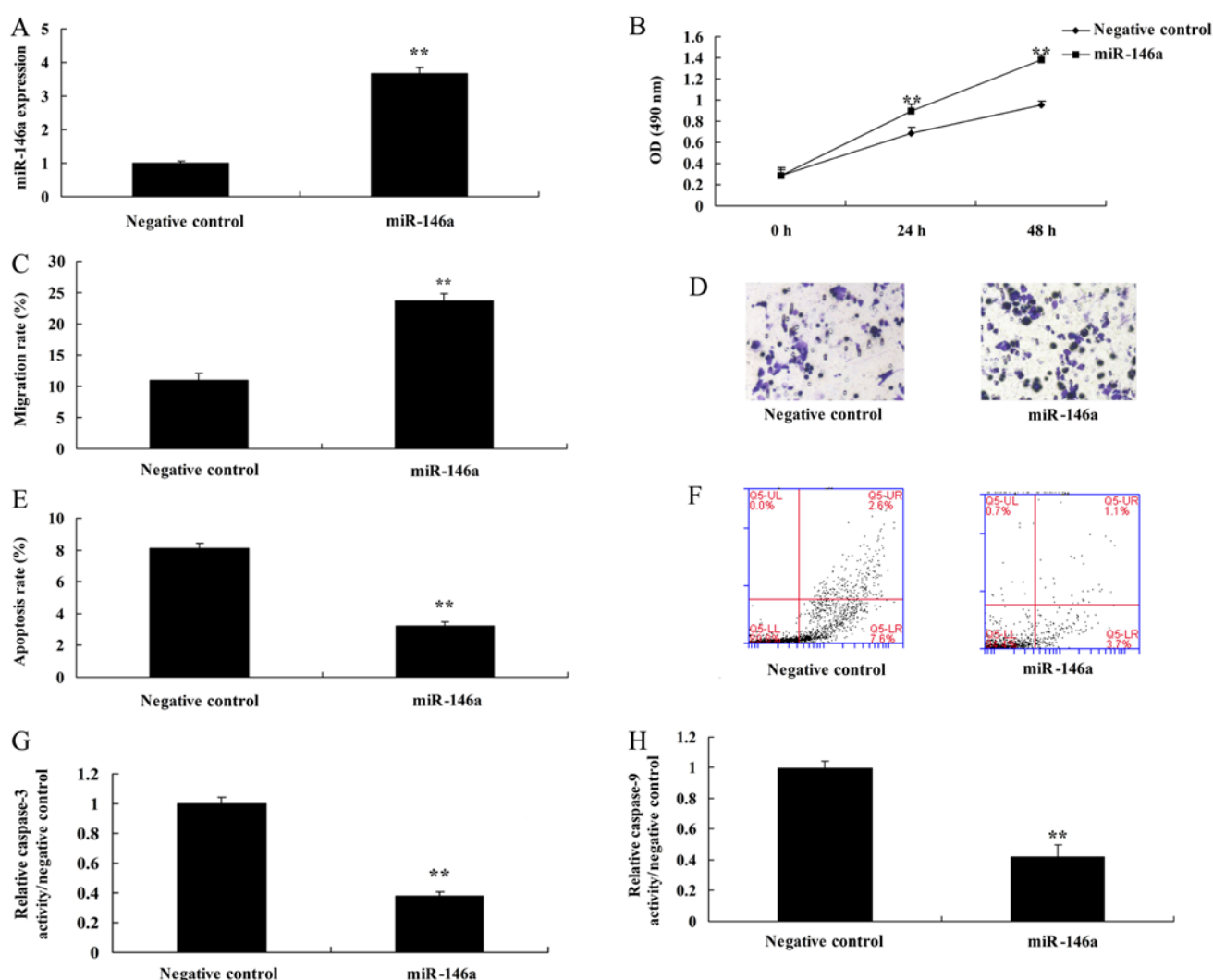


Figure 3. Overexpression of miRNA-146a promotes cell growth and inhibits apoptosis of cervical cancer cells in a co-culture of cervical cancer and CD4⁺ T cells. (A) miRNA-146a expression, (B) cell growth, (C and D) migration rate, (E and F) apoptosis rate, and (G and H) caspase-3 and -9 activities. Negative, negative control group; miR-146a, overexpression of the miRNA-146a group. **P<0.01 compared with the negative control group.

Results

miR-146a expression in human cervical cancer specimens.

We first assessed the changes of miR-146a expression in human cervical cancer tissue samples and normal control samples. The results revealed that miR-146a expression was increased in human cervical cancer tissues (Fig. 1A and B). In addition, we found that overall survival (OS) and disease-free survival (DFS) of patients with low miR-146a expression were higher than those of patients with high miR-146a expression (Fig. 1C and D).

miR-146a expression in tumor tissues and infiltrated T cells.

Next, the expression of miR-146a in tumor tissues of patients with G1-G2 grade cervical cancer was significantly lower than that in those with G3-G4 grade (Fig. 2A). However, the expression of miR-146a in infiltrated T cells in patients with G1-G2 grade was extremely higher than that in those with G3-G4 grade (Fig. 2B). The IL-17a levels were lower in patients with high miR-146a expression than those in patients with low miR-146a expression (Fig. 2C). These results revealed that

miR-146a exerted anticancer effects in human cervical cancer and regulated cell growth and apoptosis of Th17 cells.

Overexpression of miR-146a promotes cell growth and reduces apoptosis of cervical cancer cells in a co-culture of cervical cancer and CD4⁺ T cells. Next, miR-146a mimics were used to increase the expression of miR-146a in cervical cancer cells, compared with the negative control group (Fig. 3A). Overexpression of miR-146a promoted cell growth and the migration rate, and reduced the apoptosis rate and caspase-3 and -9 activities in cervical cancer cells by miR-146a mimics, compared with the negative control group (Fig. 3B-H). Additionally, we found that miR-146a regulated the function of Th17 cell differentiation, whose mechanism required further analysis.

