MEG3/MIR-376B-3P/HMGA2 axis is involved in pituitary tumor invasiveness

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OBJECTIVE To date, long noncoding RNAs (lncRNAs) have proven to function as key regulators in tumorigenesis. Among these lncRNAs, *MEG3* displays low levels in various neoplasms and tumor cell lines. However, the regulatory mechanism of *MEG3* and *MIR-376B-3P*, one of the microRNAs from downstream gene clusters of the *DLK1-MEG3* locus, remains insufficiently defined.

METHODS The authors used quantitative real-time polymerase chain reaction analysis to analyze whether decreased *MEG3* and *MIR-376B-3P* expression levels were associated with the invasiveness of clinical nonfunctioning pituitary adenomas (CNFPAs) in 30 patients. Furthermore, functional experiments unveiled the pathophysiological role of *MEG3*, *MIR-376B-3P*, and *HMGA2* in pituitary-derived folliculostellate (PDFS) cell lines. Moreover, dual-luciferase reporter assay, Western blot analysis, and immunofluorescence were applied to reveal the correlations among *MEG3*, *MIR-376B-3P*, and *HMGA2*.

RESULTS *MEG3* and *MIR-376B-3P* were decreased in patients with CNFPA, and their transcriptional levels were highly associated with invasive CNFPAs. Moreover, excessive expression of *MEG3* and *MIR-376B-3P* inhibited tumorigenesis and promoted apoptosis in PDFS cells. Importantly, the authors found that *MEG3* acted as an enhancer of *MIR-376B-3P* expression. Furthermore, as a target gene of *MIR-376B-3P*, *HMGA2* served as an oncogene in pituitary adenoma and could be negatively regulated by *MEG3* via enriching *MIR-376B-3P*.

CONCLUSIONS This study offers a novel mechanism of an *MEG3/MIR-376B-3P/HMGA2* regulatory network in CNFPAs, which may become a breakthrough for anticancer treatments.

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KEYWORDS *MEG3*; lncRNA; *MIR-376B-3P*; pituitary adenoma; invasiveness; oncology; pituitary surgery

PITUITARY adenoma (PA) is characterized by abnormal proliferation or hormone secretion of anterior glandular cells. The prevalence of PA ranges from 37.2 to 115.6 cases per 100,000 people.^{1,9,11,24,29} PA is indicated by symptoms including headaches, visual decline, endocrinopathies, and even incidental lesions.^{19,21} Representing approximately one-third of all PA types, clinical nonfunctioning PAs (CNFPAs) are typically not related to obvious clinical and biochemical signs of hormonal hypersecretion.¹⁹ Additionally, neoplasm invasiveness in-

fluences the outcome of surgery, medication, and radiotherapy.¹⁸

Growing evidence has demonstrated that the process of tumorigenesis occurs through a series of complex polygenic events. Despite the lack of coding capabilities, noncoding RNAs (ncRNAs) are vital elements in oncogenic activities.^{3,27,31,39} Long ncRNAs (lncRNAs) contain more than 200 nucleotides and are customarily up to 100 kb in size, while microRNAs (miRNAs) are usually about 23 nucleotides long. Due to their intrinsic abilities, lncRNAs

ABBREVIATIONS cDNA = complementary DNA; CNFPA = clinical nonfunctioning PA; Dox = doxycycline; FBS = fetal bovine serum; FITC = fluorescein isothiocyanate; HRP = horseradish peroxidase; lncRNA = long ncRNA; miRNA = microRNA; NC = negative control; ncRNA = noncoding RNA; NP = normal pituitary; PA = pituitary adenoma; PBS = phosphate-buffered saline; PDFS = pituitary-derived folliculostellate; PI = propidium iodide; PVDF = polyvinylidene fluoride; qRT-PCR = quantitative real-time polymerase chain reaction; TRITC = trimethylrhodamine isothiocyanate.

