

lncRNA MEG3 inhibits pituitary tumor development by participating in cell proliferation, apoptosis and EMT processes

XUEJIAN WANG^{1,2}, XIANGDONG LI¹ and ZHIFENG WANG²

¹Department of Neurosurgery, First Affiliated Hospital of Soochow University, Soochow University, Soochow, Jiangsu 225000; ²Department of Neurosurgery, Affiliated Hospital 2 of Nantong University, Nantong University, Nantong, Jiangsu 226000, P.R. China

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Abstract. Pituitary tumors do not pose a threat to life but can cause visual disturbances and serious clinical syndromes, such as infertility and metabolic syndrome. Therefore, screening of key genes involved in the occurrence and development of pituitary tumors can provide new targets for the treatment of pituitary tumors. The aim of the present study was to investigate the molecular mechanism of long non-coding (lnc) RNA maternally expressed 3 (MEG3) in cell proliferation, apoptosis and epithelial-mesenchymal transition (EMT) processes of pituitary tumor. Tissue samples were obtained from 34 patients who underwent surgical treatment of pituitary tumors. Pituitary tumor cells (GH3 and MMQ) were transfected with pcDNA3.1(+)-MEG3, short hairpin (sh)MEG3, microRNA (miR)-23-3p inhibitor or their controls using Lipofectamine[®] 2000. Reverse transcription-quantitative PCR and western blot analyses were used to detect the levels of MEG3, miR-23b-3p and FOXO4, as well as proliferation-, apoptosis- and EMT-associated genes and proteins. Cell Counting Kit-8 and flow cytometry assays were performed to detect proliferation and apoptosis, and Transwell assay was undertaken to assess invasion and migration. Luciferase reporter and RNA pulldown assays were performed to verify the binding between lncRNA MEG3, miR-23b-3p and FOXO4. Pearson's correlation analysis was used to analyze the correlation between expression levels of MEG3, miR-23b-3p and FOXO4. lncRNA MEG3 was expressed at lower levels in pituitary tumor tissues and cells. Overexpression of lncRNA MEG3 inhibited proliferation, invasion and migration and accelerated apoptosis of

pituitary tumor cells. lncRNA MEG3 negatively regulated miR-23b-3p expression levels, while miR-23b-3p negatively regulated FOXO4 expression levels. Overexpression of lncRNA MEG3 inhibited the EMT process in pituitary tumor cells. miR-23-3p inhibitor rescued the effect of shMEG3 on proliferation, invasion, migration, apoptosis and the EMT process in pituitary tumor cells. lncRNA MEG3 inhibited pituitary tumor development by participating in cell proliferation, apoptosis and the EMT process, which may present a novel target for pituitary tumor treatment.

Introduction

Pituitary adenoma (PA) occurs in anterior and posterior pituitary and residual cells of the craniopharyngeal duct epithelium and accounts for ~10% of intracranial tumors (1). The disease is more common in men than in women (1.32:1) in China (2), often affecting the patient's growth and development, as well as reproductive, learning and working ability (3). The clinical manifestation of pituitary tumors includes headaches, vision loss, visual field defects and abnormal hormone secretion; the primary manifestation in women also includes menstrual disorders, amenorrhea and galactorrhea (4). The current treatment of pituitary tumors generally includes surgery, medication and radiation therapy (5). The goal of these three treatment methods is to decrease or eliminate the impact of tumor-occupying lesions, correct excessive hormone secretion by the tumor and retain normal pituitary function (6). However, the recurrence rate following pituitary tumor surgery is high at ~7-35% (7). In addition, pituitary tumors may lead to complications following surgery, such as diabetes insipidus, sphenoid sinusitis, cerebrospinal fluid leakage, worsening visual impairment, cerebral palsy and meningitis (8). It is therefore important to develop novel effective treatments.

Long non-coding (lnc)RNA is a class of RNA molecules, a number of which have been identified as biomarkers of cancer, such as exosome H19 and Clarin 1 antisense RNA 1 (9-13). Iyer *et al* (10) performed data analysis on 7,256 tumor and normal tissue samples and cell lines, and screened 7,942 lncRNAs associated with cancer; these lncRNAs may be biomarkers for specific types of cancer tissue. Numerous lncRNAs have been

Correspondence to: Dr Xiangdong Li, Department of Neurosurgery, First Affiliated Hospital of Soochow University, Soochow University, 899 Pinghai Road, Soochow, Jiangsu 225000, P.R. China
E-mail: meilihe0513@163.com

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confirmed to be associated with the development of pituitary tumor (11). For example, exosome H19 inhibits the growth of distal pituitary tumors and the expression levels of exosome H19 in plasma can predict the prognosis and treatment effectiveness (12). D'Angelo *et al* (13) demonstrated that expression levels of ribosomal protein SA pseudogene 52 are significantly higher in pituitary tumor cells than in controls and accelerate pituitary tumor development via regulating high mobility group A. Clarin 1 antisense RNA 1 has also been confirmed to participate in the Wnt/ β -actin signaling pathway, inhibit autophagy progress and suppress pathogenesis of pituitary tumors (14). There has been research on differential expression of lncRNAs in PA and the functional mechanisms involved in PA occurrence, development and prognosis (15,16). However, the expression levels, function and regulatory mechanisms of lncRNAs in PA need further investigation.

lncRNA maternally expressed 3 (MEG3) has been reported to be expressed at significantly lower levels in a number of tumors (17,18). In the development of normal anterior pituitary tissue to PA, MEG3 expression levels gradually decrease, whereas those of proliferating cell nuclear antigen (PCNA) significantly increase in non-functional (NF)PA (19). PCNA is negatively associated with MEG3 (20). Therefore, the expression levels of MEG3 may be associated with the development and invasive ability of NFPA. Previous studies have also found that MEG3 levels are decreased in NF and corticotrophic cell PA, which may be associated with the pathogenesis of this disease (15,21). To the best of our knowledge, however, the detailed molecular mechanism underlying MEG3 in PA has not been identified. The present study aimed to investigate this mechanism, which may provide a theoretical basis for gene therapy of pituitary tumor.

Materials and methods

Analysis of GSE77517 profile. GSE77517 (ncbi.nlm.nih.gov/geo/query/acc.cgi) was downloaded from the Gene Expression Omnibus (GEO) database, which comprises 5 patients with NFPA. The relative MEG3 expression level compared with that in normal tissue was calculated.

