

# Tumor suppressor microRNA-34a inhibits cell migration and invasion by targeting MMP-2/MMP-9/FNDC3B in esophageal squamous cell carcinoma

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**Abstract.** MicroRNAs (miRNAs) are a large family of small, non-coding RNAs that play a pivotal role in tumorigenesis. miR-34a, which is a member of the miR-34 family, is a downstream target of p53. Increasing evidence shows that miR-34a dysregulation may contribute to tumor development and progression in numerous cancers, including esophageal squamous cell carcinoma (ESCC). However, the mechanism of miR-34a in the regulation of ESCC cells need to be further elucidated because of the complex regulative network of miRNAs. The miR-34a expression in ESCC samples has been

confirmed using quantitative reverse transcription polymerase chain reaction. The effects of miR-34a on cell migration and invasion were examined in ESCC cell lines using wound healing and Transwell assays, respectively. The effects of miR-34a on matrix metalloproteinase (MMP)-2 and -9 and fibronectin type III domain containing 3B (FNDC3B) expression levels were detected by luciferase reporter assays and western blot analysis. Quantitative polymerase chain reaction revealed that the miR-34a expression is significantly downregulated in the ESCC tissues compared to that in the adjacent normal tissues. miR-34a overexpression was significantly suppressed migration and invasion in the ESCC cells and simultaneously inhibited the MMP-2, MMP-9 and FNDC3B expression levels by targeting the coding and 3'-untranslated regions, respectively. The findings indicated that microRNA-34a suppresses cell migration and invasion by targeting MMP-2, MMP-9, and FNDC3B in ESCC.

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**Abbreviations:** miRNA, microRNA; miR-34a, microRNA 34a; ESCC, esophageal squamous cell carcinoma; MMP-9, matrix metalloproteinase-9; MMP-2, matrix metalloproteinase-2; FNDC3B, fibronectin type III domain containing 3B; mRNAs, messenger RNAs; 3'-UTR, 3'-untranslated region; 5'-UTRs, 5'-untranslated regions; EMT, epithelial-mesenchymal transition; YY-1, Yin Yang-1; PCR, polymerase chain reaction; real-time RT-PCR, real-time reverse transcription PCR; FFPE, formalin-fixed and paraffin-embedded; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; qRT-PCR, quantitative reverse transcription PCR; cDNA, complementary deoxyribonucleic acid; PBS, phosphate buffered saline; TGFβ1, transforming growth factor β1; CDs, coding region; Tris, trihydroxymethylaminomethane

**Key words:** esophageal squamous cell carcinoma, miR-34a, migration and invasion, matrix metalloproteinase 2/9, fibronectin type III domain containing 3B

## Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most frequent malignant tumors in China (1). Some advances have been made in the treatment of ESCC, including surgery, chemotherapy, radiation, or a combination of these options. However, the associated prognosis of ESCC patients is still not satisfactory. The five-year overall survival rate of ESCC patients after surgery is only approximately 14-22% (2-4). Tumor invasion and metastasis are the major causes of the poor prognosis of ESCC patients (5,6). Some oncogenic and tumor suppressive factors have been reported to be associated with ESCC progression. However, only a few of them are specific and conclusive (7,8). Therefore, exploring the detailed molecular mechanisms of ESCC cell progression, invasion, and metastasis will help improve disease diagnosis and therapy.

MicroRNAs (miRNAs) are endogenously expressed, small, non-coding RNAs of 19-24 nucleotides that regulate gene expression by either inhibiting translation or cleaving their

target messenger RNAs (mRNAs) (9,10). The miRNA target site is considered to be the 3'-untranslated region (3'-UTR) of mRNA. Mounting evidence shows that miRNAs may also bind coding or 5'-untranslated regions (5'-UTRs) (11-15). Evidence also shows that miRNAs are involved in the pathogenesis of most cancer, such as cell differentiation, proliferation, oncogenesis, angiogenesis, cell migration, and invasion, where some can function as tumor suppressors or oncogenes (16-19). The aberrant regulation of miRNAs has been shown involved in numerous cancers, including ESCC (4,14).

MicroRNA-34a (miR-34a), which is located in chromosome 1p36 and belongs to the miR-34 family, is directly regulated by the p53 transcription factor (20). miR-34a is more commonly downregulated than the non-malignant tissues in multiple cancers, such as tongue squamous cell carcinoma (12), non-small cell lung cancer (21), colon cancer (22), pancreatic cancer (23), and others (24-26). miR-34a in cancer has also been extensively studied in tumor growth, migration, invasion, and epithelial-to-mesenchymal transition (EMT). Accordingly, miR-34a has been reported to suppress cell proliferation, migration, invasion, and EMT in various cancer cells by targeting oncogenes (22-24,27-31). miR-34a modulates stem cell self-renewal and difference in multiple cancers (23,25,32-35). In addition, miR-34a regulates drug resistance (36-38), and has been suggested to act as a suppressor that plays a key role in tumorigenesis. However, the role that miR-34a plays in ESCC is still in question. One recent study found that miR-34a could inhibit ESCC cell migration and invasion by targeting Yin Yang-1 (YY-1). YY-1 can suppress the matrix metalloproteinase (MMP)-2 and -9 expression levels (39). However, miR-34a is reported to inhibit cell migration and invasion by directly targeting MMP-9 in tongue squamous cell carcinoma (12). Therefore, whether miR-34a can directly target MMP-9 or MMP-2 to regulate ESCC cell migration and invasion needs to be investigated. The detailed role and mechanism of miR-34a in ESCC cell regulation needs to be further elucidated.

This study evaluated the miR-34a and p53 expression in ESCC tumor tissues and adjacent normal tissues. It shows that miR-34a and p53 both significantly decreased in the ESCC tissues. Moreover, the expression of p53 and miR34a has positive correlation in ESCC tissues. We further verified the functional role for miR-34a in ESCC cell invasion and migration. The regulative network of miR-34a in ESCC cancer was also explored. The obtained data indicate that miR-34a suppressed ESCC cell invasion and migration. Our study also demonstrates that MMP-2/MMP-9/fibronectin type III domain containing 3B (FNDC3B) is a direct downstream target of miR-34a, and miR-34a overexpression in the ESCC cells decreases both mRNA production and protein expression of MMP-2/MMP-9/FNDC3B. miR-34a suppressed tumor cell invasion and migration in ESCC by suppressing MMP-2 and MMP-9/FNDC3B expression levels.

## Materials and methods

**Ethics statement.** The experiments were approved by the Institutional Ethics Review Board of the First Affiliated Hospital of the Shihezi University School of Medicine. All samples were obtained from patients who signed informed

consent forms approving the use of their tissues for research purposes after surgery.

