miR-144 suppresses cell proliferation and invasion in gastric cancer through downregulation of activating enhancer-binding protein 4

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Abstract. Gastric cancer (GC) is the most common malignant disease and its incidence rate is increasing rapidly worldwide. The molecular mechanisms underlying GC tumorigenesis require further investigation. The expression and physiological roles of microRNA-144 (miR-144) have been investigated in numerous types of tumor. However, its biological function in GC remains largely unknown. The reverse transcription-quantitative polymerase chain reaction was used to determine the expression of miR-144 in GC cells and normal gastric epithelial cells. An miR-144 mimic was transfected into HGC-27 cells. In addition, bioinformatics analysis was performed to identify the potential targets of miR-144. Protein expression, luciferase and rescue assays were used to confirm the target of miR-144. It was identified that the expression of miR-144 was significantly downregulated in GC cells compared with in normal gastric epithelial cells. Furthermore, overexpression of miR-144 suppressed HGC-27 cell proliferation, migration and invasion. Additionally, bioinformatics analysis suggested that the activating enhancer-binding protein 4 (AP4) is a target gene of miR-144. In addition, it was determined that miR-144 suppresses the expression of AP4 by binding directly to its 3'-untranslated regions. Furthermore, restoration of AP4 partially attenuated miR-144-induced inhibition of cell proliferation, migration and invasion. Therefore, the results of the present study suggest that miR-144 serves an important role in GC progression.

Introduction

Gastric cancer (GC) is one of the most prevalent types of malignancy. In 2015, 1.3 million cases of GC and 819,000 GC-associated cases of mortality were reported worldwide, which makes GC the third leading cause of cancer-associated mortality globally (1).

MicroRNAs (miRNAs or miRs) are small non-coding RNAs, ~22 nucleotides in length, which regulate the expression of multiple target mRNAs and serve crucial roles in various biological processes, including cell development, infection, immunity and carcinogenesis (2,3). Previous studies have demonstrated that a variety of miRNAs are dysregulated in many types of cancer, including GC (4,5), which provides a new molecular basis for tumorigenesis. However, the functional effects of miRNAs in GC require further investigation.

miR-144 has been reported to be downregulated in a number of types of cancer, including breast cancer, colorectal cancer, non-small cell lung cancer, osteosarcoma, thyroid cancer, hepatocellular carcinoma and bladder cancer (6-12). It has been demonstrated that miR-144 can inhibit the proliferation of colorectal cancer cells *in vitro* and *in vivo*, which was associated with downregulation of Notch-1 signaling (13). However, Zhang *et al* (14) revealed that miR-144 significantly promotes cell proliferation, migration and invasion in nasopharyngeal carcinoma by targeting phosphatase and tensin homolog. Therefore, this suggests the function of miR-144 in tumorigenesis is complicated and tissue-specific.

The aim of the present study was to investigate the expression pattern, biological significance and underlying molecular mechanisms of miR-144 in GC cells. It was identified that miR-144 was downregulated in GC cells, and miR-144 inhibited the proliferation and invasion of GC cells by directly targeting activating enhancer-binding protein 4 (AP4).

Materials and methods

Cell culture. The gastric cancer cell lines SGC-7901, AGS, MKN-45, HGC-27 and BGC-823, and the normal gastric epithelial cell line GES-1, were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cell lines using

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TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), followed by RT. qPCR was performed with an ABI 7500 system (Thermo Fisher Scientific, Inc.) using TaqMan Universal PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, and 60˚C for 1 min. The expression of U6 small nuclear RNA was determined as an internal control. The primers used for RT-qPCR were as follows: miR-144: 5'-GCGCGAATTCGAGATCTTAACAGA CCCTAGCTC-3' (forward primer) and 5'-GCGCGGATCCGT GCCCTGGCAGTCAGTAGG-3' (reverse primer); U6snRNA: 5'-CGCAAGGAUGACACGCAAAUUCGUGAAGCGUUC CAUAUUUUU-3'. Fold changes in relative gene expression were calculated using the $2^{-\Delta\Delta Cq}$ method (15). All reactions were performed in triplicate.