Patients and tissues. A total of 34 patients who underwent surgical treatment of pituitary tumors at First Affiliated Hospital of Soochow University (Nantong, China) from January to December 2019 were enrolled in the present study, including 19 males and 15 females. Patients had not been treated with radiotherapy or chemotherapy before surgery. The age range of the patients 25-67 years, with an average age of 46.5 years. The present study was approved by the ethics committee of First Affiliated Hospital of Soochow University and all patients provided written informed consent for the use of their samples in scientific research. Patient information, including age, sex and tumor size, stage and invasiveness were recorded. Pituitary tumor samples were collected during surgery. After the tissue was obtained, it was placed in liquid nitrogen to freeze and stored in a refrigerator at -80°C .

Cell culture and transfection. Pituitary tumor cell lines (GH3 and MMQ) were purchased from the American Type Culture Collection (ATCC). GH3 and MMQ cells were maintained

in RPMI-1640 medium containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) at 37°C and 5% CO_2 for routine culture and passage. Cells in the logarithmic growth phase were used for experiments. Cells were transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. When confluence reached 80%, the cells were digested by trypsin digestion solution at room temperature for 1 min and resuspended in RPMI-1640 medium. Cells were seeded in a 6-well plate at a density of $2 \times 10^5/\text{l}$ and divided into pcDNA3.1(+)-MEG3 and pcDNA3.1(+)-negative control (NC) transfection groups, with 3 replicate wells in each group. The concentration of pcDNA3.1(+)-MEG3 and pcDNA3.1(+)-negative control was 20 pmol/l. The sequence of lncRNA MEG3 was obtained from ddbj database (getentry.ddbj.nig.ac.jp/getentry/na/AB032607/?filetype=html). pcDNA3.1(+)-MEG3 and pcDNA3.1(+)-negative control (NC) were constructed by Sangon Biotech Ltd. When confluence reached 70-80%, cells were washed twice with PBS, then serum-free RPMI-1640 was added and DNA-liposome complex was made at a ratio of $1 \mu\text{g}:3 \mu\text{l}$. Following incubation at 37°C and 5% CO_2 for 5 h, the culture was replaced with RPMI-1640 containing 10% fetal bovine serum. After culturing at 37°C for 48 h, cells were collected for subsequent experiments. MicroRNA (miRNA or miR)-23b-3p mimic and controls, as well as short hairpin (sh)MEG3, miR-23b-3p inhibitor or their controls, were also transfected as aforementioned.

Reverse transcription-quantitative (RT-q)PCR assay. RNA was extracted from tissue samples and pituitary tumor cells ($\sim 100 \text{ mg}$) using TRIzol[®] (Invitrogen; Thermo Fisher Scientific, Inc.). Purity and concentration of total RNA was detected by NanoDrop 1000 (Thermo Fisher Scientific, Inc.) and A260/A280 should be ~ 2.0 . The Platinum SYBR Green qPCR super Mix-UD kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to establish a $25 \mu\text{l}$ reaction system according to the manufacturer's instructions. All primers were designed and purchased from Nanjing Kingsray Biotechnology Co., Ltd.. Primer sequences were as follows: MEG3, forward, 5'-CTGCCC ATCTACACCTCACG-3' and reverse, 5'-CTCTCCGCCGTC TTGCGCTAGGGGCT-3'; miR-23b-3p, forward, 5'-CACAAA GGCATCTCTACGCCATC-3' and reverse, 5'-TGTAGCTGC CTCGCCAGAGGC-3'; FOXO4, forward, 5'-CTTTCTGAA GACTGGCAGGAATGTG-3' and reverse, 5'-GATCTAGGT CTATGATCGCGGCAG-3'; GAPDH, forward, 5'-AGAGGC AGGGATGATGTTCTG-3' and reverse, 5'-GACTCATGACCA CAGTCCATGC-3'; and U6, forward, 5'-TGCGGGTGCTCG CTTCGGCAGC-3' and reverse, 5'-CCAGTGCAGGGTCCG AGGT-3'. The amplification conditions were as follows: Holding stage, 50°C for 2 min, 95°C for 2 min; cycling stage, 95°C for 15 sec, 60°C for 30 sec, 40 cycles; melt curve stage, 95°C for 15 sec, 60°C for 1 min. The relative expression was calculated as $2^{-\Delta\Delta\text{Cq}}$ (22). For miR-23b-3p expression, U6 was chosen as the internal reference control.

Cell Counting Kit (CCK)8 assay. The CCK8 method was used to determine cell proliferation according to the manufacturer's instructions (APeXBIO Technology LLC). Cells grown to the logarithmic phase were digested, passaged, and seeded into 96-well plates at 2×10^3 cells/well. The volume of culture medium per well was $100 \mu\text{l}$. After 24 h of incubation, $10 \mu\text{l}$

CCK8 solution was added to each well. After 1 h incubation, the absorbance of each well was measured with a microplate reader at a wavelength of 450 nm. The experiment was repeated 3 times. A proliferation curve was plotted with time as the abscissa and average absorbance value as the ordinate.

Western blot assay. Pre-chilled PBS was used to wash adherent cells (GH3 and MMQ) and centrifugation was performed at 300 x g at 4°C for 5-10 min. The cell lysate (obtained using RIPA lysis buffer; Beyotime Institute of Biotechnology) was evenly added to the entire surface of the vessel and pipetted several times with a pipette. The lysed cells were transferred to pre-cooled centrifuge tubes and the total protein was precipitated. The cells were centrifuged at 400 x g for 15 min in a 4°C centrifuge (pre-cooled) and the supernatant was collected in a 0.5-ml eppendorf (EP) tube. Total protein samples (100 µg) were separated by 10% SDS-PAGE and transferred to a PVDF membrane. After the protein was blocked with 5% BSA (BioVision, Inc.) at room temperature for 4 h, membranes were incubated with 1:1,000 antibodies from Abcam (cleaved Caspase-3, cat. no. ab32042; Survivin, cat. no. ab76424; Bcl-2, cat. no. ab182858; Bax, cat. no. ab32503; E-cadherin, cat. no. ab233611; Twist, cat. no. ab50887; Slug, cat. no. ab27568; MMP-7, cat. no. ab207299; FOXO4, cat. no. ab128908 and GAPDH, cat. no. ab8245) overnight at 4°C. The membrane was washed and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10,000, cat. no. ab150113; Abcam) at room temperature for 4 h. The protein was developed via ECL (Merck KGaA) scanned by gel imaging system and quantified by densitometry (Quantity One Version 4.6.3; both Bio-Rad Laboratories, Inc.). The expression level of each protein was expressed as the ratio of the protein optical density value of each group to that of GAPDH.