**Human tissue samples and cell lines.** A total of 15 normal esophageal and 15 human ESCC tissues were used for real-time polymerase chain reaction (PCR) analysis. All tissue specimens were obtained from the First Affiliated Hospital of Shihezi University. All tissues were formalin-fixed and paraffin-embedded (FFPE) for pathological diagnosis. The normal esophageal tissues were obtained from a distance of  $\geq 5$  cm from the tumor tissues.

The ESCC cell lines used, including EC9706 and TE-1, were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). HEK293 was a gift from the biochemical laboratory (American Type Culture Collection) of the Shihezi University School of Medicine. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BI), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified incubator containing 5% CO<sub>2</sub> at an atmosphere of 37°C.

**RNA isolation and quantitative reverse transcription PCR (qRT-PCR).** The total RNA, including miRNA, was isolated from the FFPE and cell samples using the miRNeasy FFPE (no. 73504) and miRNeasy Mini (no. 217004) kits from Qiagen (Hilden, Germany). The kits were used according to the manufacturer's instructions. The concentration and purity of the RNA samples were measured using NanoDrop2000 (Thermo Scientific). The RNA was reverse transcribed into cDNA using the miScript II Reverse Transcription (RT) kit (no. 218161) from Qiagen according to the manufacturer's protocol with 1000 ng total RNA. Subsequently, qRT-PCR was conducted using the SYBR Green PCR kit (miScript SYBR Green PCR kit for miRNA; no. 218073; Qiagen) and the QuantiFast SYBR Green PCR kit for mRNA (no. 204054; Qiagen) containing ROX as the reference dye in the ABI 7500 RT-PCR system. The PCR primers for miR-34a (Hs\_miR-34a\_1 miScript primer assay, MS00003318), U6 (Hs\_RNU6-2\_1 miScript primer assay, MS00033740), and ACTB (encoding  $\beta$ -actin) (Hs\_ACTB\_1\_SG, QT00095431) were purchased from Qiagen. The MMP-2, MMP-9, and FNDC3B primers were synthesized (Applied Sangon Biotech Co., Ltd., Shanghai, China). The sequences were as follows: MMP-2 forward, 5'-TATGGCTTCTGCCCTGAGAC-3' and MMP-2 reverse 5'-CACACCACATCTTCCGTCA-3'; MMP-9 forward, 5'-CGAACTTTGACAGCGACAAG-3' and MMP-9 reverse, 5'-CGGCACTGAGGAA TGATCTA-3'; and FNDC3B forward, 5'-TTGGAGAGGG AATGGTGTGT-3' and FNDC3B reverse 5'-CAGGTCACGAGCAAGTTAG-3'. Accordingly, U6 and  $\beta$ -actin were used as internal control for the normalization and quantification of the miRNA and mRNAs, respectively. Quantification was performed in triplicate.

**miR-34a mimic/inhibitor transfection.** The chemically synthesized miR-34a mimic (Syn-hsa-miR-34a-5p miScript miRNA mimic, MSY0000255), miR-34a inhibitor (Anti-hsa-miR-34a-5p miScript miRNA inhibitor, MIN0000255), and negative controls (AllStars Negative Control siRNA, no. 1027280; miScript inhibitor negative control, no. 1027271)

were purchased from Qiagen. The ESCC cells were transfected with miR-34a mimic (10 nM) for miR-34a overexpression or with miR-34a inhibitor (50 nM) for miR-34a inhibition by Hiperfect (no. 301705; Qiagen) according to the manufacturer's instructions.

**Wound healing assay.** A total of  $30 \times 10^4$  ESCC cells were cultured in one 6-well culture plate. These cells were transfected with different concentrations of miR-34a mimic (10 nM) for overexpression or miR-34a inhibitor (50 nM) for inhibition using Hiperfect for 24 h. The transfection allowed cells to reach full confluence. The confluent cells were subsequently wounded by scraping using a 10  $\mu$ l pipette tip. The dislodged cells were removed by washing with a serum-free medium. The cells that migrated into the wounded area or protruded from the wound border were captured using an inverted microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) after 24 h incubation at 37°C with 5% CO<sub>2</sub>. The cell migration distances were quantified by subtracting the distance between the wound edges at 24 h from the distance measured at 0 h. Each experiment was independently performed for at least three times.

**Cell invasion assays.** The 8  $\mu$ m plain (for migration) or Matrigel-coated (for invasion) (BD Biosciences, Franklin Lakes, NJ, USA) pore membrane and Transwell inserts (Corning Costar Corp., Corning, NY, USA) were placed in the wells of 24-well culture plates (Corning Costar Corp.) to assess the migrated or invasive ability of the cell sublines. Subsequently, EC9706 cells ( $9 \times 10^4$  per well) initially transfected with miR-34a mimic (10 nM) or miR-control (10 nM) were cultured for 24 h. The ESCC cells were then harvested and re-suspended in 500  $\mu$ l serum-free DMEM medium. Accordingly, 150  $\mu$ l cell suspension was seeded into the upper chamber. Thereafter, 600  $\mu$ l DMEM containing 10% FBS was added to the lower chamber to stimulate cell penetration. After 24 h incubation at 37°C with 5% CO<sub>2</sub>, the DMEM was discarded and washed thrice with 1X PBS. The non-invasive cells that remained on top of the membrane were then manually removed with a cotton wool. The invading cells that adhered to the filter's undersurface were fixed using 4% paraformaldehyde (Solarbio, Beijing, China) for 30 min at 4°C, dried under room temperature, and stained with 0.1% crystal violet for 20 min. The Transwell inserts were thoroughly washed with water. The invaded cells were counted under a microscope in five randomly chosen fields. Representative images were then taken. Data are expressed as the number of invaded cells (means  $\pm$  standard deviation) normalized to the number of control cells that migrated. Each test was performed in triplicate.

**Target gene prediction and luciferase reporter assay.** The predicted miR-34a target genes and their target binding site regions were investigated using a bioinformatics tool (TargetScan, miRanda, RNA22 software) and a literature review. We analyzed the biological function and distribution on the tissue of the target genes. We then chose the migration- and invasion-related genes and their relationship with the ESCC. We found that MMP-2, MMP-9, and FNDC3B mRNA contained putative miR-34a target sites. The miR-34a target sequences in the MMP-9 coding region and the MMP-2

and FNDC3B 3'UTR region were amplified by PCR. These sequences were then inserted into multiple cloning sites of the pMIR-REPORT that contained a firefly luciferase reporter gene (Obio Technology, Shanghai, China). The mutant reporter vectors of the MMP-9 coding region and the MMP-2 and FNDC3B 3'-UTR regions that lacked the miR-34a binding sites were obtained through site-directed mutagenesis. All constructs were verified by DNA sequencing.