Cell transfection. miR-144 mimic (5'-UACAGUAUAGAU AUGUACU-3'), miR-144 antisense (5'-AGUACAUCAUCU AUACUGUA-3') and miR-144 scramble (negative control, 5'-AUCAUCUAUACUGUAAGUAC-3') were obtained from Chang Jing Bio-Tech, Ltd. (Changsha, China). For transfection, HGC-27 cells were seeded in 6- or 24-well plates and transiently transfected at 70-80% confluence using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The final concentration of miR-144 mimic, miR-144 antisense or miR-144 scramble in the transfection system was 100 nM. After 24 h, the cells were used for the subsequent experiments.

Cell viability. HGC-27 cell proliferation was evaluated using an MTT assay. A total of $5x10^3$ cells were seeded into each well of a 96-well plate and incubated for 24, 48 or 72 h. Subsequently, 20 μl MTT (0.5 mg/ml in DMSO) was added to each well. Following removal of the medium, absorbance was determined at 570 nm using a spectrophotometer.

Cell apoptosis. The HGC-27 cells for apoptosis analysis were harvested, washed twice with ice-cold PBS, and resuspended in 1Xbinding buffer at a concentration of $6x10^5$ cells/ml. A total of 100 μl of the solution ($6x10^4$ cells) was then transferred to a 5 ml culture tube. The cells were then incubated with 5 μl of Annexin V-fluorescein isothiocyanate (BD Biosciences, San Jose, CA, USA) and 5 μl of 1 mg/ml propidium iodide (PI) at room temperature in the dark for 15 min. Following the incubation period, 400 μl of 1X binding buffer was added to each sample and the samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Results were obtained by analyzing data with FlowJo software (version 7.6.1; FlowJo LCC, Ashland, OR, USA). All assays were repeated three times.

Transwell and Matrigel assays. The migration and invasion of HGC-27 cells were assayed using Transwell chamber (Corning Inc., Corning, NY, USA). For the migration assays, $1x10^5$ cells were plated in the top chamber containing a non-coated membrane. The cells were plated in the serum-free medium (Invitrogen; Thermo Fisher Scientific, Inc.), and medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS was used as a chemoattractant in the lower chamber. For invasion assays, the filters were precoated with Matrigel (BD Biosciences) in the upper chamber prior to cell seeding. Subsequently, $2x10^4$ cells were seeded into the upper chamber and the lower chamber was filled with culture medium supplemented with 10% FBS. The cells were incubated at 37˚C in a tissue culture incubator with 5% CO_2. After 16 h, cells that had migrated and invaded to the lower membrane surface were fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 10 min and stained with 0.1% crystal violet for 10 min at room temperature. Images were captured by a phase-contrast microscope (Zeiss AG, Oberkochen, Germany). Five random fields from each membrane were photographed and cells were counted. These experiments were performed in triplicate.

Western blot analysis. HGC-27 cells were harvested and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris/HCl and 1% NP-40, pH 7.5). Protein concentration was determined using the Bradford assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts (50 μg) of proteins were separated by SDS-PAGE (8% gels) and then transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 10% non-fat milk in PBS for 1 h at room temperature, membranes were incubated overnight with anti-AP4 antibodies (cat. no. sc-166024; dilution, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C, followed by horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature. The signals were detected with the SuperSignal West Pico Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Protein levels were normalized to total GAPDH, using a monoclonal anti-GAPDH antibody (cat. no. sc-293335; dilution, 1:1,000; Santa Cruz Biotechnology).

Bioinformatics. Potential miR-144 targets were predicted and analyzed using the following two publicly available databases: TargetScan 6.2 (http://www.targetscan.org), and miRanda (http://www.microrna.org/microrna/getGeneForm.do) (16,17).