Flow cytometry assay. A total of 4x10⁵ cells (GH3 and MMQ cell lines) were seeded in 6-well plates for 24 h, then digested with trypsin without EDTA at room temperature for 1 min for collection. Following centrifugation at 400 x g at 4°C for 5 min, the supernatant was discarded. The cells were washed twice with pre-chilled PBS. A total of 3 µl Annexin V-FITC and 3 µl PI (Sigma-Aldrich; Merck KGaA) were added and mixed gently, then 300 µl combined buffer solution was added and the reaction was allowed to progress for 15 min at room temperature in the dark. Flow cytometry (FACSCalibur; BD Biosciences) was used to measure cell apoptosis. Software used for analysis was MultiSET V3.0.1 (BD Biosciences).

Transwell assay. Transwell experiments were used to verify cell invasion and migration ability. Cells from each group were as aforementioned collected and resuspended in serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) (cell density, 5x10⁴/ml). The upper Transwell chamber was pre-coated with Matrigel at 37°C for 1 h, then cell suspension (100 µl/well) was added. A total of 600 µl medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) was added to the lower chamber and incubated at 37°C in a 5% CO₂ incubator for 24 h. The Transwell chamber was obtained, washed with PBS, fixed with 5% glutaraldehyde at room temperature for 2 h and stained with 0.01% crystal violet

at room temperature for 20 min. The number of transmembrane cells in 5 random fields of view was counted under a light microscope (magnification, x200). A total of three parallel wells was set up for each group of cells and the experiment was repeated 3 times. For migration experiments, the same procedure was followed but Matrigel was not used.

Luciferase reporter experiment. Starbase (starbase.sysu.edu.cn/) was used to predict the binding sites between miR-23b-3p and MEG3 or FOXO4. In addition, MEG3-mutant (Mut) (5'-GGGACUGACU-ACUUACACAA-3') and FOXO4-Mut were designed (5'-AGCAAUGCUGUUGGA AUACAC AA-3'). In order to confirm that MEG3 can target miR-23b-3p, the 3' untranslated region (UTR) of MEG3 was inserted into a luciferase reporter gene vector and co-transfected with miR-23b-3p mimic into 293T cells. pMIR-REPORT Luciferase plasmid was purchased from Promega Corporation. miR-23b-5p mimics and control were synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd.. The sequences were as follows: miR-23b-3p mimics, AUCACAUUGCCA GGGAUUACC and miR-NC, CAGUACUUUUGUGUAGUA CAA. The carrier contained two luciferase reporter genes (*Renilla* and firefly luciferase reporter gene). The 3'UTR of the lncRNA MEG3 was inserted directly downstream of the *Renilla* luciferase reporter gene. If the 3'UTR was recognized by miR-23b-3p, MEG3 expression would be suppressed. The 293T cells were seeded in 24-well plates and transfected with reporter gene vector and miR-23b-3p mimic by Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C. After 24 h, the luciferase report was performed using a dual luciferase assay system (Promega Corporation) according to the manufacturer's instructions. The binding of miR-23b-3p and FOXO4 was detected in the same manner.

Pull-down assay. Pull-down assay was performed according to the manufacturer's instructions (cat. no. KT103-01; Guangzhou Sai Cheng Biotechnology Co., Ltd.). Cells were collected into an RNase-free EP tube and were centrifuged with 300 x g at 4°C for 5 min. The supernatant was discarded, and cells were washed 1-2 times with PBS solution. Then, 1 ml Cell lysis buffer containing protease inhibitor was added. The cells were centrifuged at 4°C at 14,000 x g for 15 min and the supernatant was transferred to a new 1.5-ml EP tube. The magnetic beads were suspended upside down slightly, 50 µl suspended magnetic bead suspension was placed in an RNase-free 1.5-ml EP tube and the supernatant was removed. Binding Buffer (500 µl) was added to each tube, gently inverted and mixed to wash the magnetic beads twice. Centrifugation was performed at 5,000 x g at 4°C for 1 min and the supernatant was removed. Binding Buffer (500 µl) was used to resuspend the magnetic beads and 2 µg probe was added to the EP tube. Samples were placed in a refrigerator at 4°C and incubated for 8 h. The magnetic bead-probe mixture was centrifuged at 5,000 x g at 4°C for 2 min and the supernatant was removed. Magnetic beads were washed once with 500 µl binding buffer, and then 1 ml binding buffer, 5 µl RNase Inhibitor and 300 µl cell lysate were added in sequence, mixed slightly, sealed, placed in a refrigerator at 4°C and incubated overnight. The mixture was centrifuged at 5,000 x g at 4°C for 2 min, the supernatant was removed and 1 ml washing buffer was added. The magnetic

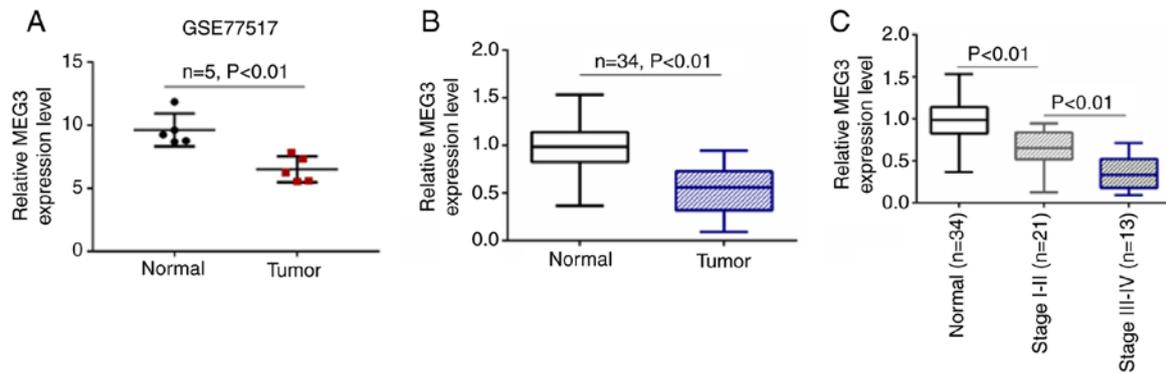


Figure 1. MEG3 expression levels are lower in PA tumor tissue. (A) In profile of GSE77517, MEG3 expression levels in patients with NFPA are decreased compared with those in normal tissue. (B) MEG3 expression levels are significantly lower in PA tumor tissue. (C) MEG3 expression levels are lower in patients with stage III-IV NFPA than in those with stage I-II NFPA. MEG3, maternally expressed 3; PA, pituitary adenoma; NF, non-functional.

beads were washed 5 times, centrifuged at $5,000 \times g$ at 4°C for 1 min, and the supernatant was removed. Finally, $50 \mu\text{l}$ 2X Loading buffer was added to each group, boiled at 100°C for 10 min, centrifuged at $5,000 \times g$ at 4°C for 1 min and the supernatant was transferred to a new EP tube, then a $20 \mu\text{l}$ sample was used for western blotting, as aforementioned. The primary antibody FOXO4 was purchased from Abcam (1:1,000; cat. no ab154520).