Downregulation of miR-146a inhibits cell growth and induces apoptosis of cervical cancers in a co-culture of cervical cancer cells and CD4⁺ T cells. In determination of the function of anti-miR-146a in a co-culture of cervical cancer and CD4⁺ T cells, we found that miR-146a expression was reduced

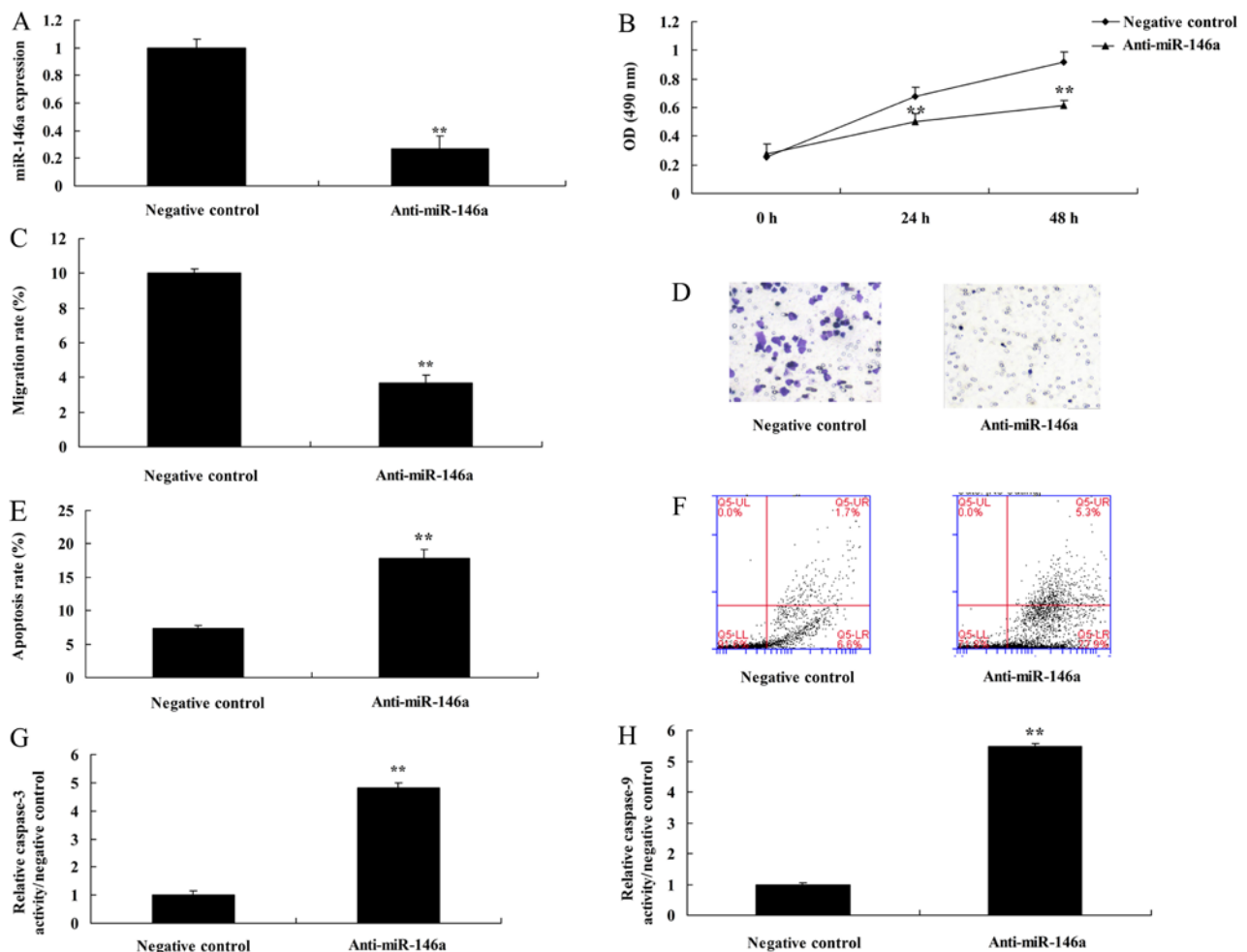


Figure 4. Downregulation of miRNA-146a suppresses cell growth and induces apoptosis of cervical cancer in a co-culture of cervical cancer and CD4⁺ T cells. (A) Anti-miRNA-146a expression, (B) cell growth, (C and D) migration rate, (E and F) apoptosis rate, and (G and H) caspase-3 and -9 activities. Negative, negative control group; anti-miR-146a, downregulation of the miRNA-146a group. **P<0.01 compared with the negative control group.

in cervical cancer cells by anti-miR-146a mimics, compared with the negative control group (Fig. 4A). Downregulation of miR-146a promoted cell growth and the migration rate, and inhibited the apoptosis rate and caspase-3 and -9 activities in cervical cancer cells, compared with the negative control group (Fig. 4B-H).

Downregulation of miR-146a induces NF- κ B signaling by targeting TRAF6. We investigated the mechanism of anti-miR-146a in cervical cancer cells, and this study revealed miR-146a putative binding sites and corresponding mutant sites of TRAF6 (Fig. 5A). Downregulation of miR-146a induced TRAF6 and NF- κ B protein expression, as well as Bax protein expression in cervical cancer cells. In addition, it promoted the protein expression of p-Stat3 in CD4⁺ T cells (Fig. 5B-F). Furthermore, in medium, downregulation of miR-146a increased the levels of IL-6, IL-21 and IL-17A, compared with the negative control group (Fig. 5H and I).

Overexpression of miR-146a induces NF- κ B signaling by targeting TRAF6. In addition, we also found that overexpression of miR-146a suppressed TRAF6 and NF- κ B protein expression, as well as Bax protein expression in cervical

cancer cells. In addition, it reduced p-Stat3 protein expression in CD4⁺ T cells (Fig. 6A-E). Then, in medium, overexpression of miR-146a decreased the levels of IL-6, IL-21 and IL-17A, compared with the negative control group (Fig. 6F-H). These results confirmed that miR-146a/NF- κ B signaling played a critical role in determining the Th17 cell differentiation by TRAF6.

si-TRAF6 reduces the function of miR-146a in a co-culture of cervical cancer and CD4⁺ T cells. Next, we further investigated the role of TRAF6 in the mechanism of miR-146a in cervical cancer cells. To this end, TRAF6 protein expression was downregulated using si-TRAF6 plasmid. The inhibition of TRAF6 suppressed TRAF6 and NF- κ B protein expression as well as Bax protein expression in cervical cancer cells by anti-miR-146a. In addition, it also suppressed p-Stat3 protein expression in CD4⁺ T cells (Fig. 7A-E). The inhibition of TRAF6 decreased the levels of IL-6, IL-21 and IL-17A, compared with the anti-miR-146a group (Fig. 7F-H). The inhibition of TRAF6 attenuated the functions of anti-miR-146a on cell growth, the migration rate, and the apoptosis rate as well as caspase-3 and -9 activities in cervical cancer cells, compared with the anti-miR-146a group (Fig. 8).