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TABLE 1. Information of clinical patients (n = 30)

Case No.	Sex	Age (yrs)	Max Diameter of Tumor (mm)	Knosp Grade
1	M	28	29	1
2	F	18	25	1
3	F	36	13	1
4	F	47	29	1
5	M	38	24	1
6	M	46	11	1
7	F	54	30	2
8	M	46	18	2
9	F	57	39	2
10	F	61	23	2
11	F	53	20	2
12	M	51	31	2
13	F	59	34	3
14	M	42	30	3
15	M	56	29	3
16	M	72	35	3
17	F	60	32	3
18	F	45	27	3
19	M	52	26	3
20	F	58	23	3
21	F	33	22	3
22	F	76	28	3
23	F	59	37	4
24	M	49	24	4
25	M	38	35	4
26	F	46	46	4
27	F	62	37	4
28	M	53	52	4
29	F	34	20	4
30	F	55	31	4

regulate biological processes through interacting with DNA, RNA, and proteins. Additionally, miRNAs have the ability to match the 3'-UTR sequence of certain genes to suppress their expression.¹²

As an imprinted gene in the *DLK1-MEG3* locus, *MEG3* has been identified as a tumor suppressor in PA^{34,35} and other types of tumors.^{8,13,16,33} Interestingly, selective suppression of the *DLK1-MEG3* locus has been shown in human CNFPAs.^{17,36} The miRNA cluster in the *DLK1-MEG3* locus consists of more than 40 miRNAs.²⁵ Among these miRNAs, the *MIR-376* family exhibits a low transcriptional level similar to that of *MEG3* in neoplasms.^{2,4} However, the correlation between them has yet to be investigated.

In this study, we found that transcriptional levels of *MEG3* and *MIR-376B-3P* had connections with tumor invasiveness in CNFPA patients. Then, we also confirmed that *MEG3* and *MIR-376B-3P* could serve as tumor suppressor genes during proliferation of a pituitary-derived folliculostellate (PDFS) cell line. Moreover, *MEG3* was

found to be involved in *MIR-376B-3P* expression regulation. We further validated that *MEG3* and *MIR-376B-3P* could both downregulate *HMGA2*. In summary, these data suggest that *MEG3* and *MIR-376B-3P* synergistically regulate tumor invasiveness by targeting *HMGA2* and therefore serve as potential therapies for refractory CNFPAs.

Methods

Clinical Samples

In total, 30 patients with PA (Table 1) who underwent transsphenoidal surgery at our department were recruited into this study, and 12 normal pituitary (NP) tissues were provided by the Forensic Authentication Center of Sun Yat-sen University. Clinical invasiveness was defined by experienced neurosurgeons using the Knosp classification system¹⁴ (noninvasive, n = 12; invasive, n = 18). All surgical specimens were clinically and pathologically confirmed as hormone-negative tumors. We also confirmed that the recruited patients who provided informed consent did not receive transsphenoidal surgery or other therapies prior to surgery. This study was authorized and supervised by the ethics committee.

Cell Culture

PDFS, a folliculostellate cell line, originates from a clinically nonfunctioning pituitary macroadenoma.⁷ PDFS exhibited no expression of hypophyseal hormone or Pit-1, which is a secretory pituitary cell marker. However, PDFS exhibited expression of vimentin and S100 protein, which is a folliculostellate-specific biomarker in the anterior pituitary gland. Due to the expression of activin A and follistatin, PDFS has an integral activin signaling pathway that regulates the autocrine/paracrine function of the anterior pituitary gland. Furthermore, PDFS can promote the proliferation of co-cultured primary pituitary tumor cells, and colony formation of this cell was found in soft agar.⁷ Therefore, PDFS is an ideal cell model for studying the biological behavior of CNFPAs. PDFS cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% antibiotics (Invitrogen). A human embryonic kidney cell line (HEK-293T) was cultured in medium containing 10% FBS, non-essential amino acids, sodium pyruvate, and L-glutamine. Cells were cultured in a CO₂ incubator.

Construction of Stable Cell Lines

MEG3 plasmid construction and stable transfection were performed as previously described.⁵ Briefly, for constructing an inducible *MEG3* transcription system, namely pTRE-*MEG3*, the cDNA sequence of *MEG3* was cloned into pTRE-Tight (ClonTech). In addition, pCMV-rtTA-puro was generated using the Tet-On system (ClonTech) as previously reported.⁵ Then, the above two plasmids were cotransfected into PDFS cells at a ratio of 3 to 1 using Lipofectamine 3000 reagent (Invitrogen). Successfully constructed cells were separated and propagated by puromycin selection. Transfection efficiency was then measured through quantitative real-time polymerase chain reaction (qRT-PCR) after adding doxycycline (Dox) to induce *MEG3* expression.