Statistical analysis. SPSS19.0 software (IBM Corp.) was used for statistical analysis. The experimental data are expressed as the mean \pm SD ($n=3$). The two-tailed paired t-test was used to compare differences between two groups being compared once. For comparisons in datasets containing multiple groups or single groups being compared more than once, one-way ANOVA with Tukey's post hoc test was applied to analyze the difference. Differences in patient information, including age, sex, tumor size, stage and invasiveness between two groups were analyzed by χ^2 test. Pearson correlation analysis was used to analyze the correlation between expression levels of MEG3, miR-23b-3p and FOXO4. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MEG3 expression levels are lower in PA tumor tissue. GSE77517 was downloaded from the GEO database and 5 patients with NFPA were included. Based on the profile of GSE77517, expression levels of lncRNA MEG3 were lower compared with those in normal tissue (Fig. 1A). Tumor and adjacent tissue samples ($n=34$) were collected from patients with PA and the expression levels of MEG3 were detected by RT-qPCR. MEG3 expression was significantly lower in PA tumor tissue compared with normal samples (Fig. 1B). MEG3 expression levels were assessed in samples from 34 patients with NFPA (21 patients with stage I-II and 13 with stage III-IV). MEG3 expression levels were lower in patients with stage III-IV than those with stage I-II NFPA (Fig. 1C). Based on the median value of MEG3, these patients were divided into lncRNA MEG3 high and low expression groups. Tumor size, invasiveness and clinical stage were significantly different but there was no significant difference between sex and age in these two groups (Table I).

MEG3 inhibits proliferation and improves apoptosis of PA tumor cells. PA cell lines GH3 and MMQ (purchased from ATCC) were transfected with pcDNA3.1(+)-NC, pcDNA3.1(+)-MEG3. RT-qPCR was used to detect the transfection efficiency. MEG3 was overexpressed, indicating that the transfection was successful in both GH3 and MMQ cells (Fig. 2A). Following transfection, proliferation significantly decreased, whereas the rate of cell apoptosis significantly increased (Fig. 2B and C). Survivin, cleaved-Caspase-3, Bax and Bcl-2 are proliferation- and apoptosis-associated proteins (23). Western blot analysis was performed to detect expression levels of these proteins. The expression levels of Survivin and Bcl-2 significantly decreased, whereas those of cleaved-Caspase-3 and Bax were increased following transfection (Fig. 2D). These results suggested that MEG3 inhibited proliferation and improved apoptosis of PA tumor cells.

MEG3 inhibits invasion, migration and the EMT process. Following transfection, migration and invasion was detected by Transwell assay. Cell migration and invasion significantly decreased in both GH3 and MMQ cell lines (Fig. 3A). Expression levels of E-cadherin, Twist, Slug and MMP-7 were also detected. The levels of these EMT markers were regulated by overexpressed MEG3: E-cadherin was upregulated, while Twist, Slug and MMP-7 were downregulated by overexpression of MEG3 (Fig. 3B). This suggested that MEG3 inhibited pituitary tumor development by participating in the EMT process.

MEG3 targets and downregulates miR-23b-3p. Based on starbase (starbase.sysu.edu.cn/), binding sites were predicted between MEG3 and miR-23b-3p. The binding was shown in Fig. 4A. Dual luciferase reporter gene assay showed that, when co-transfected miR-23b-3p entered 293T cells, the relative luciferase activity of wild-type (WT) MEG3 reporter gene was significantly inhibited. By contrast, the luciferase activity of the Mut MEG3 reporter gene was not affected by miR-23b-3p co-transfection (Fig. 4B). RNA pull-down assay also confirmed the binding interaction between MEG3 and miR-23b-3p (Fig. 4C). RT-qPCR was performed to detect the expression levels of miR-23b-3p in cells following transfection and in tissue. miR-23b-3p was

Table I. Correlation between MEG3 expression levels and clinical pathology in patients with pituitary adenoma.

Characteristic	Total patients (n=34)	MEG3 expression levels		P-value
		High (≥median, n=15)	Low (<median, n=19)	
Age, years				0.620
<50	16	7	9	
≥50	18	8	10	
Sex				0.468
Female	15	6	9	
Male	19	9	10	
Tumor size, cm				0.045
≤3	16	10	6	
>3	18	5	13	
Invasiveness				0.018
Non-IPA	17	11	6	
IPA	17	4	13	
Clinical stage				0.009
I-II	21	13	8	
III-IV	13	2	11	

MEG3, maternally expressed 3; IPA, invasive pulmonary adenocarcinoma.

significantly decreased in cells following MEG3 overexpression (Fig. 4D). miR-23b-3p was more highly expressed in PA tumor than normal tissue (Fig. 4E). Pearson's correlation analysis was used to analyze the correlation between expression levels of MEG3 and miR-23b-3p; the results showed that MEG3 and miR-23b-3p were negatively correlated (Fig. 4F). This indicated that MEG3 targeted miR-23b-3p.

miR-23b-3p targets FOXO4. Based on the starbase, binding sites between FOXO4 and miR-23b-3p were predicted. The binding was shown in Fig. 5A). When co-transfected with miR-23b-3p mimic, the relative luciferase activity in the FOXO4-WT group was significantly inhibited (Fig. 5B). RNA pull-down assay also confirmed binding between miR-23b-3p and FOXO4 (Fig. 5C). RT-qPCR and western blot were used to detect the expression levels of FOXO4 in cells following miR-23b-3p mimic or MEG3 transfection. Following miR-23b-3p mimic transfection, miR-23b-3p expression was significantly increased in both GH3 and MMQ cells, indicating that transfection was successful (Fig. 5D). FOXO4 was downregulated following miR-23b-3p mimic transfection (Fig. 5D) but upregulated following MEG3 transfection (Fig. 5E). Moreover, relative FOXO4 expression levels were lower in pituitary tumor than in normal tissue samples (Fig. 5F). Pearson's correlation analysis showed that FOXO4 was negatively correlated with miR-23b-3p but positively correlated with MEG3 (Fig. 5G and H).