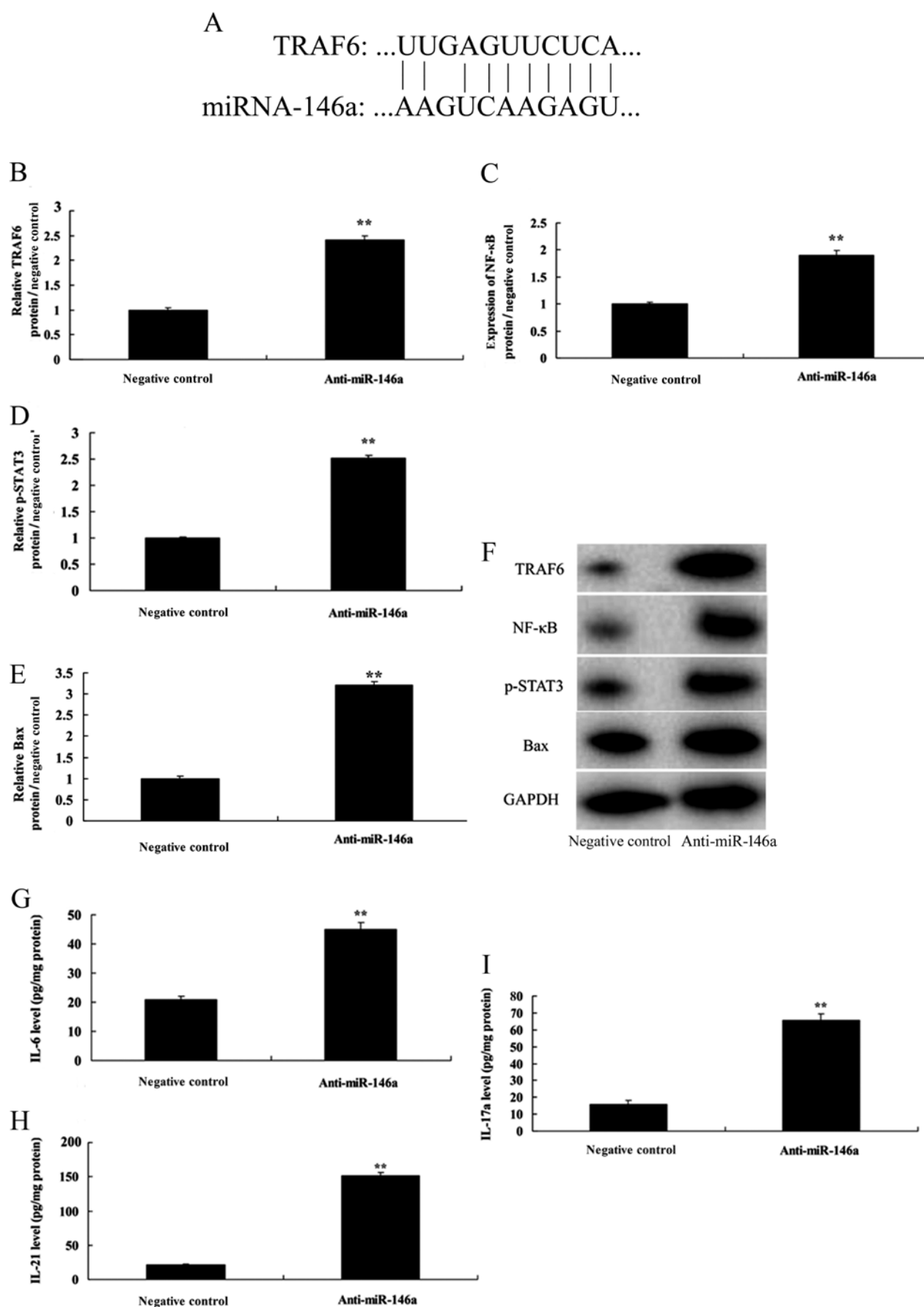


Figure 5. Downregulation of miRNA-146a induces NF-κB signaling by targeting TRAF6. (A) miRNA-146a putative binding sites and corresponding mutant sites of TRAF6. (B-F) TRAF6, NF-κB, p-STAT3 and Bax protein expression, using western blot and statistical analyses. The protein levels of (G) IL-6, (H) IL-21 and (I) IL-17A. Negative, negative control group; anti-miR-146a, downregulation of the miRNA-146a group. **P<0.01 compared with the negative control group.

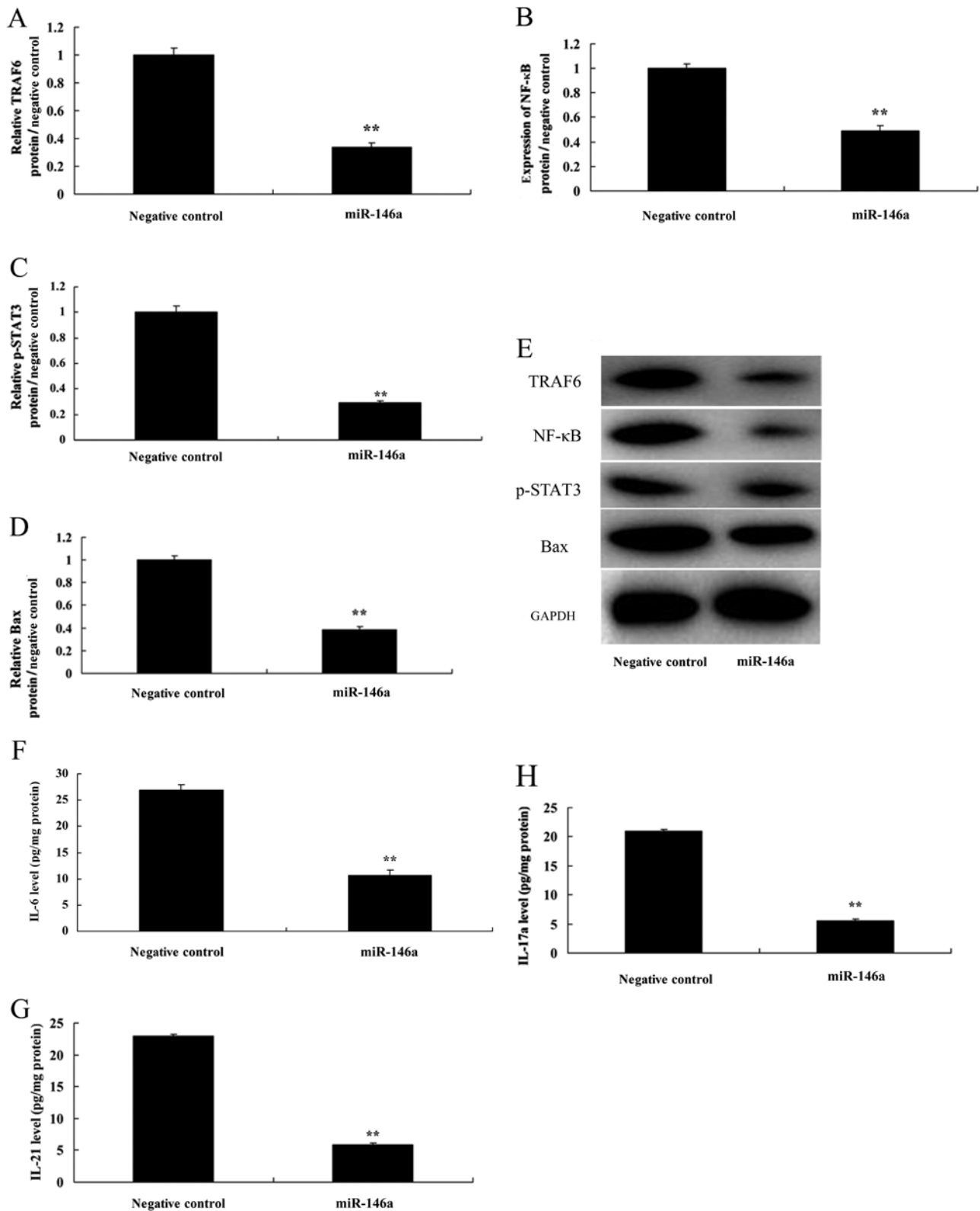


Figure 6. Overexpression of miRNA-146a suppresses NF-κB signaling by targeting TRAF6. (A-E) TRAF6, NF-κB, p-STAT3 and Bax protein expression, using western blot and statistical analyses. The protein levels of (F) IL-6, (G) IL-21 and (H) IL-17A. Negative, negative control group; miR-146a, overexpression of the miRNA-146a group. **P<0.01 compared with the negative control group.