Cell Transfection

PDFS cells (1×10^6 cells/well) were grown in 6-well plates until attaining 60%–70% density. Then, we transfected *Hsa-MIR-376B-3P* mimics, *Hsa-MIR-376B-3P* inhibitor, si-*HMGA2*-1, si-*HMGA2*-2, and their corresponding blank reagent (RiboBio) into PDFS cells. Forty-eight hours later, qRT-PCR was performed to analyze the transfection efficiency.

Quantitative Real-Time PCR

TRIzol Reagent (Life Technologies) was used for RNA extraction. Synthesis of complementary DNA (cDNA) was conducted with the Prime Script RT Reagent Kit (Takara) and Mir-X miRNA First-Strand Synthesis Kit (Takara). The forward and reverse primers for *MEG3* were 5'-CCTTCCATGCTGAGCT-3' and 5'-TGTTGGTGGGATCCAGGAAA-3', those for *HMGA2* were 5'-CTACCTCAGCAGCAGTTGGA-3' and 5'-CCTGGGACTGTGAAGGGATT-3', the 5' primer for *MIR-376B-3P* was 5'-GCATCATAGAGGAAAATC CATG-3', and the corresponding 3' primer was the mRQ 3' primer supplied by the kit. The primers for *U6* and *GAPDH* were synthesized by Sangon Biotech Co., Ltd. The qRT-PCR detection was used to determine the transcriptional level of certain genes.

CCK-8 Assay

Cell viability was monitored using a CCK-8 Kit (Sigma). The transfected PDFS cells were cultured in 96-well plates (2×10^4 cells/well). Ten microliters of CCK-8 detection reagent was applied and incubated for 2–4 hours, followed by the detection of the 450-nm optical density value (microplate spectrophotometer).

Flow Cytometry Assay

The cell apoptotic rate was measured by an Annexin V-FITC/PI (fluorescein isothiocyanate/propidium iodide) Apoptosis Detection Kit (KeyGen Biotech). Specifically, 1×10^5 treated cells were incubated in serum-free DMEM. Forty-eight hours later, 500 μ l of binding buffer was used to resuspend collected cells. After adding 5 μ l of Annexin V-FITC and 5 μ l of PI, cells were cultured and protected from light for 15 minutes. Stained cells were analyzed using a flow cytometer (CytoFLEX, Beckman Coulter).

TUNEL Staining

The treated cells were seeded on coverslips for at least 24 hours. After washing twice with phosphate-buffered saline (PBS), 4% paraformaldehyde fixation was applied to treat cells for 15 minutes and 0.25% Triton-X 100 permeation for 20 minutes. TUNEL assay was performed in accordance with the kit's specification (KeyGen Biotech).

Cell Colony Formation Assay

PDFS cells (1×10^2 per well) were cultured in a 6-well plate for at least 1 week after the transfection with corresponding reagent. The medium was replaced every 2 days. Cells were stained with crystal violet for 30 minutes after 4% formaldehyde fixation. The colonies (with a diameter

$\geq 100 \mu\text{m}$) were calculated to measure the proliferation ability of cells.

Transwell Assay

We used Matrigel chambers (BD Biosciences) to detect the cells' invasion ability. Briefly, transfected PDFS was seeded in FBS-free DMEM in the hydrated Matrigel chambers, while 10% FBS was applied in the lower chambers. After 24 hours, invaded cells on the bottom surface were fixed and dyed with 0.1% crystal violet for 30 minutes. A microscope was used to count invaded cell numbers in three different fields.

Wound Healing Assay

A straight scratch was slightly scored on each treated cell monolayer in 6-well plates using a sterile Eppendorf tip. After washing away the detached cells with PBS buffer, FBS-free culture medium was added. Cells moving toward the scratch wound were monitored and imaged at 0, 24, 48, and 72 hours using a microscope (Zeiss Axio Observer Z1).