Rescue assay. For the rescue assay, GH3 cells were divided into 3 groups (shNC + miR-NC, shMEG3 + miR-NC and shMEG3 + miR-23b-3p inhibitor). shMEG3 significantly

increased proliferation, migration and invasion of GH3 cells and inhibited apoptosis, whereas miR-23b-3p inhibitor rescued the effects of shMEG3 on these processes (Fig. 6A-C). Then, western blot analysis was performed to detect expression levels of apoptosis and EMT-associated proteins, including Survivin, Cleaved-Caspase-3, Bax, Bcl-2, E-cadherin, Twist, Slug, MMP-7 and GAPDH. Expression levels of Survivin, Twist, Slug and MMP-7 were upregulated by shMEG3, whereas those of Cleaved-Caspase-3, Bax, Bcl-2 and E-cadherin were down-regulated (Fig. 6D). When miR-23-3p was co-transfected, the expression levels of these proteins were rescued. These results indicated that shMEG3 facilitated pituitary tumor cell proliferation, migration and invasion by participating in the EMT process, whereas miR-23b-3p inhibitor rescued the effect of shMEG3.

Discussion

Pituitary tumor is a common type of intracranial tumor (24). Due to its complicated pathogenesis, the treatment of pituitary tumor presents a clinical problem (25). Therefore, seeking effective therapeutic targets is key for the treatment of pituitary tumors. In the present study, MEG3 expression levels were lower in pituitary tumor tissues and cells than in normal samples. Overexpression of MEG3 inhibited proliferation, invasion and migration of pituitary tumor cells and accelerated apoptosis.

MEG3, a maternally expressed imprinted gene, encodes an lncRNA with a length of ~1,600 nt (26). Previous studies have demonstrated that MEG3 stimulates transcriptional activity mediated by the tumor suppressor p53, causes p53 protein to aggregate in the cell, decreases expression levels of MDM2 protein and selectively activates growth

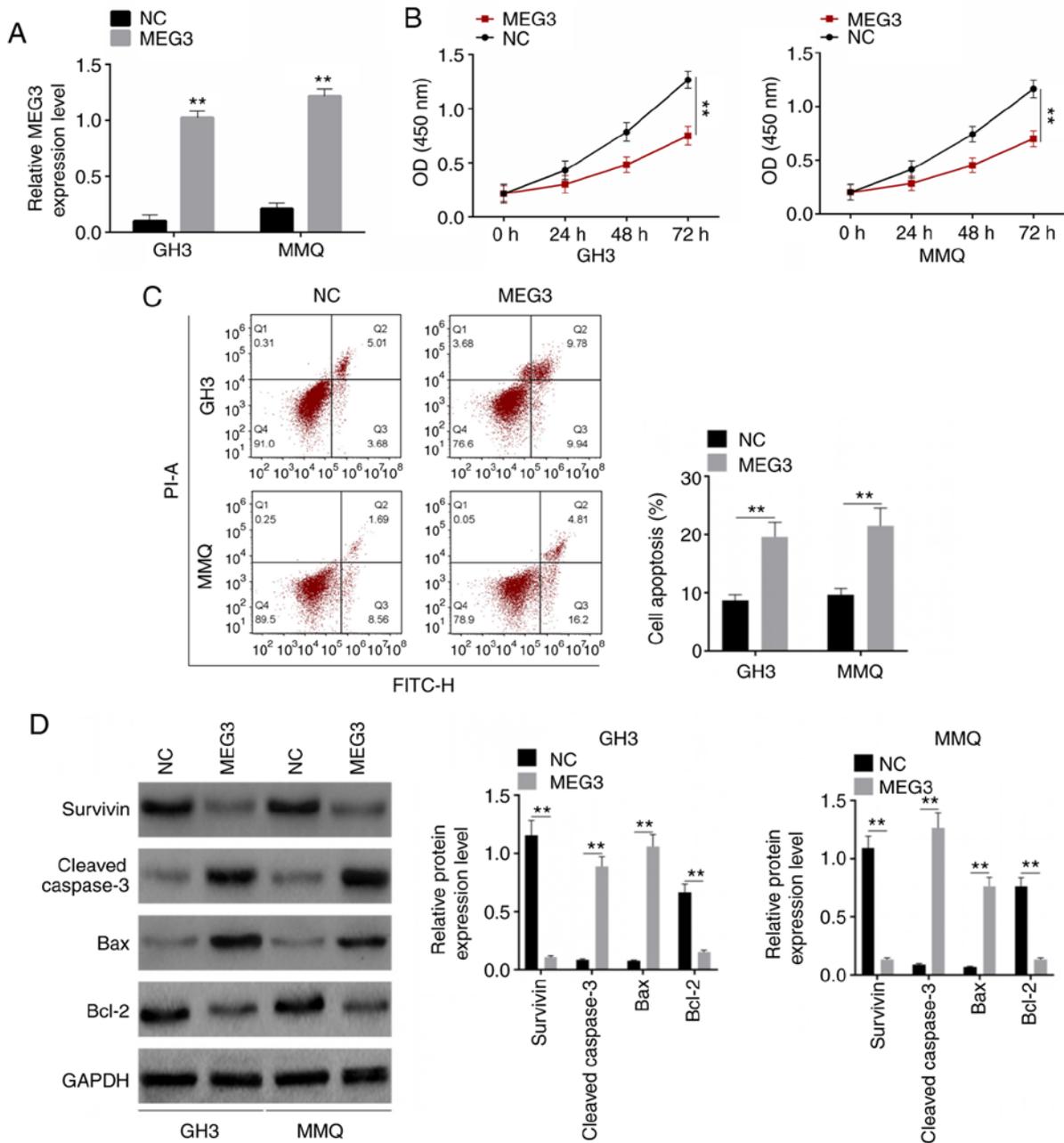


Figure 2. MEG3 inhibits proliferation and improves apoptosis of PA tumor cells. (A) PA cell lines GH3 and MMQ were transfected with pcDNA3.1(+)-NC or pcDNA3.1(+)-MEG3. Reverse transcription-quantitative PCR was used to detect the transfection efficiency. (B) Following transfection, proliferation was detected by Cell Counting Kit-8 assay. (C) Apoptosis was verified by flow cytometry assay. (D) Western blot analysis was performed to detect expression levels of proliferation- and apoptosis-associated proteins (Survivin, cleaved-caspase-3, Bax and Bcl-2). ** $P < 0.01$. MEG3, maternally expressed 3; PA, pituitary adenoma; NC, negative control; OD, optical density.

differentiation factor 15 (27,28). The expression of MEG3 is controlled by epigenetics and abnormal CpG methylation of MEG3 occurs in various types of tumor, such as cervical cancer and pituitary adenoma (23,29,30). In a mouse model, MEG3 has been shown to regulate cerebral angiogenesis via the VEGF pathway and inactivation of MEG3 expression levels in NFPA has been demonstrated clinically (31,32). In the present study, patients with stage III-IV pituitary tumor exhibited lower MEG3 mRNA expression levels than those with I-II pituitary tumor. Therefore, lncRNA MEG3 was considered to be a critical inhibitor of pituitary tumor development.