STAT3 inhibits the function of miR-146a in a co-culture of cervical cancer and CD4⁺ T cells. Finally, we examined the role of STAT3 in the mechanism of miRNA-146a in cervical cancer by Th17 cells and used STAT3 decoy ODN to induce

the protein expression. STAT3 decoy ODN induced the protein expression of p-STAT3 in CD4⁺ T cells, and induced Bax protein expression in cervical cancer cells by miR-146a, compared with the miR-146a group (Fig. 9A-C). However,

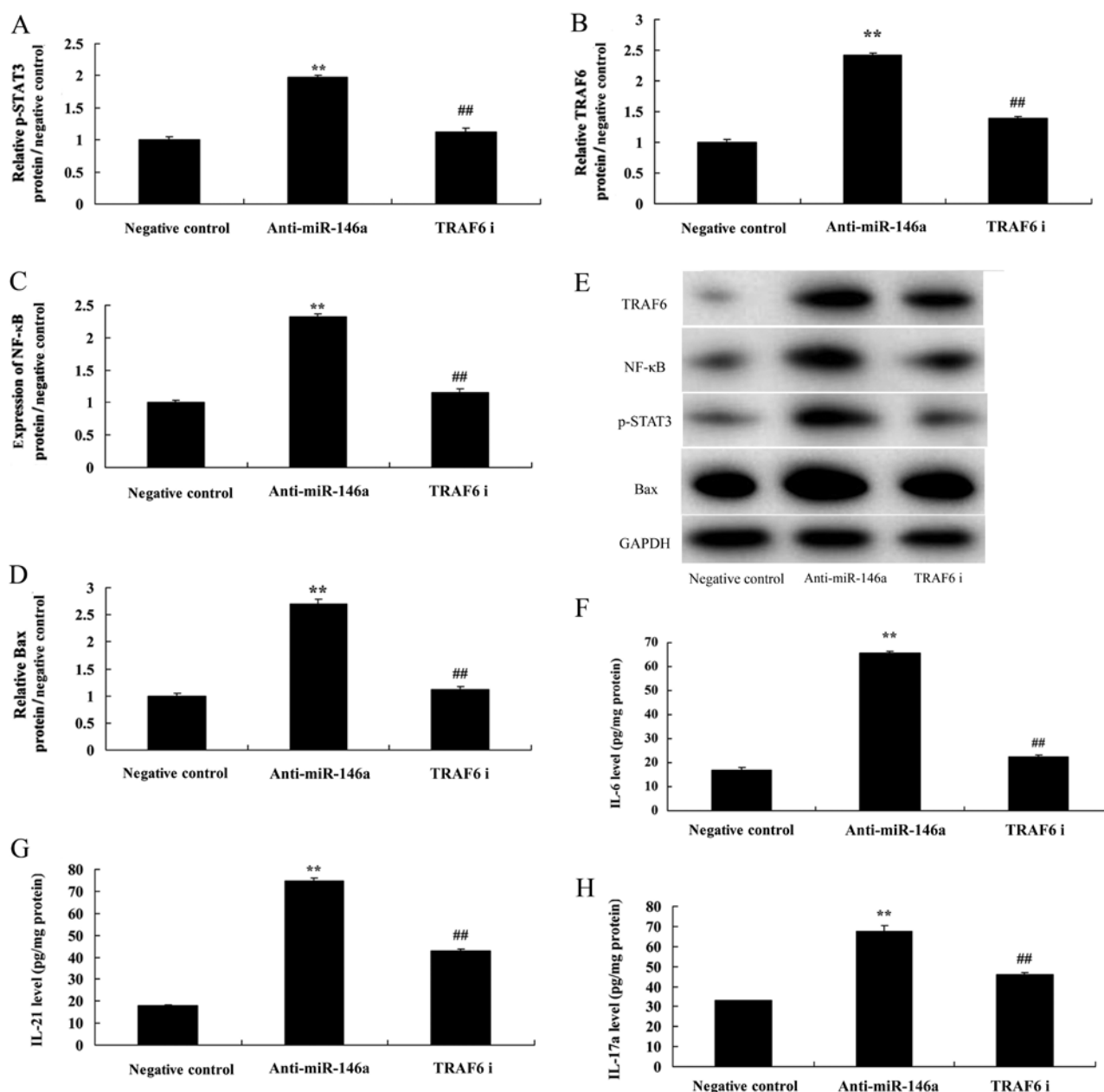


Figure 7. The inhibition of TRAF6 attenuates the functions of miRNA-146a in a co-culture of cervical cancer and CD4⁺ T cells. (A-E) TRAF6, NF-κB, p-STAT3 and Bax protein expression, using western blot and statistical analyses. The protein levels of (F) IL-6, (G) IL-21 and (H) IL-17A levels. Negative, negative control group; anti-miR-146a, downregulation of the miRNA-146a group; TRAF6 i, downregulation of the TRAF6 group. **P<0.01 compared with the negative control group; ##P<0.01 compared with the anti-miR-146a group.

STAT3 decoy ODN failed to affect IL-6 and IL-21 levels, and reduced IL-17a levels in the medium, compared with the miR-146a group (Fig. 9D-F). In addition, STAT3 decoy ODN attenuated the functions of miR-146a on cell growth, the migration rate, the apoptosis rate and caspase-3 and -9 activities in cervical cancer cells by miR-146a, compared with the miR-146a group (Fig. 10).

Discussion

Cervical cancer is one of the common malignancies in females worldwide (1). In recent years, the cervical cancer screening technique has been improved and popularized in developed areas (1). Most precancerous lesions of the uterine cervix can

be timely discovered and treated. Therefore, the morbidity of cervical cancer has been markedly reduced (11). However, in developing countries, especially remote and less developed areas, morbidity ranks at the top among all female malignancies (11). This can be ascribed to insufficient screening popularization and backward medical technology (11). In addition, with the social development cervical cancer patients exhibit a younger trend (12). Cervical cancer is also associated with high mortality. It is ranked in the 3rd place among all malignancies worldwide (12). Therefore, it has severely affected the lives and health of women worldwide. In the present study, miRNA-146a expression was increased in human cervical cancer. Wang *et al* revealed that miRNA-146a expression was upregulated in cervical carcinogenesis (13).