For the luciferase reporter assay, the EC9706 and 293T cells ( $14 \times 10^4$ ) were seeded into 24-well plates the day before transfection to ensure 80% confluence at the time of transfection. Accordingly, 1  $\mu$ g of firefly luciferase reporter plasmid, including the wild-type or mutant coding regions of MMP-9, 3'UTR of MMP-2, and 3'UTR of FNDC3B, and 0.05  $\mu$ g of pRL-TK, which is a plasmid expressing the *Renilla* luciferase, were transfected into EC9706 and 293T cells cultured in 24-well plates together with 50 nM miR-34a mimic or negative control and 100 nM of miR-34a inhibitor or negative control using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were subjected to lysis 48 h post-transfection. The luciferase activities were determined using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. The relative firefly luciferase activity (i.e., firefly luciferase activity/*Renilla* luciferase activity) for each construct was compared to the negative control. The luciferase activity was averaged in triplicate for each transfection.

**Western blot analysis.** The ESCC cells were transfected with miR-34a mimic or miR-34a inhibitor using the previously described method. The cells were washed three times with 1X PBS 48 h post-transfection and lysed with RIPA lysis buffer (Solarbio) in ice. The total proteins were extracted following standard protocol. Equal amounts of protein from whole cell lysates of each sample were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. These proteins were then transferred to a polyvinylidene difluoride membrane (Solarbio). The membranes were blocked in 5% non-fat dried milk at room temperature for 2 h. They were then incubated with primary antibodies at 4°C overnight and extensively washed. The membranes were further incubated with a corresponding secondary antibody at room temperature for 2 h after washing with 1X Tris-buffered saline buffer containing 0.1% Tween-20 six times (5 min  $\times$  6). The primary antibodies against  $\beta$ -actin (diluted 1:1000; Zhongshan Golden Bridge Biotechnology, Beijing, China), MMP-9 (diluted 1:500; Abcam, Cambridge, MA, USA), MMP-2 (diluted 1:500; Cell Signaling Technology, Danvers, MA, USA), and FNDC3B (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were employed. The secondary antibodies were used at 1:4000 to 1:5000 concentrations. The relative protein expression levels were quantified by the Gelpro analyzer software (GelPro32 4.0) using  $\beta$ -actin as the internal reference.

**Statistical analysis.** The statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The experimental data are presented as mean  $\pm$  standard deviation based on the results of at least three repeats. The between-group comparisons were all based on Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

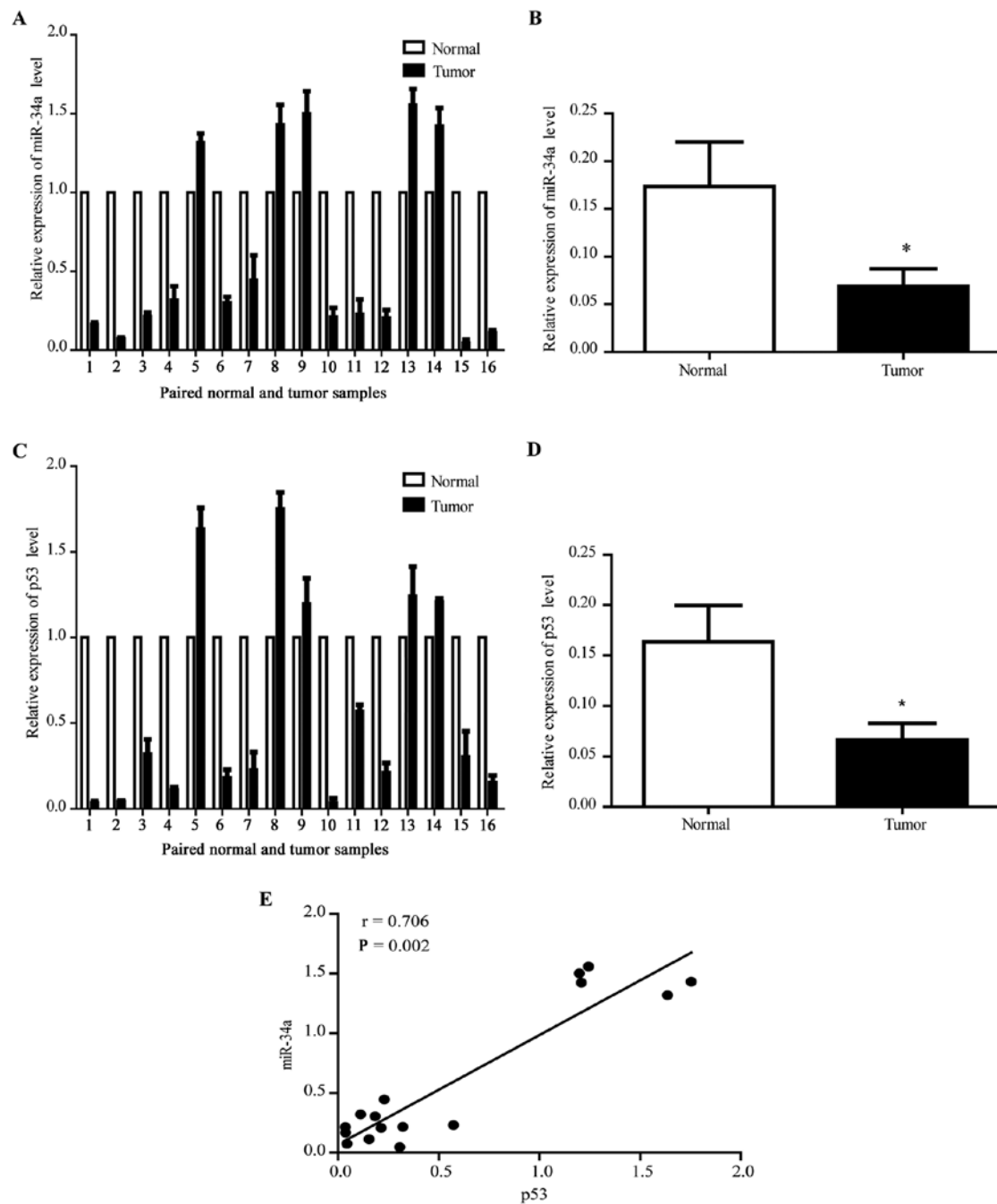


Figure 1. Decreased miR-34a and P53 expression in esophageal cancer. (A and C) Relative miR-34a and P53 levels in 16 ESCC specimens and matched normal tissues are determined by qRT-PCR using U6 as the internal reference. Data are presented as  $2^{-\Delta\Delta CT(CT_{miR-34a-U6tumor})-(CT_{miR-34a-U6normal})}$  values. (B and D) Average miR-34a and P53 expression in the ESCC and matched normal tissues. All values are normalized to U6 RNA levels. Data are presented as  $2^{-\Delta CT(miR-34a-U6)}$  values (\* $P < 0.05$ ). (E) A potential positive correlation between p53 and miR-34a in ESCC ( $P < 0.05$ ).