Here, MEG3 negatively regulated miR-23b-3p expression levels. The role of miR-23b-3p has been confirmed in multiple types of cancer. For example, miR-23b-3p is a carcinogenic miRNA that inhibits PTEN expression levels in renal cell carcinoma (33). PTEN inactivation has been demonstrated in invasive pituitary tumor tissue; decreased expression levels of PTEN protein result in a decreased tumor suppressive effect of PTEN protein and enhance tumor invasion (34). Additionally, miR-23b-3p is upregulated in radiation-induced thymic lymphoma (35). Downregulation of miR-23b-3p increases autophagy, which promotes resistance to radiation in pancreatic cancer cells (36). The present

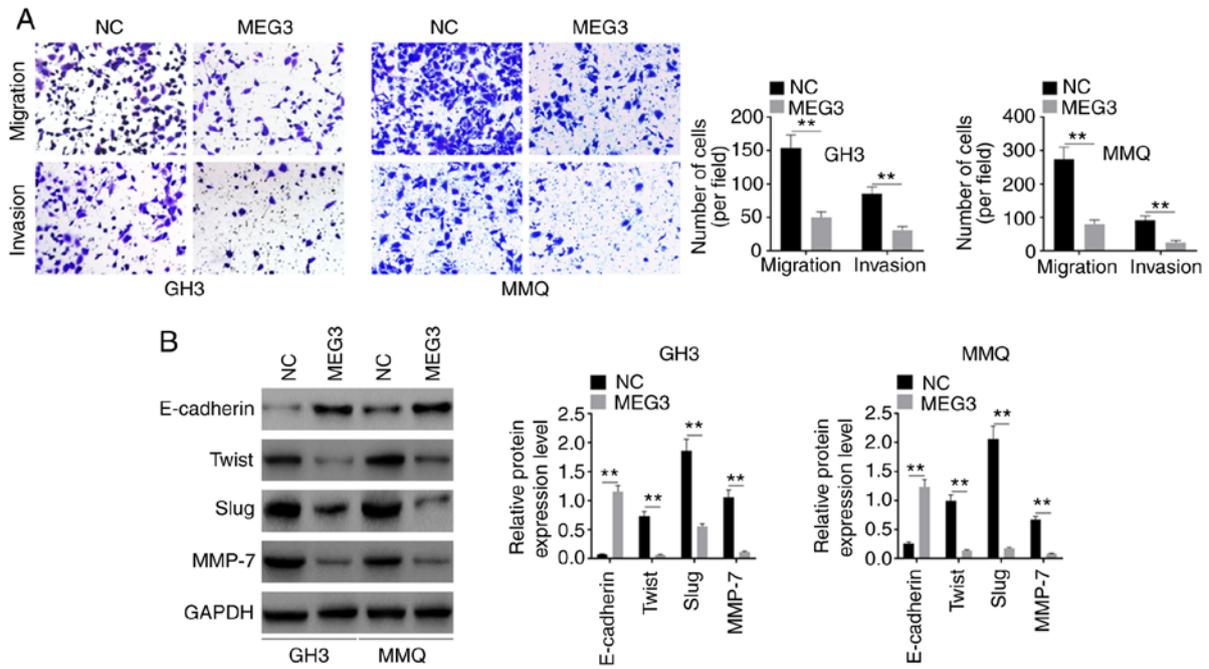


Figure 3. MEG3 inhibits invasion, migration and the EMT process. (A) Following transfection, migration and invasion were detected by Transwell assay. (B) Expression levels of EMT markers (E-cadherin, Twist, Slug and MMP-7) were detected by western blot analysis. **P<0.01. MEG3, maternally expressed 3; EMT, epithelial-mesenchymal transition; NC, negative control.