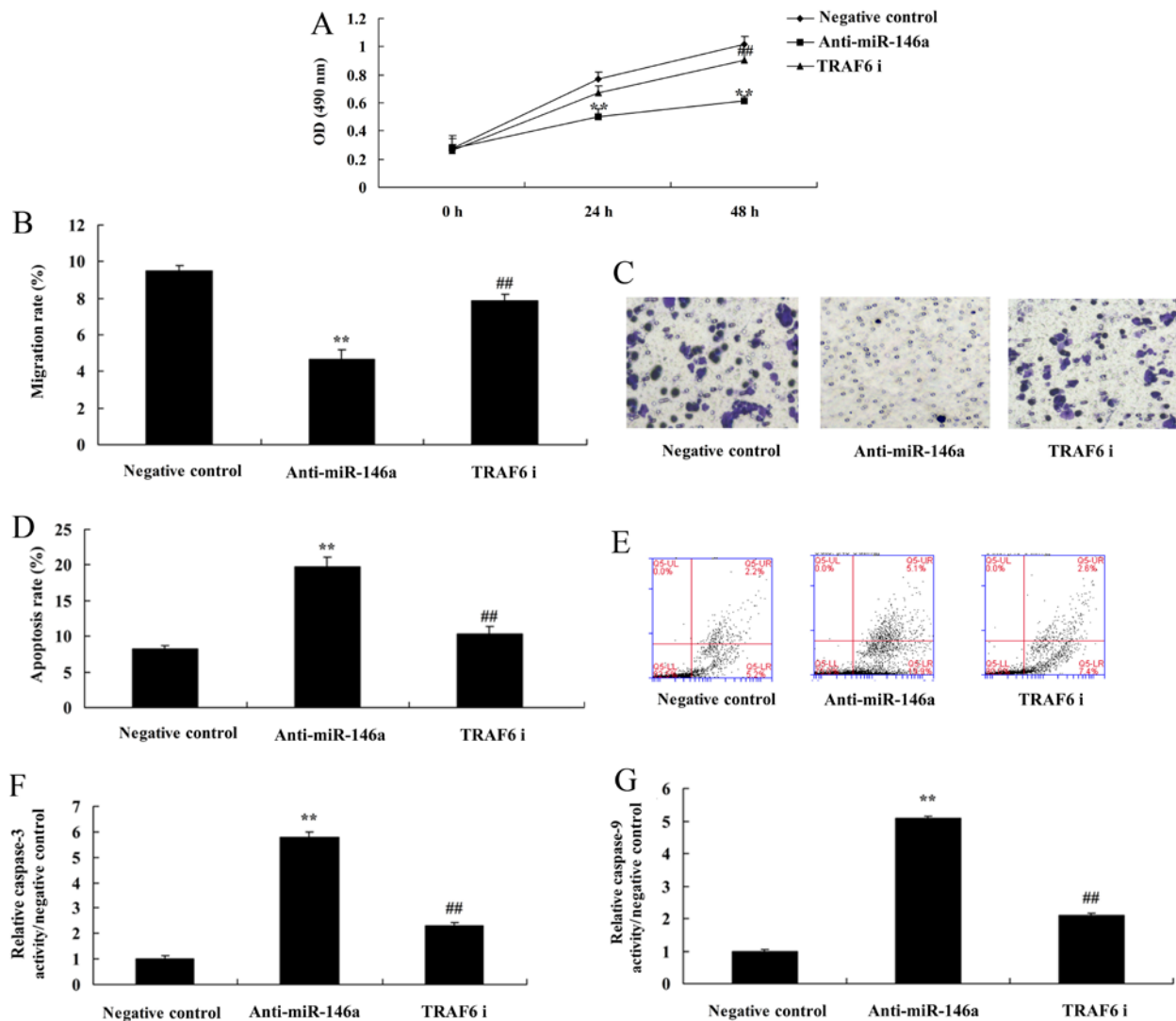


Figure 8. The inhibition of TRAF6 attenuates the functions of miRNA-146a on cervical cancer cell growth and apoptosis in a co-culture of cervical cancer and CD4⁺ T cells. (A) Cell growth, (B and C) migration rate, (D and E) apoptosis rate, and (F and G) caspase-3 and -9 activities. Negative, negative control group; miR-146a, downregulation of the miRNA-146a group; TRAF6 i, downregulation of the TRAF6 group. **P<0.01 compared with the negative control group; ##P<0.01 compared with the anti-miR-146a group.

TRAF6 is an important NF- κ B regulatory factor that has important biological functions. TRAF6 not only possesses an immune regulatory function but in addition, it is also involved in osteoclast maturation and differentiation. TRAF6 also plays a key role in tumor genesis, development, invasion and metastasis. NF- κ B activation by TRAF6 is necessary in lung cancer and esophageal carcinoma. Our study revealed that downregulation of miRNA-146a induced NF- κ B signaling by targeting TRAF6. He *et al* revealed that upregulation of miR-146a protects the small intestine against small intestine ischemia and I/R injury by downregulating the TLR4/TRAF6/NF- κ B pathway (14).

Th17 cells are a subgroup of CD4⁺ T cells that can mediate cytokines. Specifically, they can guarantee that cells exert different immune effects (7). Research has revealed that there is a dynamic balance in such cells. Imbalances play vital roles in the genesis and development of multiple inflammations, autoimmune diseases and tumors (15). Among the Th17-related cytokines, IL-6, IL-17 and IL-23 have a positive response value in the autoimmune regulation of the body (15). In addition, it displays high detection significance in local lesion response (7).

Therefore, it is vital to detect Th17 cells in the blood and tissues of such patients. Our data demonstrated that downregulation of miRNA-146a promoted Th17 cytotoxicity to induce apoptosis of cervical cancer cells. Li *et al* revealed that miR-146a blocks the autocrine IL-6- and IL-21-induced Th17 differentiation pathways in autoreactive CD4⁺ T cells (16).