Luciferase reporter assay. The 3'-untranslated region (3'-UTR) of the AP4 gene that was predicted to interact with miR-144 was amplified and inserted into pMIR-Report Luciferase vector (Ambion; Thermo Fisher Scientific, Inc.). Mutations within potential miR-144-binding sites were generated by nucleotide replacement. Co-transfection of indicated plasmids plus 1 ng pRL-TK *Renilla* was performed using Lipofectamine® 2000 (Promega Corporation, Madison, WI, USA). After 24 h, the activities of firefly luciferase and *Renilla* luciferase in the cell lysates were measured with the Dual-Luciferase reporter assays (Promega Corporation, Madison, Wisconsin, USA), and values for cells with reporter genes containing the wild-type AP4 3'-UTR were set equally to 1. Experiments were performed three times.

Co-transfection of miR-144 and AP4. AP-4 DNA sequences were amplified from human genomic DNA, subcloned into the pcDNA3.1+ vector (Invitrogen; Thermo Fisher Scientific, Inc.), and verified by DNA sequencing. miR-144 mimics/mimic control and the pc-AP4 plasmids were co-transfected into

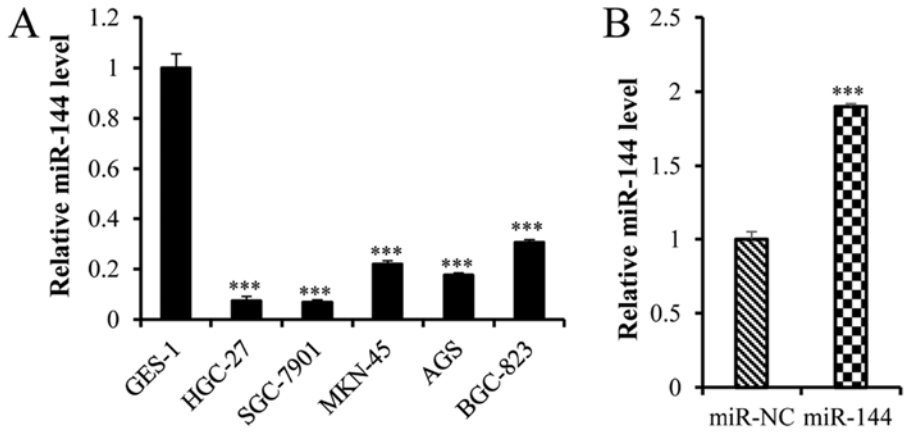

Figure 1. Expression levels of miR-144 in gastric cancer cells. (A) miR-144 expression was determined by RT-qPCR in gastric cancer cell lines (HGC-27, SGC-7901, MKN-45, AGS and BGC-823) and a normal gastric epithelial cell line (GES-1). ***P<0.001 vs. GES-1. (B) RT-qPCR was performed to confirm the effect of transfection with miR-144 mimic. ***P<0.001 vs. miR-NC. Data are from three independent experiments and are presented as the mean ± standard deviation. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control.

cultured HGC-27 cells by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 48 h.

Statistical analysis. Data are presented as the mean ± standard deviation from at least three separate experiments. Statistical differences were analyzed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Comparisons between two groups were performed using Student's t-test. Comparisons among three or more groups were performed using one-way analysis of variance followed by a least significant difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR-144 in GC cells. To delineate the function of miR-144 in gastric epithelial cell malignant transformation, its expression levels were analyzed in GC cells and normal gastric epithelial cells. As presented in Fig. 1A, results from RT-qPCR analysis revealed that the expression level of miR-144 was significantly lower in the five GC cell lines SGC-7901, AGS, MKN-45, HGC-27 and BGC-823 compared with the normal gastric epithelial cell line GES-1. These data suggest that the expression of miR-144 is downregulated in GC.