Luciferase Reporter Assay

For *HMGA2* 3'-UTR analysis, the sequence containing regulatory sites of *MIR-376B-3P* was synthesized and cloned into psiCHECK TM-2 vector (Promega). One hundred nanograms of constructed vector and 200 nmol/L *MIR-376B-3P* mimics or negative control (NC) were cotransfected into HEK-293T cells. A Dual-Luciferase Assay Kit (Promega) was used to detect cell lysate after 48 hours. We performed the experiment in triplicate.

Immunohistochemistry and Immunofluorescence

Paraffin-embedded slides of clinical adenomas and subcutaneous tumors were incubated with primary antibodies of *HMGA2* (1:100, Cloud-Clone) and Ki-67 (1:50, Cloud-Clone) at 4°C overnight after the processes of dewaxing, dehydration, endogenous peroxidase suppression, and blocking. The slides were treated with biotin-labeled immunoglobulin G (CWBIO) for 10 minutes on the 2nd day. Streptavidin-horseradish peroxidase (HRP) and DAB dye liquor (CWBIO) were used sequentially to treat samples. Finally, sections were photographed under a microscope (Olympus BX63). Treated cells on coverslips were subjected to immunofluorescence staining after a similar fixation process. Then, the fixed cells were processed with 0.1% Triton X-100 and 5% bovine serum albumin buffer for 10 and 30 minutes, respectively. The coverslips were treated with *HMGA2* primary antibody (1:10, Cloud-Clone) before incubating with trimethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit secondary antibody (KeyGen Biotech). DAPI was used for nuclear counterstaining, and images were captured using an Olympus camera (Olympus BX63).

Western Blot Analysis

Proteins were isolated using lysis buffer (Beyotime) with the addition of 1% phenylmethylsulfonyl fluoride (Amresco). Twelve percent sodium dodecyl sulfate–

polyacrylamide gel electrophoresis was used to separate proteins (50 µg) under constant voltage, followed by the transfer of samples onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). We blocked PVDF with 5% defatted milk before incubating rabbit polyclonal antibodies to HMGA2 (1:1000, Cloud-Clone), BAX (1:2500, Proteintech), C-MYC (1:2000, Proteintech), BCL2 (1:2000, Proteintech), and GAPDH (1:5000, Cloud-Clone) at 4°C overnight. We used Tris-buffered saline–Tween solution to wash the membranes 3 times and treated them with secondary antibody (1:3000, Proteintech) on the following day. PVDF membranes were photographed covered by Chemiluminescent HRP Substrate (Millipore Corporation) using the Amersham Imager 600 (GE).

Tumor Xenograft In Vivo

Forty-five 4-week-old nude mice and treated PDFS cells were assigned to 9 groups: 1) M-NC group (NC plasmid), 2) M-*MEG3* group (*MEG3* plasmid), 3) M-MIR-mimics-NC group (NC of mimics reagent), 4) M-MIR-mimics group (*MIR-376B-3P* mimics), 5) M-MIR-inhibitor-NC group (NC of inhibitor reagent), 6) M-MIR-inhibitor group (*MIR-376B-3P* inhibitor), 7) M-si-NC group (si-NC), 8) M-si-1 group (si-*HMGA2-1*), and 9) M-si-2 group (si-*HMGA2-2*). Cell suspensions (200 µl, 5×10^6 cells) of the abovementioned groups were injected subcutaneously into each mouse. Tumor parameters were measured with a digital caliper weekly. The volume (V) was calculated through assessing length (l) and width (w) as follows: $V = lw^2/2$. The mice were killed and subcutaneous neoplasms were weighed on day 28. All animal research was verified by the Institutional Animal Ethics Committee and conducted under institutional guidelines.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 20.0, IBM Corp.). Unless otherwise specified, differences between 2 and more than 2 groups were calculated using the Student t-test and 1-way ANOVA, respectively. A p value < 0.05 was considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001, ns = no significance).