The signal transduction and transcription activator protein family is a class of important cytokine signaling proteins (17). Multiple cytokines can transfer signals to the cells through the classical STAT signaling pathway (17). Thus, gene expression of specific target cells can be altered. Activation of the STAT signaling pathway determines the differentiation direction of T cells. Thus, it regulates a series of physiological and pathological processes (18). Of them, the STAT3 protein has been indicated to play a critical role in regulating Th17 cell differentiation (18). Abnormalities in the STAT3 signaling pathway is related to the genesis of multiple diseases, such as infections, tumors and autoimmune diseases (19,20). In the present study, we demonstrated that the downregulation of miRNA-146a induced TRAF6 and NF- κ B protein expression, increased IL-6, IL-17A and IL-21

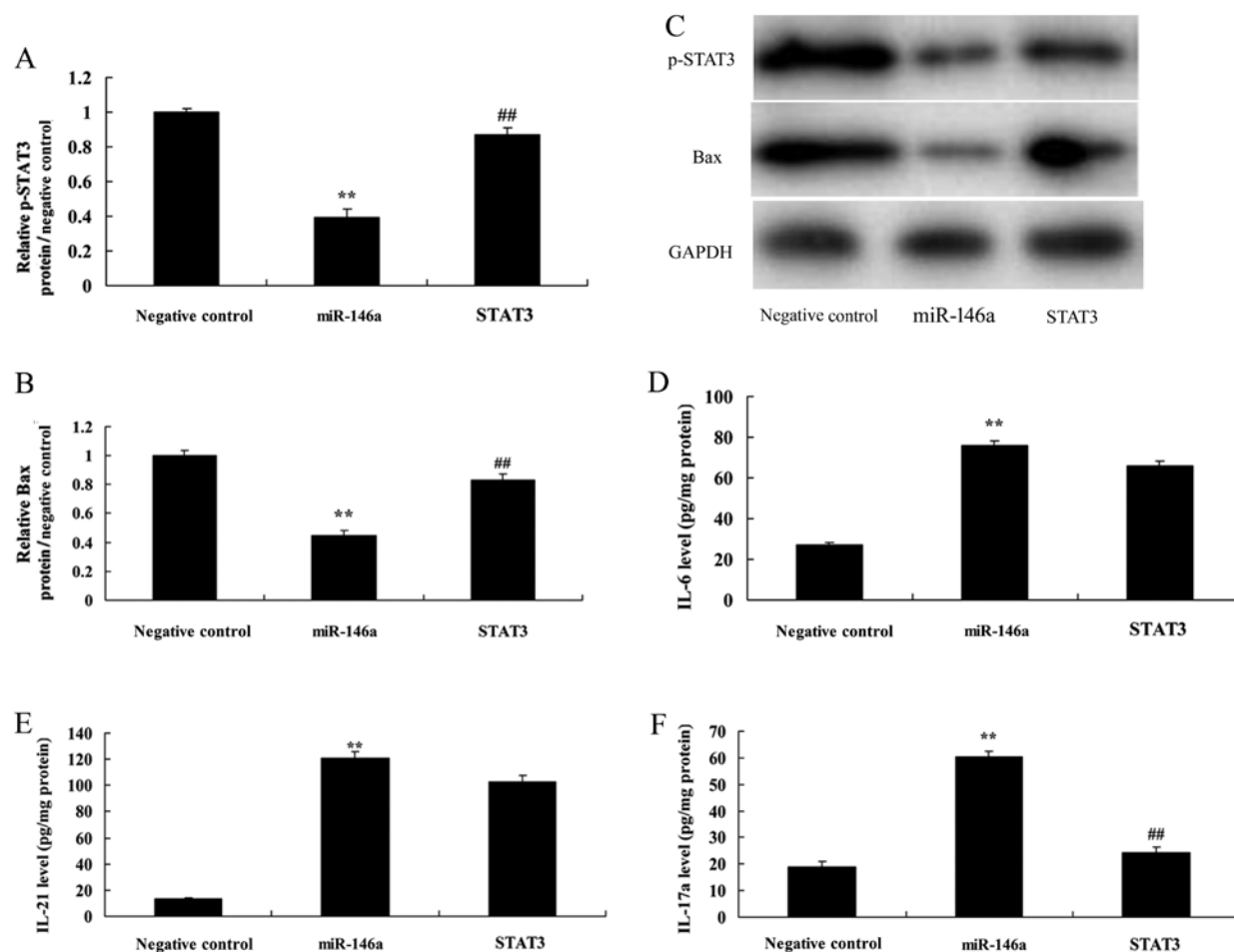


Figure 9. STAT3 attenuates the functions of miRNA-146a in a co-culture of cervical cancer and CD4⁺ T cells. (A-C) p-STAT3 and Bax protein expression, using western blot and statistical analyses of p-STAT3 and Bax protein expression. The protein levels of (D) IL-6, (E) IL-21 and (F) IL-17A. Negative, negative control group; miR-146a, overexpression of the miRNA-146a group; STAT3, STAT3 decoy ODN. **P<0.01 compared with the negative control group, ##P<0.01 compared with the miRNA-146a group.

levels, and promoted p-STAT3 protein expression. Zhou *et al* revealed that microRNA-146a mediated the IL-6/STAT3 signaling mechanism of microRNA-146a-mediated IL-6/STAT3 signaling in lumbar intervertebral disc degeneration (21).

Cytokines IL-6, IL-21 and IL-23 play major roles in Th17 cell differentiation. In addition, signal transfer is mainly achieved through the STAT3 signaling pathway (22). Typically, IL-6 is regarded as the initial factor of Th17 cell differentiation. TGF- β plays a synergistic part. IL-6 activates the STAT3 protein (22). This can enhance the expression of IL-21, IL-6 and IL-23 genes, so that cells secrete IL-21 and IL-23 (23). IL-21 activates the STAT3 protein and further promotes Th17 cell differentiation and secretion (24). The STAT3 protein regulates the TH17 cell differentiation-related cytokines (25). Briefly, the STAT3 protein directly binds with IL-17 and IL-17F, and regulates IL-17F secretion and promotes Th17 cell differentiation (25). In this study, we found STAT3 inhibited the function of miRNA-146a in a co-culture of cervical cancer and Th17 T cells. Ye *et al* suggests that miR-146a suppresses the STAT3/VEGF pathways in primary human retinal microvascular endothelial cells (26).

Collectively, this study demonstrated that miRNA-146a regulates the function of Th17 cell differentiation to modulate

cervical cancer cell growth and apoptosis through NF- κ B signaling by targeting TRAF6 (Fig. 11). miRNA-146a may functions as an oncogene in cervical cancer via Th17 cell differentiation by targeting TRAF6.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

TL designed the experiment. ML, CX, XX, JD, LC and RO performed the experiment. TL and ML analyzed the data.