miR-144 suppresses the proliferation of GC cells. To clarify the role of miR-144 in GC progression, a miR-144 mimic was transfected into HGC-27 cells, which had the lowest expression level of miR-144. The effect of miR-144 mimic was confirmed by RT-qPCR (Fig. 1B). miR-144 overexpression significantly suppressed cell proliferation as revealed using an MTT assay (Fig. 2A). In addition, the proportion of apoptotic cells significantly increased in miR-144-transfected cells (Fig. 2B and C). Therefore, the results suggest that upregulation of miR-144 may inhibit GC cell proliferation and promote apoptosis *in vitro*.

miR-144 inhibits the migration and invasion of GC cells. Subsequently, the role of miR-144 in regulating GC cell migration and invasion was investigated using cells transfected with miR-144 mimic or control. As demonstrated by Transwell and Matrigel assays, overexpression of miR-144 significantly inhibited the migratory and invasive capabilities of GC cells (Fig. 2D and E).

miR-144 targets AP4 directly. The aforementioned results suggested an inhibitory role for miR-144 in GC. Therefore, to investigate the target of miR-144 in GC and reveal the underlying molecular mechanisms, miR-144 targets were searched for using TargetScan. The conserved target gene AP4, which has been identified as a transcription factor, was selected for subsequent investigation (Fig. 3A). As presented in Fig. 3B, overexpression of miR-144 significantly inhibited AP4 expression, whereas inhibition of miR-144 resulted in an increased protein level of AP4, which suggests that AP4 is a target of miR-144. To further confirm that AP4 is a direct target of miR-144, a luciferase reporter assay was performed. Notably, miR-144 overexpression significantly suppressed the luciferase activity of the wild-type AP4 3'-UTR, which was fully rescued when the potential miR-144-binding site was mutated (Fig. 3C).

AP4 overexpression partially reverses the tumor-suppressive effects of miR-144. To further verify the functional association between miR-144 and AP4, the present study assessed whether ectopic expression of AP4 could reverse the inhibitory effects of miR-144 on GC cells. As presented in Fig. 4A, transfection with pc-AP4 could increase AP4 expression levels. The increased expression of AP4 significantly attenuated the tumor-suppressive effect of miR-144, underlining the importance of AP4 for miR-144 action in cell proliferation, migration and invasion (Fig. 4B-D).

Discussion

It is understood that a variety of miRNAs are involved in GC tumorigenesis (4,5). The present study identified that miR-144 was downregulated in GC cells compared with normal cells. Furthermore, it was demonstrated that increased expression of miR-144 suppressed the proliferation, migration and invasion