Results

MEG3 and *MIR-376B-3P* Expression Levels in CNFPA Patients

In the current study, the expression levels of *MEG3* and *MIR-376B-3P* in 30 CNFPAs and 12 NP tissues were determined. *MEG3* expression was found to be decreased in CNFPAs compared to NP tissues (Fig. 1A). The transcription level of *MIR-376B-3P*, which belongs to the downstream gene cluster of *MEG3*, was significantly different between CNFPAs and NP tissues (Fig. 1B). We also investigated the expression of other genes in the downstream gene cluster of *MEG3* between CNFPAs and NP tissues and found that *MIR-376B-3P* expression ranked the lowest among them (Fig. 1C). The average relative expression of *MEG3* was set as the margin, and 30 tumor tissues were segregated into high- and low-expression groups (n = 8 and 22, respectively). Correlations between *MEG3* and the clinical parameters of patients were analyzed. As sug-

gested in Supplemental Table 1, a low *MEG3* level was closely connected with tumor invasiveness, but no statistical differences were found with indexes of age, sex, or tumor diameter. The comparison between the high (n = 10) and low (n = 20) expression groups of *MIR-376B-3P* produced a similar result (Supplemental Table 2). Moreover, we defined tumor invasiveness using the Knosp classification system¹⁴ (Fig. 1D). Significantly lower transcriptional levels of *MEG3* and *MIR-376B-3P* were determined in the noninvasive group compared to the invasive group (Fig. 1E and F).

MEG3 Overexpression, Cell Apoptosis, and Tumorigenesis In Vitro

We supposed that disordered *MEG3* expression might have a bearing on tumor phenotype in CNFPAs on the basis of the aforementioned findings. We generated a stable clone of *MEG3* in PDFS cells as reported⁵ and named it PDFS-*MEG3*, and the negative control was named PDFS-NC. Overexpression efficiency was determined after treatment with Dox for 48 hours (Fig. 2A). A significant decline in cellular proliferation was detected in PDFS-*MEG3* cells via CCK-8 assay (Fig. 2B). Next, we investigated cell apoptosis using flow cytometry analysis and TUNEL staining. A marked increase in apoptotic rate was shown in cells overexpressing *MEG3* (Fig. 2C and D). Western blot analysis and cell colony formation assay showed similar results of a proliferative state with increased expression of apoptotic proteins and reduced colony formation in PDFS-*MEG3* cells (Fig. 2E and F). Migration ability was also suppressed in PDFS-*MEG3* (Fig. 2G). Moreover, a significant decline of cell invasive ability was found following *MEG3* overexpression (Fig. 2H). All these assays reveal that *MEG3* exerts antineoplastic effects in CNFPAs.

Inhibitory Role of *MIR-376B-3P* in the CNFPA Cell Line

We used miRNA mimics to elucidate the role of *MIR-376B-3P* in CNFPAs. After 24 hours of transfection with miRNA reagents, qRT-PCR was performed to ascertain the transfection efficiency (Fig. 3A). Accordingly, CCK-8 assay demonstrated that *MIR-376B-3P* also exhibited an inhibitory role in PDFS cell vitality (Fig. 3B). As expected, increased *MIR-376B-3P* expression promoted cell apoptosis (Fig. 3C and D). Western blot analysis was used to determine BAX, BCL2, and C-MYC levels (Fig. 3E). Similarly, cell colony formation assay revealed that *MIR-376B-3P* could inhibit cell proliferation (Fig. 3F). Additionally, cell migration and invasiveness characteristics were inhibited in PDFS cells overexpressing *MIR-376B-3P* (Fig. 3G and H). As illustrated in Fig. 3, *MIR-376B-3P* functions as a suppressor similar to *MEG3* in PDFS cells.

MEG3 and *MIR-376B-3P* Expression

Given the tumor-suppressive roles of *MEG3* and *MIR-376B-3P* in PDFS cells, we examined whether *MEG3* was involved in regulating *MIR-376B-3P* expression. First, an increased *MIR-376B-3P* transcriptional level was found in PDFS-*MEG3* (Fig. 4A). As previously demonstrated, the *MEG3* promoter is hypermethylated in CNFPAs.³⁶ There-