## Results

**miR-34a expression is decreased in the ESCC.** The miR-34a expression in the ESCC was confirmed by determining the miR-34a levels in the ESCC and normal esophageal tissues through qRT-PCR using U6 as the internal control. Fig. 1A shows that the miR-34a expression decreased in 11 of 16 (68.8%) tumor samples. The average miR-34a expression also decreased in tumor tissues (Fig. 1B). In addition, the expression of p53 in ESCC tissues declined (Fig. 1C and D) with the miR34a expression level, the positive regulation expression between p53 and miR34a (Fig. 1E) indicate that miR34a may

be a downstream target gene of p53 in ESCC similarly to other cancers.

**miR-34a inhibits ESCC cell migration and invasion.** The effects of miR-34a in the ESCC were investigated by transfecting the miR-34a mimics or the miR-34a inhibitor into the EC9706 and TE-1. The transfection efficiency was confirmed using qRT-PCR (Fig. 2). The cell migration assays were then performed within 48 h after miR-34a mimic transfection. The wound-healing assay showed that cell migration was significantly inhibited in the miR-34a mimic-transfected EC9706 cells compared with the negative control. A comparison to

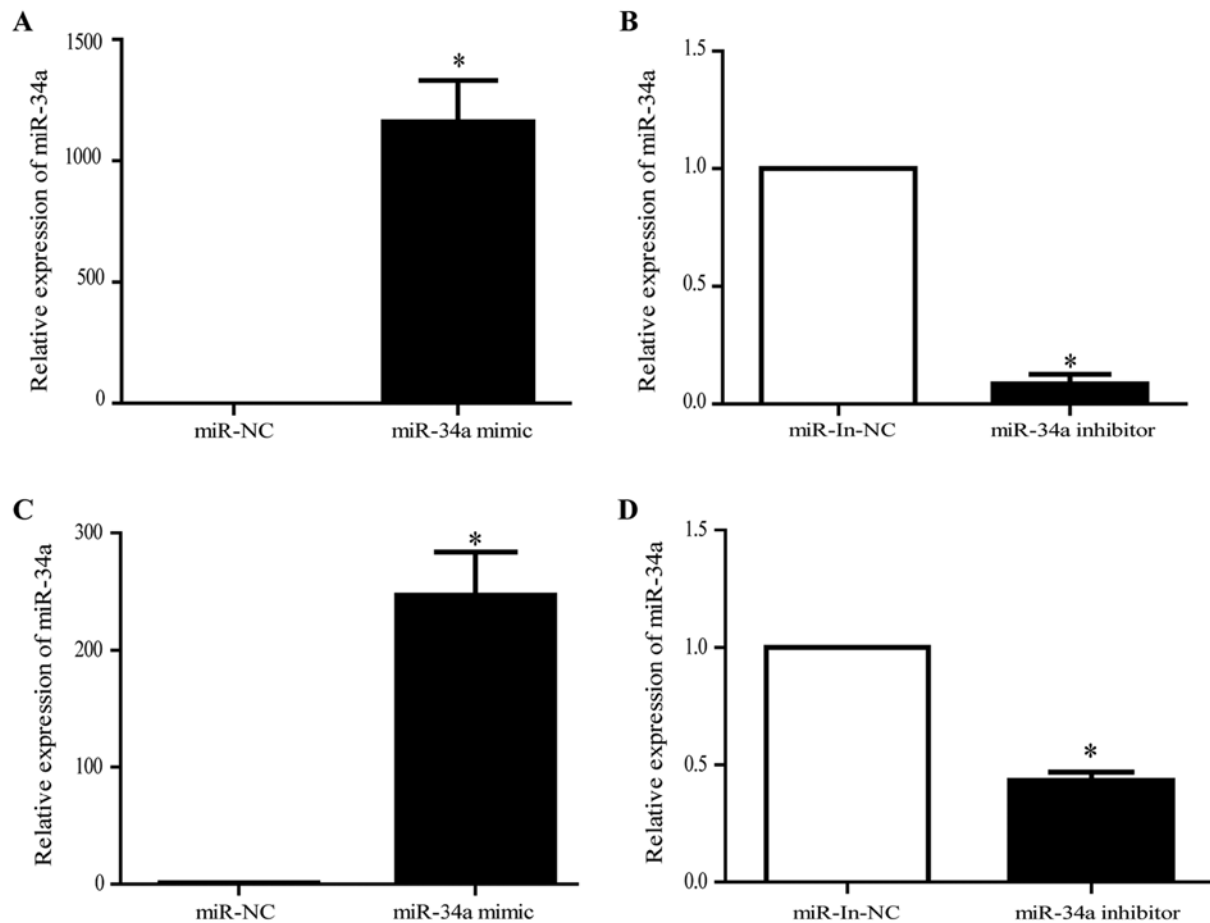


Figure 2. Transfection efficiency of miR-34a in esophageal cancer cells. (A and B) miR-34a expression in EC9706 cells was determined by qRT-PCR after transfecting miR-34a mimic, inhibitor, or negative control (\* $P < 0.05$ ). (C and D) miR-34a expression in TE-1 cells was determined by qRT-PCR after transfecting miR-34a mimic, inhibitor, or negative control (\* $P < 0.05$ ). NC, normal control; In, inhibitor.

the negative control shows that the inhibited miR-34a expression significantly promoted EC9706 cell migration (Fig. 3A). Similarly, cell migration was significantly inhibited in the miR-34a mimic-transfected TE-1 cells. miR-34a inhibition also promoted TE-1 cell migration (Fig. 3B). We conducted Transwell migration and invasion assays. Consequently, the results demonstrated that EC9706 cell migration and invasion were inhibited by the miR-34a overexpression. In contrast, the miR-34a inhibition promoted EC9706 cell migration and invasion (Fig. 3C and D). These results suggested that miR-34a could inhibit ESCC cell migration and invasion, and inhibiting the miR-34a expression can increase ESCC cell migration and invasion.

**miR-34a directly targets and suppresses MMP-2/MMP-9/FNDC3B in ESCC cells.** The prediction results obtained using the bioinformatics tool and the literature review indicated that the human miR-34a may target the MMP-9 coding region and the MMP-2 and FNDC3B 3'-UTR regions. Fig. 4A, C and E) showed the miR-34a putative binding sites and corresponding mutant sites of MMP-9, MMP-2, and FNDC3B. We constructed luciferase reporter plasmids containing putative sequences for MMP-2, MMP-9, and FNDC3B or their corresponding mutant sequences as controls to further confirm that miR-34a directly targeted MMP-2, MMP-9, and FNDC3B. At 48 h post-trans-

fection, the luciferase activity of the reporter containing the miR-34a-targeted wild-type sequences of MMP-9 was significantly suppressed in the 293T cells (Fig. 4B) and EC9706 cells (data not shown) with miR-34a overexpression but not their corresponding mutant sequences. Similarly, the luciferase activity of the reporter containing the miR-34a-targeted wild-type sequences of MMP-2 and FNDC3B was significantly suppressed in the 293T cells (Fig. 4D and F) and EC9706 cells (data not shown) with miR-34a overexpression but not their corresponding mutant sequences.