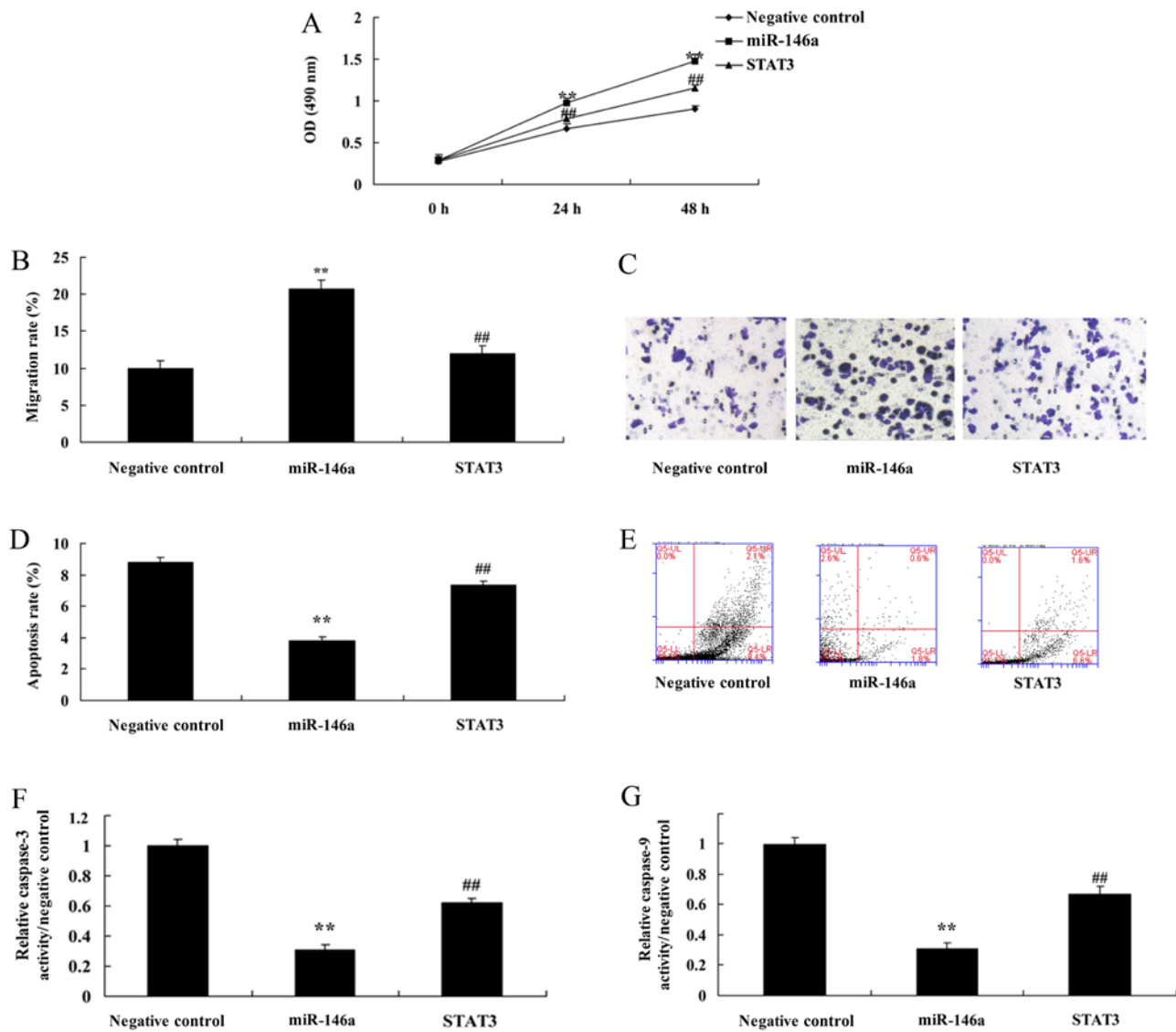


Figure 10. STAT3 attenuates the functions of miRNA-146a on cervical cancer cell growth and apoptosis in a co-culture of cervical cancer and CD4⁺ T cells. (A) Cell growth, (B and C) migration rate, (D and E) apoptosis rate, and (F and G) caspase-3 and -9 activities. Negative, negative control group; miR-146a, overexpression of the miRNA-146a group; STAT3, STAT3 decoy ODN. **P<0.01 compared with the negative control group, ##P<0.01 compared with the miRNA-146a group.

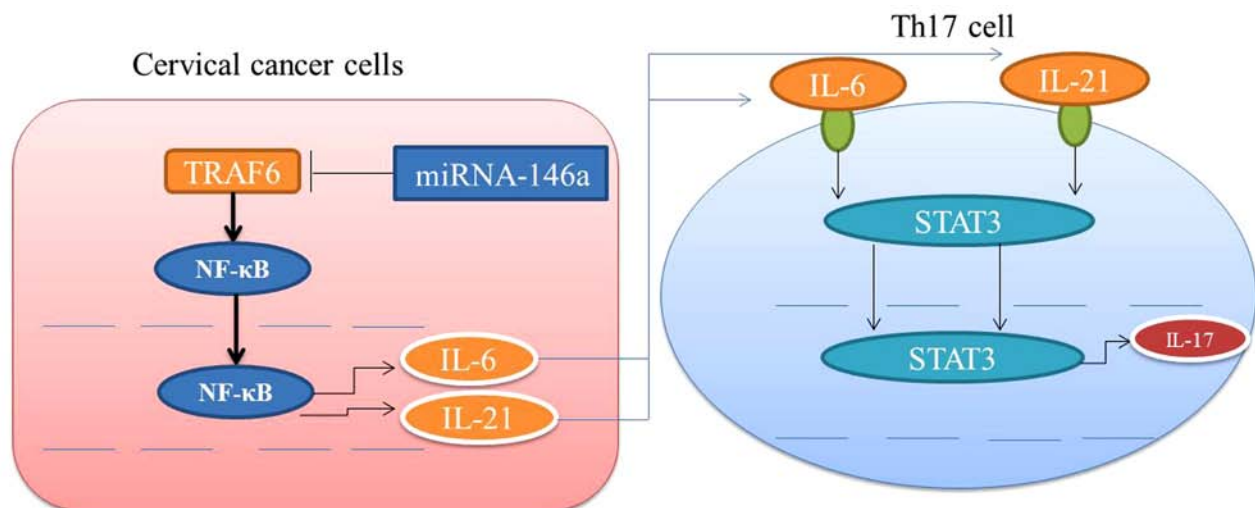


Figure 11. miRNA-146a regulates the function of Th17 cell differentiation to modulate cervical cancer cell growth and apoptosis through NF-κB signaling by targeting TRAF6.