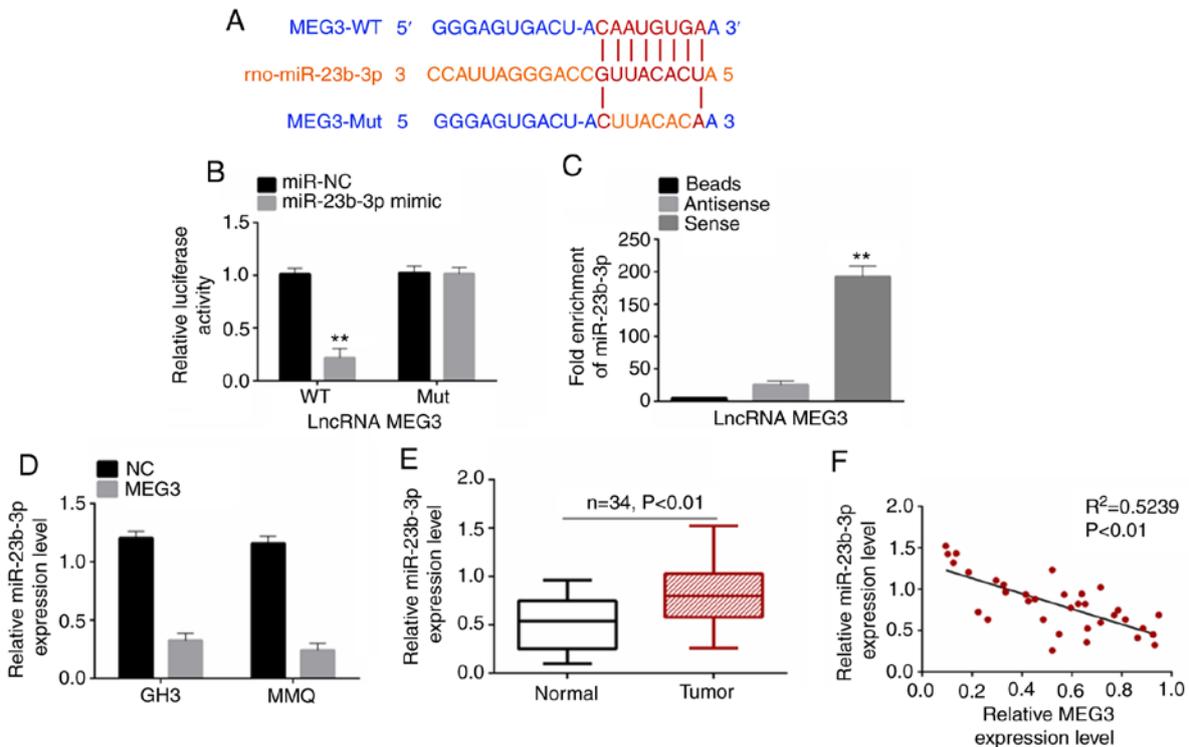


Figure 4. MEG3 targets and downregulates miR-23b-3p. (A) Starbase predicted binding sites between MEG3 and miR-23b-3p. (B) Dual luciferase reporter gene and (C) RNA pull-down assay were performed to verify the binding of MEG3 and miR-23b-3p. Reverse transcription-quantitative PCR was used to detect the expression levels of miR-23b-3p in (D) cells following transfection and (E) tissue. (F) Pearson's correlation analysis was used to analyze the correlation between expression levels of MEG3 and miR-23b-3p. **P<0.01 vs. NC. MEG3, maternally expressed 3; miR, microRNA; WT, wild-type; Mut, mutant; NC, negative control; lnc, long non-coding.

study verified that miR-23b-3p inhibitor rescued the effect of shMEG3 on proliferation, invasion, migration and apoptosis of pituitary tumor cells. Based on the aforementioned

evidence, it was concluded that MEG3 inhibits pituitary tumor development by downregulating miR-23b-3p expression levels.

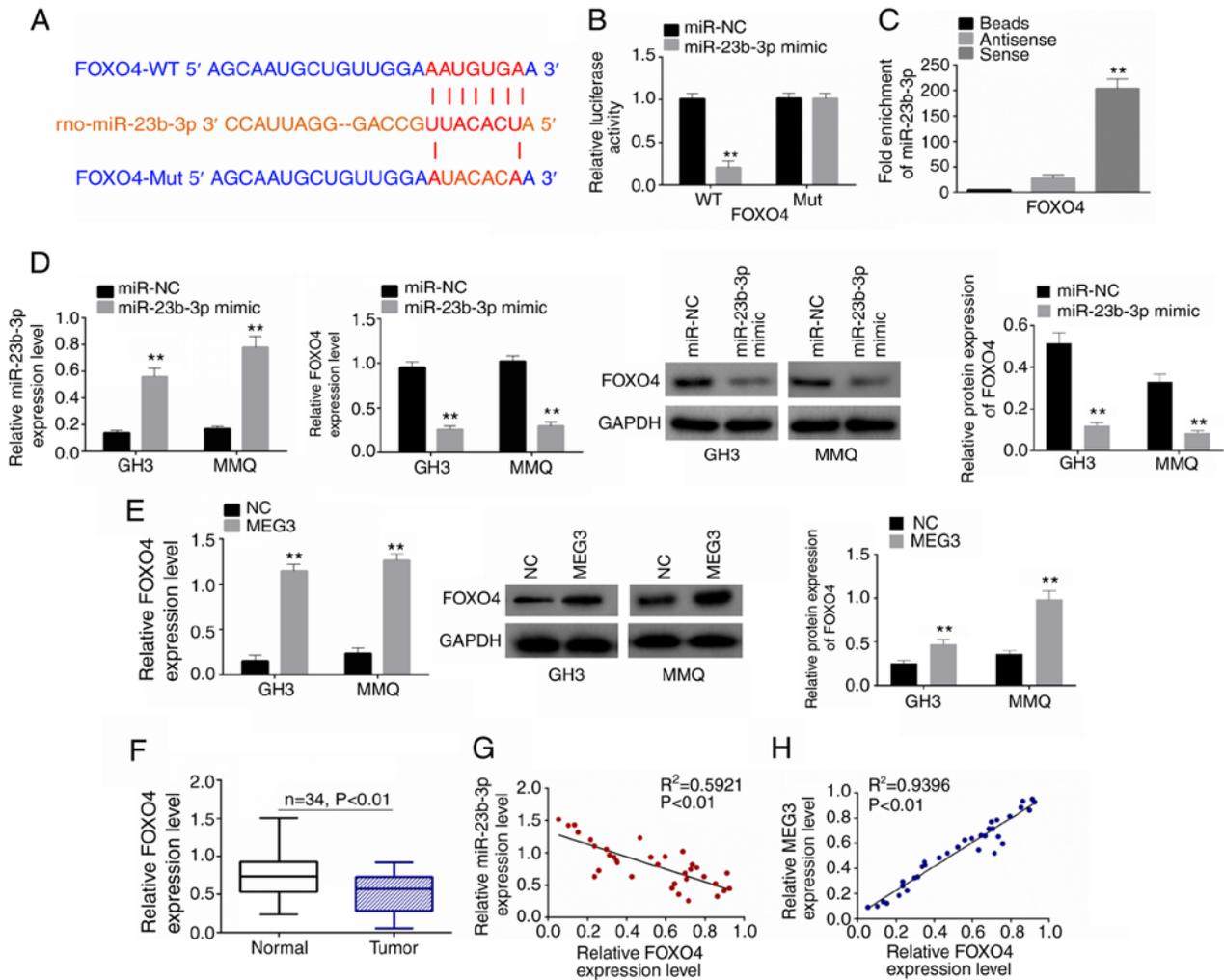


Figure 5. miR-23b-3p targets FOXO4. (A) Starbase predicted binding sites between FOXO4 and miR-23b-3p. (B) Following co-transfection with miR-23b-3p mimic, relative luciferase activity in the FOXO4-WT group was significantly inhibited. (C) RNA pull-down assay confirmed the binding between miR-23b-3p and FOXO4. (D) GH3 and MMQ cells were transfected with miR-NC and miR-23b-3p mimic. FOXO4 was downregulated following miR-23b-3p mimic transfection but (E) upregulated following MEG3 transfection. (F) Relative FOXO4 expression levels were lower in pituitary tumor than in normal tissue samples. Pearson's correlation analysis of (G) miR-23b-3p and FOXO4 and (H) MEG3 and FOXO4 expression levels. ** $P<0.01$. miR, microRNA; WT, wild-type; NC, negative control; MEG3, maternally expressed 3; Mut, mutant.

miR-23b-3p was verified to negatively regulate FOXO4 expression levels in the present study. Previous studies have found that FOXO4 is a target of NAD⁺-dependent deacetylase silencing information regulator 1 (Sir1) to regulate cell growth under normal and non-stress conditions (37,38). FOXO4 regulates apoptosis by inhibiting expression levels of Bcl-x1 (39). FOXO4 specifically binds to the Bcl-6 promoter to increase Bcl-6 transcription, while Bcl-6 directly binds to the Bcl-x1 promoter to inhibit its expression. Under stress, FOXO4 promotes Bcl-2 transcription by binding to the Bim promoter and further promotes endogenous apoptosis (40). In addition to inducing apoptosis, FOXO4 overexpression blocks the cell cycle at the G₁ phase, which is associated with p27 (41). Therefore, changes in apoptosis induced by abnormal expression of FOXO4 may be an underlying mechanism of tumor pathogenesis.