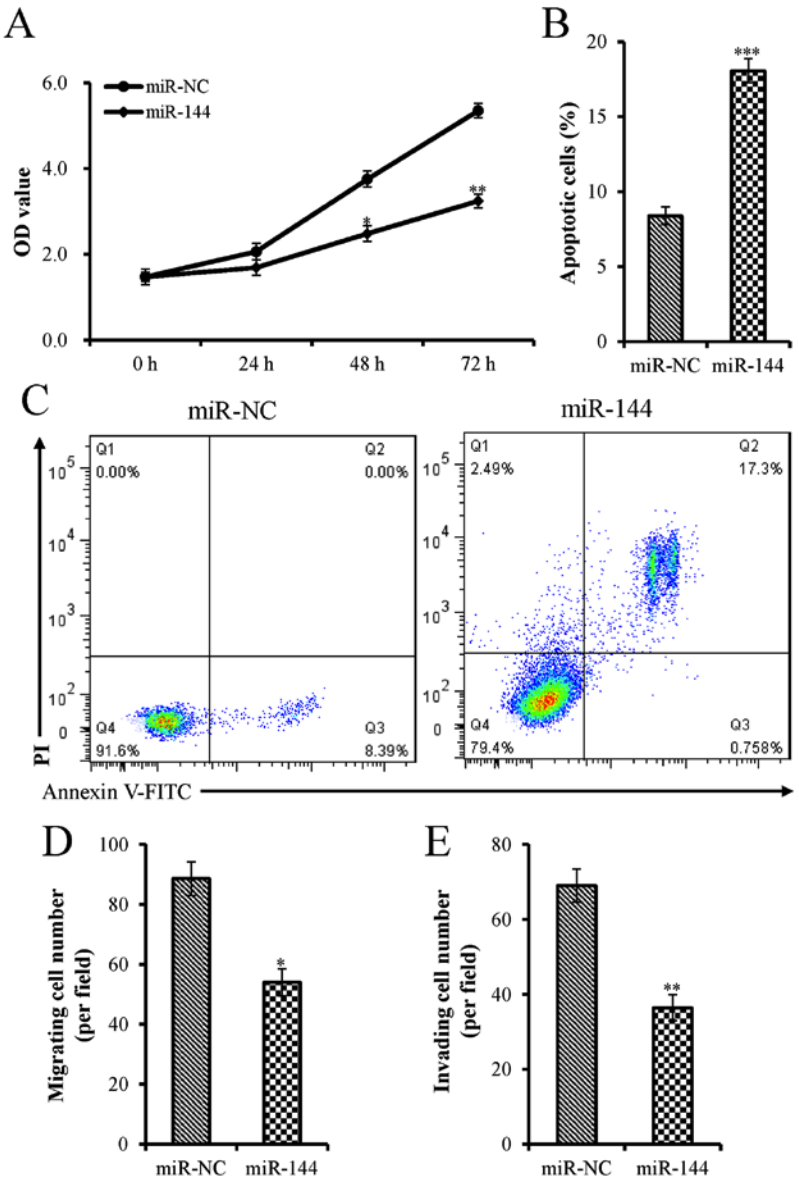

Figure 2. miR-144 suppresses the proliferation, migration and invasion of gastric cancer cells. (A) An MTT assay was performed to examine proliferation. (B) Quantitative analysis of the percentage of apoptotic cells. (C) Representative histograms of apoptosis analysis. In each plot, viable cells are in the Q4 quadrant, early apoptotic cells are in the Q3 quadrant, late apoptotic cells are in the Q2 quadrant and necrotic cells are in the Q1 quadrant. (D) Migration assay of HGC-27 cells transfected with miR-144 or miR-NC. (E) Invasion assay of HGC-27 cells transfected with miR-144 or miR-NC. Data are from three independent experiments and are presented as the mean ± standard deviation *P<0.05, **P<0.01, ***P<0.001 vs. miR-NC. miR, microRNA; NC, negative control; OD, optical density; PI, propidium iodide; FITC, fluorescein isothiocyanate; Q, quadrant.

of GC cells, which indicates that miR-144 may serve as a tumor suppressor in GC.

Previously, miR-144 has attracted much attention for its role in carcinogenesis and cancer treatment. A number of studies have reported that downregulation of miR-144 is involved in the tumorigenesis of various cancer types. A study by Pan *et al* (6) identified that miR-144 exhibits potent tumor suppressor activity in breast cancer by downregulating the zinc finger E-box-binding homeobox (ZEB)1/2-mediated epithelial-mesenchymal transition process. It has also been observed in colorectal cancer that loss of miR-144 expression predicts a poor prognosis (7). Zhou *et al* (11) demonstrated that miR-144 could potentially serve a role in the reversal of chemoresistance in hepatocellular carcinoma cells, partly by suppressing the nuclear factor (erythroid-derived 2)-like 2-dependent antioxidant pathway. Furthermore, Guo *et al* (12) suggested that the expression miR-144 was significantly decreased in bladder cancer and its inhibition promoted bladder cancer cell proliferation by decreasing the repression of enhancer of zeste homolog 2. Notably, it also has been demonstrated that the expression of miR-144 is decreased in GC (18-20). However, the biological function and underling molecular mechanisms of miR-144 in GC are not fully understood. The results of the present study indicate that miR-144 serves as a tumor suppressor in GC cells.