On the contrary, the luciferase activity of the reporter containing the miR-34a-targeted wild-type sequences of MMP-2, MMP-9, and FNDC3B increased in the miR-34a inhibitor-transfected 293T cells (Fig. 4B, D and F) and EC9706 cells (data not shown) but not their corresponding mutant sequences. The influence of miR-34a on MMP-2, MMP-9, and FNDC3B expression levels was further confirmed by measuring the MMP-2, MMP-9, and FNDC3B levels in the EC9706 cells and TE-1 cells with miR-34a overexpression or miR-34a inhibition. The result showed that MMP-9, MMP-2, and FNDC3B presented an inverse expression trend to miR-34a in the EC9706 cells (Fig. 5A-C) and TE-1 cells (Fig. 5D-F). On the one hand, western blot analysis demonstrated that the miR-34a overexpression decreased the protein levels of MMP-2, MMP-9, and FNDC3B in the EC9706 cells (Fig. 6A)



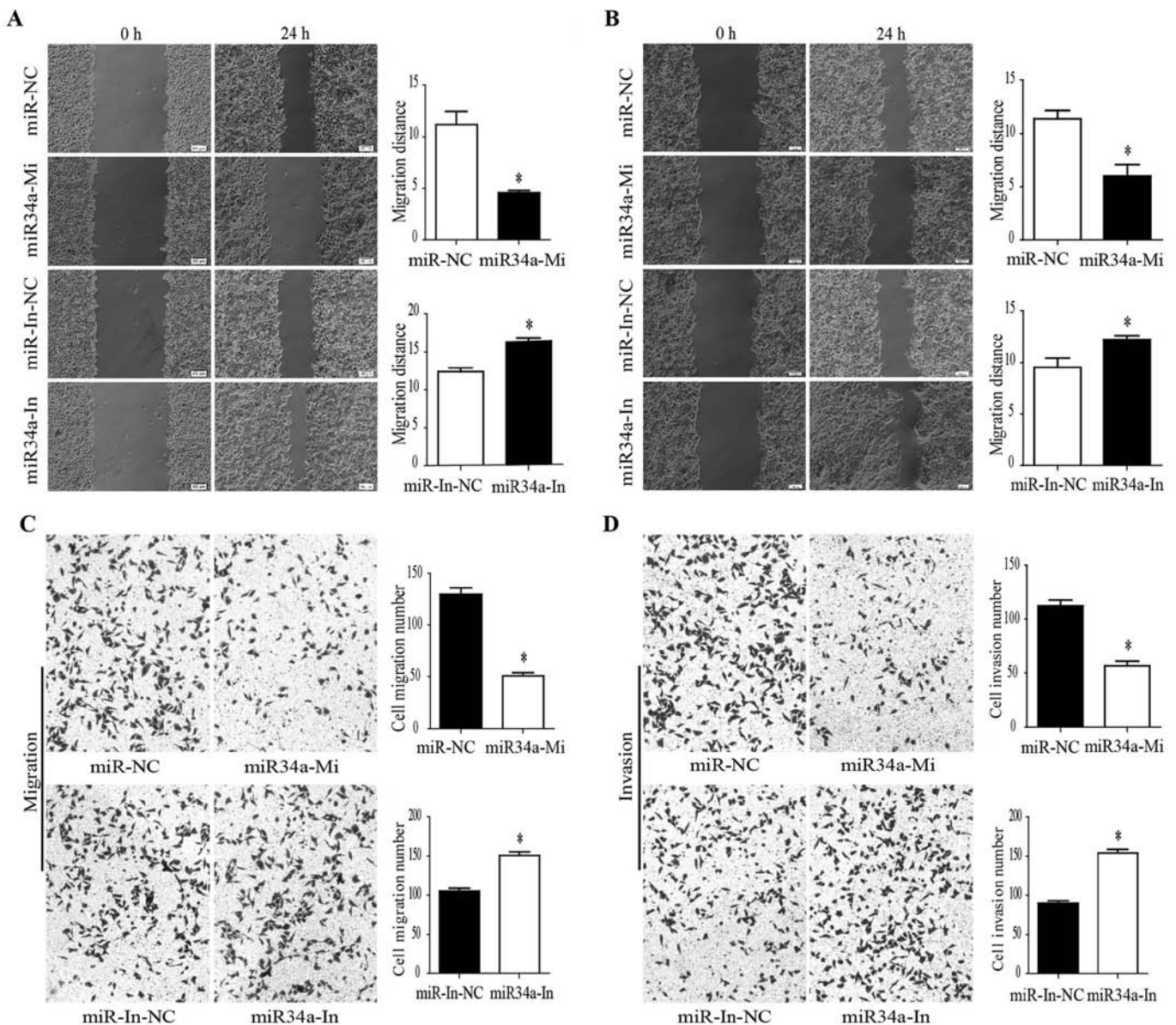


Figure 3. miR-34a inhibits ESCC cell migration and invasion. (A and B) EC9706 and TE-1 cells were transfected with miR-34a mimic, inhibitor, and negative controls and were assessed for migration by wound-healing assay at 0 h and 24 h. (\* $P < 0.05$ ). (C and D) Effects of miR-34a mimic or miR-34a inhibitor on EC9706 cell migration and invasion obtained using the Transwell assay (\* $P < 0.05$ ). NC, normal control; In, inhibitor; Mi, mimic.

and TE-1 cells (Fig. 6B). On the other hand, the miR-34a inhibitor transfection increased the protein levels of MMP-2, MMP-9, and FNDC3B in the EC9706 cells (Fig. 6A) and TE-1 cells (Fig. 6B). These data demonstrated that miR-34a directly binds to MMP-2/MMP-9/FNDC3B and represses the MMP-2/MMP-9/FNDC3B translation in ESCC cells.

## Discussion

ESCC is the leading cause of mortality in digestive tract malignancies, with a poor five-year overall survival rate (2-4). Therefore, a better understanding of the mechanisms involved in ESCC progression is urgent. miRNAs have demonstrated far-reaching effects on cellular biology and cancer development (40,41). A number of studies have reported a relatively low miR-34a expression level in various cancer types and cancer cell lines, including ESCC (12,21-26,39,42,43).

The present study found that the miR-34a as well as p53 expression was significantly reduced in human esophageal tumor tissues compared to adjacent normal tissues. It may be a potential positive control correlation between p53 and miR-34a in ESCC as previously reported in other cancers. Epigenetic mechanisms, including methylation and histone modification, chromosome deficiency, and transcriptional regulation, can influence the miRNA expression (44). Furthermore, the abnormal miR-34a expression is reported in multiple cancers (e.g., breast, lung, colon, and bladder cancers, and pancreatic carcinoma) due to aberrant CpG methylation of its promoter (45,46). A study found that the miR-34a promoter is more frequently methylated in the ESCC than in controls. Accordingly, the miR-34a expression decreases in patients with a high level of methylation compared to that in normal tissues (42). It indicates that the mechanisms of miR-34a repression may be aberrant from the CpG methylation of its promoter in ESCC.