In the present study, overexpression of MEG3 inhibited the EMT process in pituitary tumor. EMT refers to the biological process in which epithelial cells are transformed into cells with mesenchymal phenotype via a specific procedure (42). In

this process, the adhesion structure, polarity and cytoskeleton between epithelial cells change, thereby increasing the ability of epithelial cells to deform, migrate and invade, as well as enhancing anti-apoptotic ability (43). EMT is an important biological process by which malignant tumors derived from epithelial cells acquire migration and invasion capabilities and serves a key role in tumor metastasis.

There were certain limitations in the present study. Firstly, the number of clinical samples included was relatively small; the role of MEG3 must be verified using a larger patient cohort. Secondly, the regulatory mechanism of MEG3 is not unidirectional-it may simultaneously regulate multiple miRNAs, genes and pathways. Therefore, it is necessary to identify a regulatory network to further study the molecular mechanism underlying the development of pituitary tumors. Finally, the effects of MEG3 *in vivo* need be confirmed using animal models.

In conclusion, MEG3 inhibited pituitary tumor development by participating in cell proliferation, apoptosis and the EMT process, which may be a novel target for treatment of pituitary tumors.

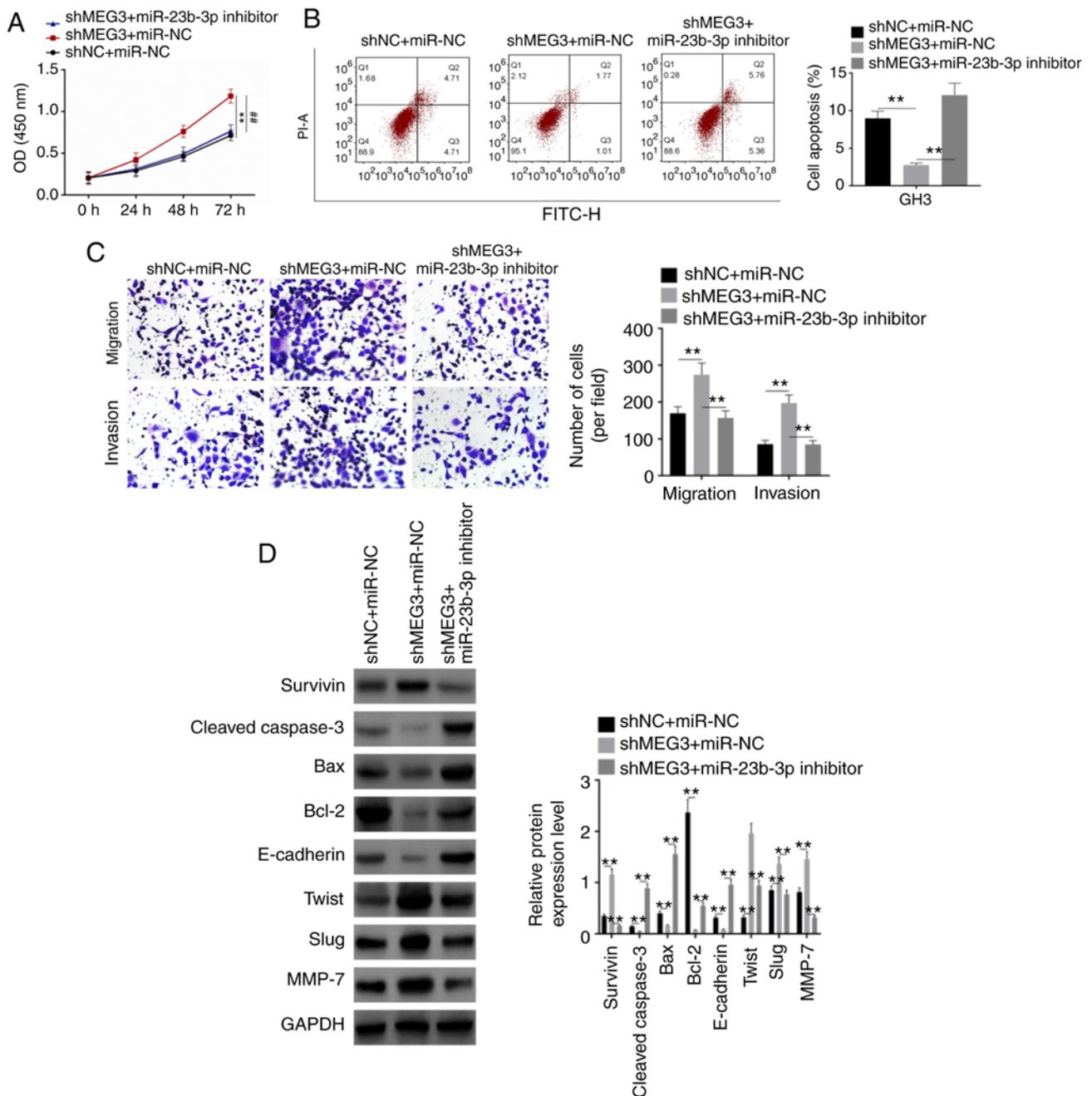


Figure 6. Rescue assay. (A) GH3 cells were divided into 3 groups (shNC + miR-NC, shMEG3 + miR-NC and shMEG3 + miR-23b-3p inhibitor). Cell Counting Kit-8 assay was performed to assess proliferation. (B) Apoptosis was verified by flow cytometry assay. (C) Transwell assay was performed to detect cell migration and invasion (magnification, x200). (D) Western blot analysis was performed to assess the levels of apoptosis- and EMT-associated proteins. **P<0.01. sh, short hairpin; NC, negative control; miR, microRNA; OD, optical density.

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Availability of data and materials

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

Authors' contributions

XW contributed to the conception of the study. XL contributed significantly to analysis and manuscript preparation; ZW performed the data analysis. XW, XW and ZW confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of First Affiliated Hospital of Soochow University (approval

no. 2018013). All patients provided written informed consent for the use of their samples in scientific research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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