To improve understanding of the molecular mechanisms involved in miR-144-mediated inhibition of proliferation, migration and invasion, AP4 was selected for further study as it was predicted to be a target of miR-144 by bioinformatics analysis. Transcription factor AP4 belongs to the helix-loop-helix

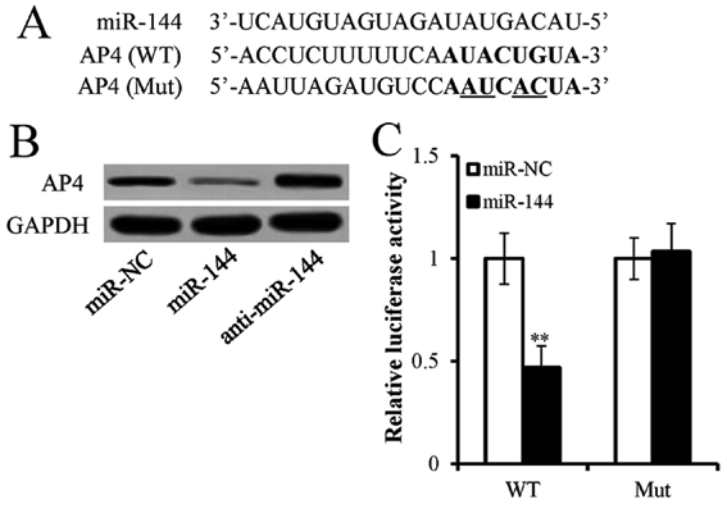

Figure 3. AP4 is a direct target of miR-144. (A) WT AP4 3'-UTR and Mut AP4 3'-UTR sequences. (B) The protein expression level of AP4 in HGC-27 cells transfected with miR-NC, miR-144 or anti-miR-144 was determined by western blot analysis. (C) The luciferase activity of the WT and Mut AP4 3'-UTR co-transfected with miR-144 or miR-NC was determined. Data are from three independent experiments and are presented as the mean ± standard deviation **P<0.01 vs. miR-NC. WT, wild-type; AP4, activating enhancer-binding protein 4; 3'-UTR; 3'-untranslated region; miR, microRNA; NC, negative control.