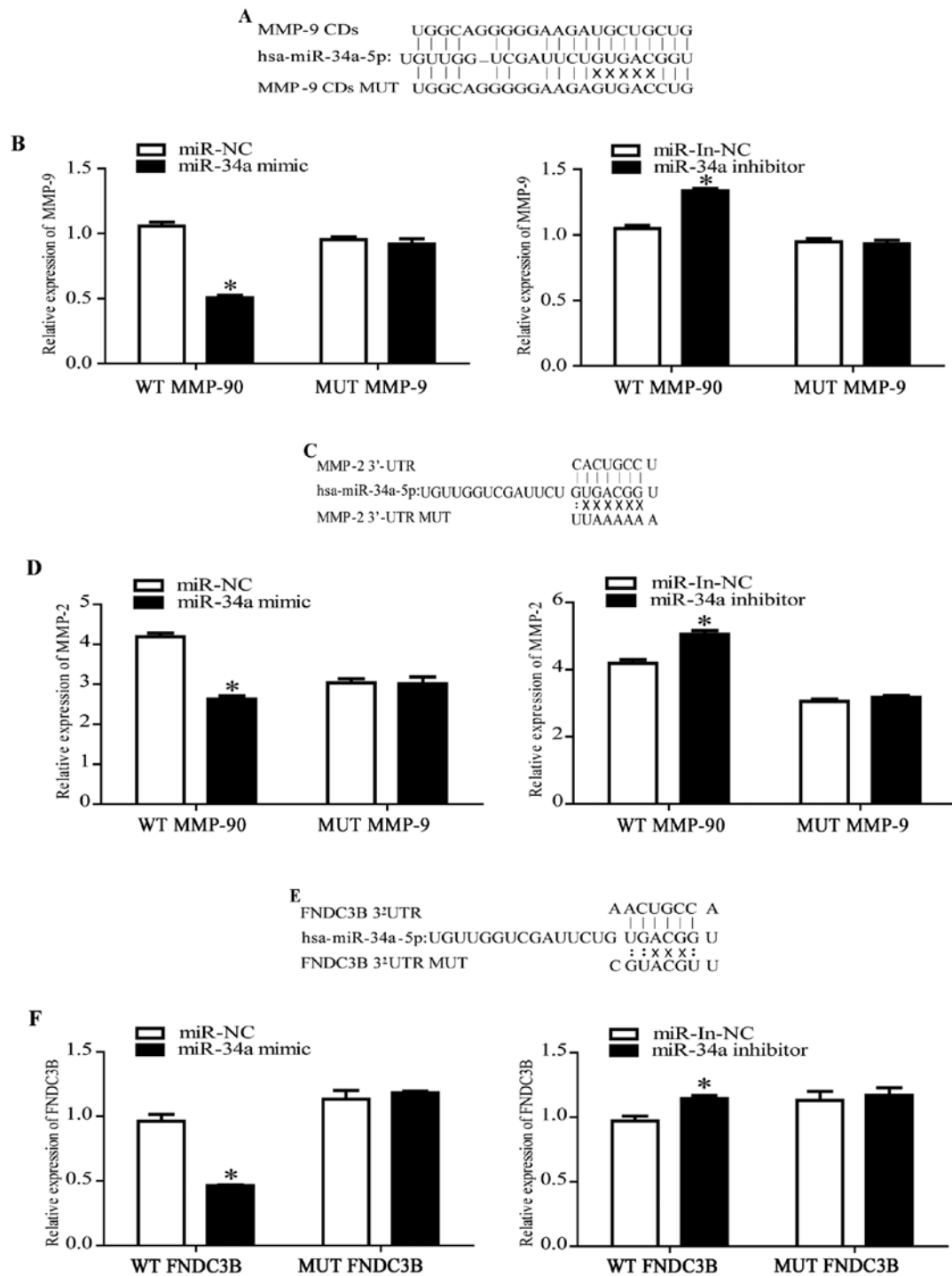


Figure 4. miR-34a suppresses luciferase activity of the reporter containing the miR-34a-targeted wild-type sequences of MMP-2, MMP-9, and FNDC3B. (A) Diagrams show the miR-34a putative binding sites and corresponding mutant sites of MMP-9. (B) Relative luciferase activity was determined after wild-type or mutant MMP-9 CDs reporter plasmids were co-transfected with miR-34a mimic or inhibitor in 293T cells. (C) The MMP-2 3'UTR regions containing the wild-type or mutant binding site for miR-34a are shown. (D) Relative luciferase activity was determined after wild-type or mutant MMP-2 3'UTR reporter plasmids were co-transfected with miR-34a mimic or inhibitor in 293T cells. (E) The FNDC3B 3'UTR regions containing the wild-type or mutant binding site for miR-34a are shown. (F) Relative luciferase activity was determined after wild-type or mutant FNDC3B 3'UTR reporter plasmids were co-transfected with miR-34a mimic or inhibitor in 293T cells. Data were normalized to the luciferase activity after transfection with miR-NC or miR-In-NC. (\*P<0.05). NC, normal control; In, inhibitor; WT, wild-type; MUT, mutant type.

Studies have found that miR-34a overexpression can suppress cell proliferation, migration, invasion, and EMT (22-24,27-31,43). Furthermore, miR-34a has significant relationships with node metastases, clinical stage, and patient mortality in tongue squamous cell carcinoma (12) and

ESCC (43). This finding indicates that miR-34a has a crucial role in tumor development, progression, and prognosis. We observed that miR-34a inhibits ESCC cell migration and invasion, which is consistent with previous results (39). Cell migration and invasion are normal events in cancer processes