TL wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Review Board of The Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, Guangdong), and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Tsunoda AT, Marnitz S, Soares Nunes J, Mattos de Cunha Andrade CE, Scapulatempo Neto C, Blohmer JU, Herrmann J, Kerr LM, Martus P, Schneider A, *et al*: Incidence of histologically proven pelvic and Para-aortic lymph node metastases and rate of upstaging in patients with locally advanced cervical cancer: Results of a prospective randomized trial. *Oncology* 92: 213-220, 2017.
2. de Azevedo C, Thuler LCS, de Mello MJG, de Oliveira Lima JT, da Fonte ALF, Fontão DFS, Carneiro VCG, Chang TMC and Ferreira CG: Phase II trial of neoadjuvant chemotherapy followed by chemoradiation in locally advanced cervical cancer. *Gynecol Oncol* 146: 560-565, 2017.
3. Matijevic M, Hedley ML, Urban RG, Chicx RM, Lajoie C and Luby TM: Immunization with a poly (lactide co-glycolide) encapsulated plasmid DNA expressing antigenic regions of HPV 16 and 18 results in an increase in the precursor frequency of T cells that respond to epitopes from HPV 16, 18, 6 and 11. *Cell Immunol* 270: 62-69, 2011.
4. Varghese VK, Shukla V, Kabekkodu SP, Pandey D and Satyamoorthy K: DNA methylation regulated microRNAs in human cervical cancer. *Mol Carcinog* 57: 370-382, 2018.
5. Shishodia G, Verma G, Das BC and Bharti AC: miRNA as viral transcription tuners in HPV-mediated cervical carcinogenesis. *Front Biosci* 10: 21-47, 2018.
6. Liang C, Ding J, Yang Y, Deng L and Li X: MicroRNA-433 inhibits cervical cancer progression by directly targeting metadherin to regulate the AKT and β -catenin signalling pathways. *Oncol Rep* 38: 3639-3649, 2017.
7. Hou F, Li Z, Ma D, Zhang W, Zhang Y, Zhang T, Kong B and Cui B: Distribution of Th17 cells and Foxp3-expressing T cells in tumor-infiltrating lymphocytes in patients with uterine cervical cancer. *Clin Chim Acta* 413: 1848-1854, 2012.
8. Yu Q, Lou XM and He Y: Preferential recruitment of Th17 cells to cervical cancer via CCR6-CCL20 pathway. *PLoS One* 10: e0120855, 2015.
9. Zhang W, Tian X, Mumtahana F, Jiao J, Zhang T, Croce KD, Ma D, Kong B and Cui B: The existence of Th22, pure Th17 and Th1 cells in CIN and cervical cancer along with their frequency variation in different stages of cervical cancer. *BMC Cancer* 15: 717, 2015.
10. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25: 402-408, 2001.
11. Wakatsuki M, Kato S, Ohno T, Karasawa K, Kiyohara H, Tamaki T, Ando K, Tsujii H, Nakano T, Kamada T, *et al*: Clinical outcomes of carbon ion radiotherapy for locally advanced adenocarcinoma of the uterine cervix in phase 1/2 clinical trial (protocol 9704). *Cancer* 120: 1663-1669, 2014.
12. Sewali B, Okuyemi KS, Askhir A, Belinson J, Vogel RI, Joseph A and Ghebre RG: Cervical cancer screening with clinic-based Pap test versus home HPV test among Somali immigrant women in Minnesota: A pilot randomized controlled trial. *Cancer Med* 4: 620-631, 2015.
13. Wang X, Tang S, Le SY, Lu R, Rader JS, Meyers C and Zheng ZM: Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. *PLoS One* 3: e2557, 2008.
14. He X, Zheng Y, Liu S, Shi S, Liu Y, He Y, Zhang C and Zhou X: MiR-146a protects small intestine against ischemia/reperfusion injury by down-regulating TLR4/TLR6/NF-kappaB pathway. *J Cell Physiol* 233: 2476-2488, 2018.
15. Tristão FS, Rocha FA, Carlos D, Ketelut-Carneiro N, Souza COS, Milanezi CM and Silva JS: Th17-inducing cytokines IL-6 and IL-23 are crucial for granuloma formation during experimental paracoccidioidomycosis. *Front Immunol* 8: 949, 2017.
16. Li B, Wang X, Choi IY, Wang YC, Liu S, Pham AT, Moon H, Smith DJ, Rao DS, Boldin MP *et al*: miR-146a modulates autoreactive Th17 cell differentiation and regulates organ-specific autoimmunity. *J Clin Invest* 127: 3702-3716, 2017.
17. Yoon JH, Sudo K, Kuroda M, Kato M, Lee IK, Han JS, Nakae S, Imamura T, Kim J, Ju JH, *et al*: Phosphorylation status determines the opposing functions of Smad2/Smad3 as STAT3 cofactors in Th17 differentiation. *Nat Commun* 6: 7600, 2015.
18. Lee SY, Lee SH, Yang EJ, Kim JK, Kim EK, Jung K, Jung H, Lee K, Lee HH, Lee BI, *et al*: Coenzyme Q10 inhibits Th17 and STAT3 signaling pathways to ameliorate colitis in mice. *J Med Food* 20: 821-829, 2017.
19. Lee SH, Park JS, Byun JK, Jhun J, Jung K, Seo HB, Moon YM, Kim HY, Park SH and Cho ML: Corrigendum: PTEN ameliorates autoimmune arthritis through down-regulating STAT3 activation with reciprocal balance of Th17 and Tregs. *Sci Rep* 7: 42267, 2017.
20. Betts BC, Sagatys EM, Veerapathran A, Lloyd MC, Beato F, Lawrence HR, Yue B, Kim J, Sebt SM, Anasetti C, *et al*: CD4⁺ T cell STAT3 phosphorylation precedes acute GVHD, and subsequent Th17 tissue invasion correlates with GVHD severity and therapeutic response. *J Leukoc Biol* 97: 807-819, 2015.
21. Zhou T, Lin H, Cheng Z, Ji C, Zhang C and Tian J: Mechanism of microRNA-146a-mediated IL-6/STAT3 signaling in lumbar intervertebral disc degeneration. *Exp Ther Med* 14: 1131-1135, 2017.
22. Qu N, Xu M, Mizoguchi I, Furusawa J, Kaneko K, Watanabe K, Mizoguchi J, Itoh M, Kawakami Y and Yoshimoto T: Pivotal roles of T-helper 17-related cytokines, IL-17, IL-22, and IL-23, in inflammatory diseases. *Clin Dev Immunol* 2013: 968549, 2013.
23. Wang J, Xu K, Wu J, Luo C, Li Y, Wu X, Gao H, Feng G and Yuan BZ: The changes of Th17 cells and the related cytokines in the progression of human colorectal cancers. *BMC Cancer* 12: 418, 2012.
24. Wan CK, Andraski AB, Spolski R, Li P, Kazemian M, Oh J, Samsel L, Swanson PA II, McGavern DB, Sampaio EP, *et al*: Opposing roles of STAT1 and STAT3 in IL-21 function in CD4⁺ T cells. *Proc Natl Acad Sci USA* 112: 9394-9399, 2015.
25. Halwani R, Sultana A, Vazquez-Tello A, Jamhawi A, Al-Masri AA and Al-Muhsen S: Th-17 regulatory cytokines IL-21, IL-23, and IL-6 enhance neutrophil production of IL-17 cytokines during asthma. *J Asthma* 54: 893-904, 2017.
26. Ye EA and Steinle JJ: miR-146a suppresses STAT3/VEGF pathways and reduces apoptosis through IL-6 signaling in primary human retinal microvascular endothelial cells in high glucose conditions. *Vision Res* 139: 15-22, 2017.