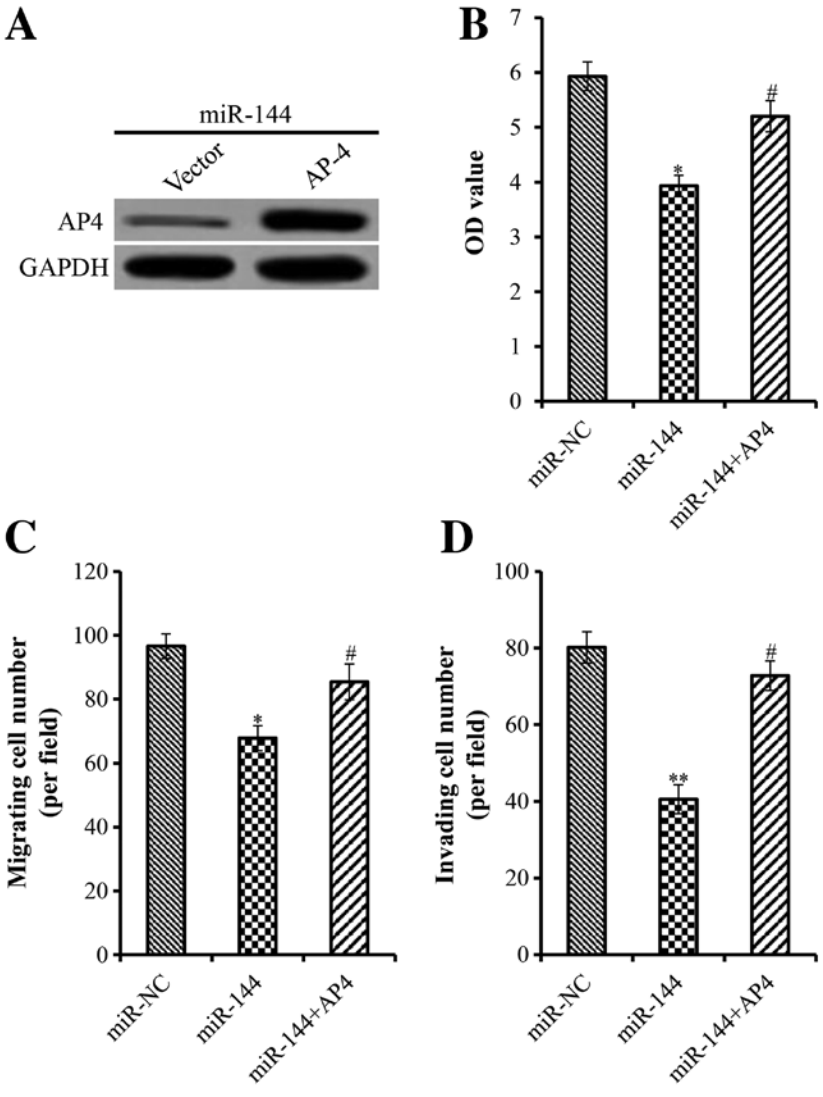

Figure 4. Overexpression of AP4 partially reverses the tumor-suppressive effects of miR-144. (A) HGC-27 cells were transfected with miR-144 with/without AP4 overexpression plasmid. (B) MTT, (C) migration (D) and invasion assays were performed to determine the proliferation, migration and invasion of HGC-27 cells transfected with miR-144 with/without AP4. Data are from three independent experiments and are presented as the mean ± standard deviation *P<0.05, **P<0.01 vs. miR-NC; $^{\#}$P<0.05 vs. miR-144-transfected cells. AP4, activating enhancer-binding protein 4; miR, microRNA; OD, optical density; NC, negative control.

family (21), is involved in cell proliferation and differentiation (22,23), affects cell cycle progression and apoptosis (24), and regulates the expression of certain genes (25,26). Previously, the overexpression of AP4 has been identified in breast cancer, prostate cancer and colorectal cancer (27-29). In addition, AP4 overexpression in GC indicated a worse prognosis for patients and positive correlations were revealed between AP4 overexpression and the depth of tumor invasion, the degree of tumor differentiation, lymphatic vessel invasion and pathological Tumor-Node-Metastasis stage (30). Previous studies have suggested that AP4 may suppress apoptosis of GC cells, and serve an important role in the tumorigenesis and progression of stomach cancer (24,31). The results of the present study identified an important molecular association between miR-144 and AP4. Results of luciferase reporter assays accompanied by western blot analysis demonstrated that miR-144 could directly target the 3'-UTR of AP4 and thereby decrease the expression of AP4 protein. Finally, increased expression of AP4 could partially reverse the miR-144-induced inhibition of cell proliferation, migration and invasion, suggesting that AP4 acts as a functionally relevant target of miR-144 in GC cells. Further studies are required to identify other candidate target genes that may be regulated by miR-144 and be involved in tumorigenesis of GC.

In conclusion, the results of the present study highlight the roles of miR-144, which demonstrates an ability to suppress proliferation, migration and invasion of GC cells by directly targeting AP4. Therefore, miR-144 may act as a tumor suppressor in GC and may exhibit a therapeutic potential for GC treatment in the future.

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Availability of data and materials

The datasets used or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

FM and JL conceived and designed the present study. FM was responsible for data acquisition, and FM, JZ and JL analyzed and interpreted the data. FM and JL wrote the manuscript. All authors are accountable for all aspects of the work. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

This work has been approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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