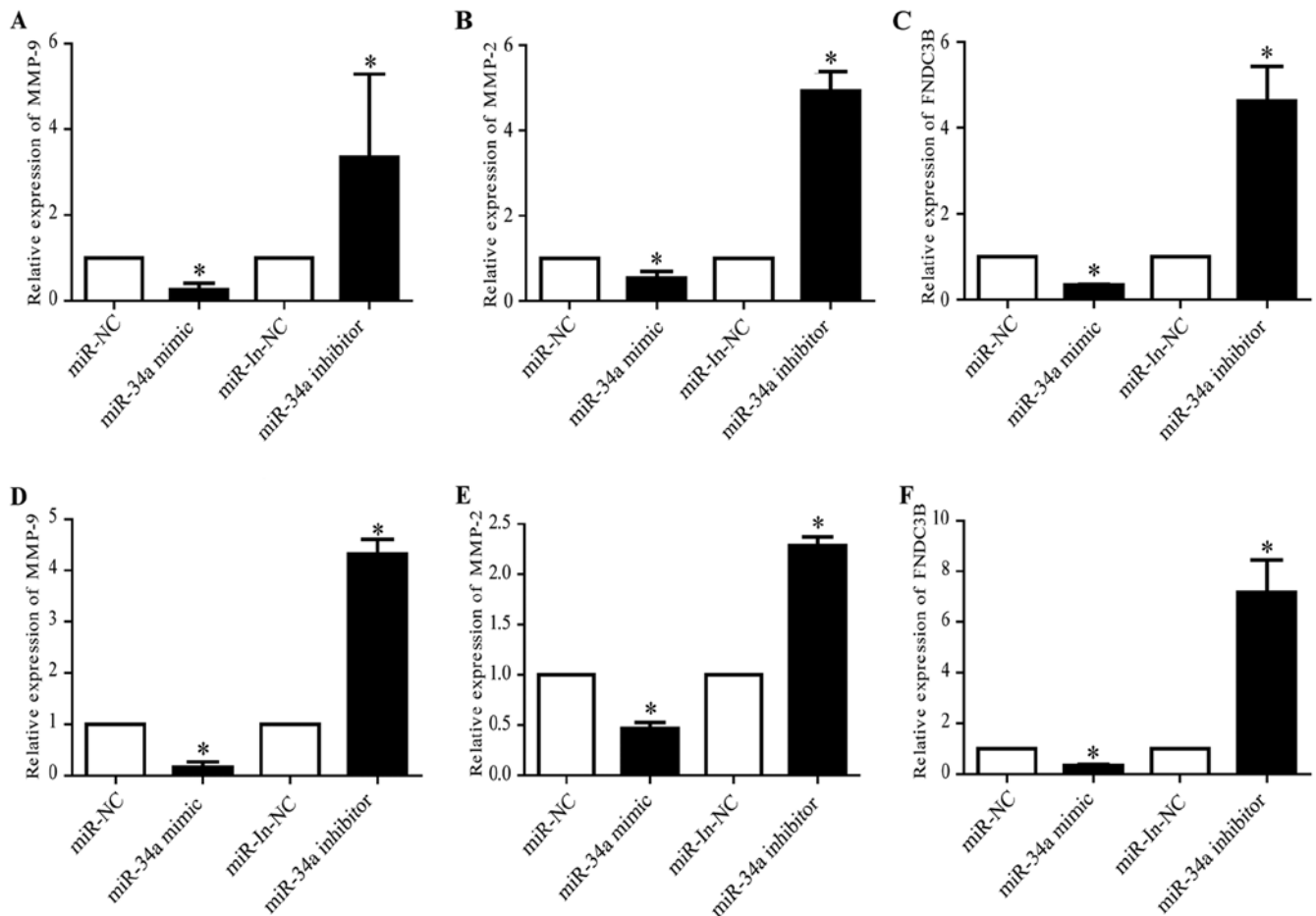


Figure 5. miR-34a overexpression decreases the MMP-2, MMP-9, and FNDC3B mRNA levels. (A-C) qRT-PCR detection of MMP-9, MMP-2, and FNDC3B mRNA expression in EC9706 cells transfected with miR-34a mimic or inhibitor. miR-34a overexpression decreases the MMP-2, MMP-9, and FNDC3B mRNA levels, whereas miR-34a inhibition increases them (\* $P < 0.05$ ). (D-F) qRT-PCR detection of MMP-9, MMP-2, and FNDC3B mRNA expression in TE-1 cells transfected with miR-34a mimic or inhibitor. miR-34a overexpression decreases the MMP-2, MMP-9, and FNDC3B mRNA levels, whereas miR-34a inhibition increases them (\* $P < 0.05$ ). NC, normal control; In, inhibitor.

and are two important elements that lead to metastases. Metastasis is a major cause of death in patients with esophageal cancer (6). miR-34a has been reported to suppress cell migration and invasion by targeting various oncogenes (22,24,25,30).

Previous studies reveal that miR-34a can inhibit ESCC cell migration and invasion by targeting YY-1. However, miR-34a might still directly modulate other genes simultaneously inhibiting ESCC cell migration and invasion because of the complex regulative network of miRNAs. We have found that MMP-2 and MMP-9 contain putative miR-34a target sites using a bioinformatics tools and a literature review. The MMP family, especially MMP-2 and MMP-9 known as gelatinases, is involved in cancer migration and invasion by degrading type-IV collagen, which is the major component of the basement membrane. MMP-2 and MMP-9 play vital roles in the early stages of tumor invasion. They are secreted during tumor growth, and can affect the surrounding micro-environment, thereby causing dynamic changes in the tumor bio-behavior (47).

MMP-2 and MMP-9 are reportedly overexpressed in ESCC tissues compared to that in the paired normal esophageal tissues. They are also related to tumor invasion and metastasis in the ESCC (48,49). The luciferase reporter

assays in this study reveal that miR-34a could directly interact with MMP-2 and MMP-9. Both mRNA and protein levels of MMP-2 and MMP-9 significantly decrease when miR-34a is overexpressed in ESCC cells. This finding is consistent with a report of an indirect negative correlation of miR-34a with MMP-2 and MMP-9 in ESCC (39). However, our results show that miR-34a directly targets MMP-2 or MMP-9. This finding seems to be different from the results of a previous report (39), where miR-34a indirectly downregulates MMP-2 or MMP-9 suppressing YY-1 in the ESCC. The current study also confirms that miR-34a directly targets MMP-9 in tongue squamous cell carcinoma (12) and Fra-1 in colon cancer (22). miR-34a may downregulate mRNA expression through direct and indirect regulatory mechanisms.

We also found that FNDC3B is the most likely direct target gene of miR-34a using a bioinformatics software and a literature review. FNDC3B is located at 3q26.31 and covers a large area (360 kb). FNDC3B is a member of the fibronectin family (50) with biological functions that remain largely unclear. The gene, which was initially discovered with another name (i.e., factor for adipocyte differentiation 104), is upregulated in the early stages of adipocyte differentiation. This upregulation indicates its potential role as a positive regulator



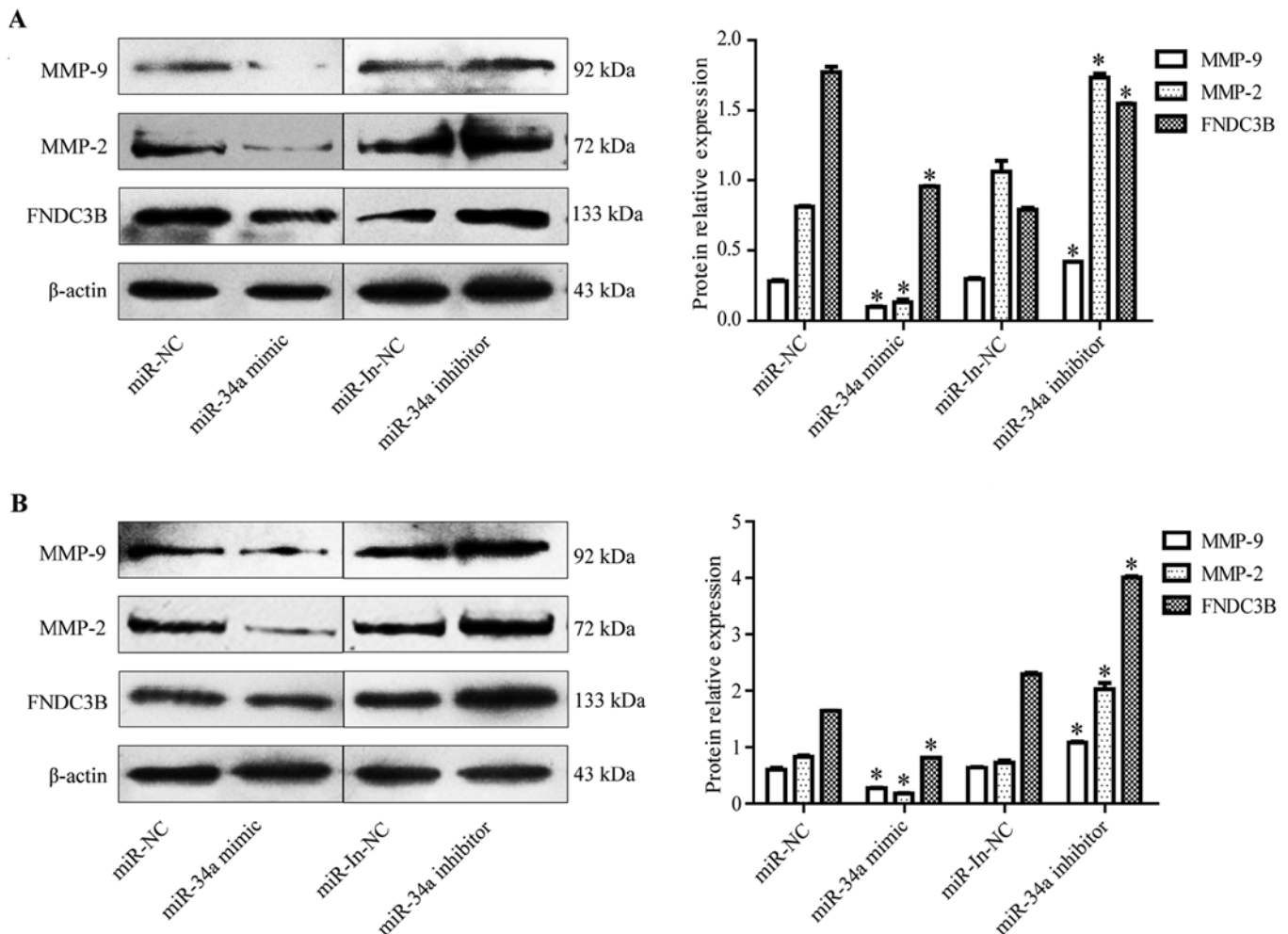


Figure 6. miR-34a overexpression decreases the MMP-2, MMP-9, and FNDC3B protein levels. (A) The MMP-9, MMP-2, and FNDC3B protein levels in the EC9706 cells transfected with miR-34a mimic or miR-34a inhibitor were detected by western blotting. miR-34a overexpression decreases the MMP-2, MMP-9, and FNDC3B protein levels, whereas miR-34a inhibition increases them ( $P<0.05$ ). (B) Western blot detection of MMP-9, MMP-2, and FNDC3B mRNA expression in TE-1 cells transfected with miR-34a mimic or inhibitor. miR-34a overexpression decreases the MMP-2, MMP-9, and FNDC3B protein levels, whereas miR-34a inhibition increases them ( $P<0.05$ ). NC, normal control; In, inhibitor.

of adipogenesis (51,52). However, FNDC3B has been recently identified as an important oncogenic driver gene of the 3q amplicon, thereby adding to the growing list of oncogenic drivers within this amplified region (53).

Previous studies have reported that miR-143-targeted the oncogene FNDC3B, regulating hepatocarcinoma metastasis (54). Furthermore, FNDC3B amplification could increase cell proliferation and promote tumorigenesis of hepatocellular carcinoma (50). The amplification and overexpression of FNDC3B are found in over 20% of cancers including ESCC (50,53,55). However, the role of FNDC3B in ESCC is presently still unconfirmed. Studies have found that the FNDC3B expression is significantly altered in ESCC and targeted by most miRNAs (56). In addition, FNDC3B overexpression induces EMT and activates several cancer pathways, including TGF $\beta$ 1 signaling, which contributes to cancer metastasis (53). miR-34a acts as a suppressor that regulates TGF $\beta$ 1 signaling by targeting PDGFRA in glioblastoma (57) and Smad4 inhibits EMT in extrahepatic cholangiocarcinoma (58). The TGF $\beta$ 1 signaling is an activity in ESCC that could induce EMT and contribute to ESCC

metastasis (59). Therefore, whether miR-34a has a connection with the TGF $\beta$ 1 signaling by regulating FNDC3B in ESCC needs to be determined. We have performed luciferase reporter assays to confirm that miR-34a could directly interact with FNDC3B. The results revealed that miR-34a could suppress the luciferase activity of the reporter containing the miR-34a-targeted wild-type sequences of FNDC3B. Both mRNA and protein levels of FNDC3B also significantly decrease when miR-34a is overexpressed in ESCC cells. In view of these results, FNDC3B is a direct target gene of miR-34a and may be important in the regulatory network. FNDC3B may also be involved in the progression of ESCC. We detected that FNDC3B could promote the ESCC cell invasion and migration (data not shown), combining this result with previous reports (53,59), we propose a hypothesis that miR-34a inhibits ESCC cell migration and invasion by targeting FNDC3B and reduces EMT by inhibiting the activity of the TGF $\beta$ 1 signaling pathway. This hypothesis needs further research.

Previous studies have confirmed that miR-34a is a downstream target of p53 (60). However, few studies have

reported the downstream targets of miR-34a in ESCC. Only one study found that miR-34a could directly target YY-1 in ESCC (39). In the present study, we found that miR-34a could directly target MMP-2, MMP-9, and FNDC3B in ESCC. miR-34a can directly and simultaneously modulate multiple genes in ESCC because of the complex regulative network of miRNAs.

This study confirmed that miR-34a expression significantly decreased in ESCC tissues and could inhibit the ESCC cell line migration and invasion. Accordingly, MMP-2, MMP-9, and FNDC3B are the genes directly targeted by miR-34a. miR-34a may have a therapeutic value in ESCC treatment. Therefore, further studies on the anticancer mechanisms of miR-34a may contribute to the development of new therapeutic strategies for ESCC.

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