FIG. 1. *MEG3* and *MIR-376B-3P* expression levels are decreased and correlate with invasiveness in patients with CNFPA. **A:** The expression level of *MEG3* was detected in 30 CNFPA patients and 12 NP tissues by qRT-PCR (Student t-test, $p < 0.0001$). **B:** Graph of the relative expression level of *MIR-376B-3P* between CNFPAs and NP tissues (Student t-test, $p < 0.0001$). **C:** The expression levels of 11 miRNAs from downstream gene clusters of *MEG3* (1-way ANOVA, $F = 65.23$, $p < 0.0001$). **D:** Coronal MRI in different patients (CNFPA cases 3, 9, 19, and 26). In grade 1, the tumor pushes into the medial wall of the cavernous sinus (green line) but does not go beyond a hypothetical line extending between the centers of the two segments of the internal carotid artery (red line). In grade 2, the tumor does not pass a line tangent to the lateral margins of the artery (yellow line). In grade 3, the tumor extends laterally to the internal carotid artery (yellow line) within the cavernous sinus. Grade 4 involves total encasement of the intracavernous internal carotid artery. **E and F:** Relative expression levels of *MEG3* (Student t-test, $p = 0.001$) and *MIR-376B-3P* (Student t-test, $p = 0.0025$) were analyzed in 12 noninvasive and 18 invasive CNFPAs. Data of all quantitative results are presented as the mean $\pm$ SEM for three independent experiments. ** $p < 0.01$, *** $p < 0.001$. Figure is available in color online only.

fore, the effect of 5-Aza-CdR, a DNA-demethylating agent, on *MEG3* and *MIR-376B-3P* was evaluated. Under DNA-demethylating agent treatment, their expression levels were significantly increased (Fig. 4B). Interestingly, both *MIR-376B-3P* mimics and the inhibitor could not significantly change the expression level of *MEG3* (Fig.

4C). These findings illuminated a positive role for *MEG3* in the transcriptional regulation of *MIR-376B-3P*.

MEG3* and the Transcriptional Activity of *HMGA2

In vivo and in vitro experiments indicated that *HMGA2* played an oncogenic role in PA (Supplemental Figs. 1 and

FIG. 2. *MEG3* overexpression induces cell apoptosis and depresses tumorigenesis in vitro. **A:** The relative expression level of *MEG3* in PDFS cells transfected with the corresponding plasmid. Data are presented as the fold change of PDFS-*MEG3* to PDFS-NC cells (Student *t*-test, $p = 0.0018$). **B:** Cell proliferation assay of PDFS-*MEG3* and PDFS-NC cells after Dox treatment using CCK-8 (Student *t*-test, 24 hours, $p = 0.0017$; 48 hours, $p = 0.0002$; 72 hours, $p = 0.0017$). **FIG. 2.** (continued)→

FIG. 2. C: Flow cytometry was applied to examine the apoptosis of PDFS-*MEG3* and PDFS-NC cells. FITC-A = strength of the FITC channel for detecting FITC-stained cells; PI PE-A = strength of phycoerythrin (PE) channel for detecting PI-stained cells; Q1-LL = lower-left quadrant, living cells; Q1-LR = lower-right quadrant, early apoptotic cells; Q1-UL = upper-left quadrant, necrotic cells; Q1-UR = upper-right quadrant, late apoptotic cells. **D:** TUNEL assay was used to detect the apoptotic effect of *MEG3* in PDFS cells. DAPI was used to locate the nuclei; red cells indicated TUNEL-positive apoptotic cells (TUNEL stain, original magnification $\times 100$). Quantitative results of the TUNEL assay are shown on the graph (Student t-test, $p < 0.0001$). **E:** The protein levels of BAX, BCL2, and C-MYC in PDFS-*MEG3* were detected by Western blot. **F:** Ectopic expression of *MEG3* suppressed PDFS cell colony formation. The quantities of relative colony formation are shown on the graph (Student t-test, $p = 0.0003$). **G:** Wound healing assays of PDFS-*MEG3* and PDFS-NC cells were performed after Dox treatment (original magnification $\times 100$). **H:** Transwell invasion assay indicated a significant reduction in cell invasiveness following *MEG3* overexpression (crystal violet stain, original magnification $\times 100$). The quantitative result is shown on the graph (Student t-test, $p = 0.0021$). ** $p < 0.01$, *** $p < 0.001$. Figure is available in color online only.

2). Then, we observed that the upregulation of *HMGA2* was highly correlated with tumor invasiveness (Fig. 5A and B, Supplemental Table 3). Interestingly, using the online software TargetScan, we found that *MIR-376B-3P* showed base complementarity with the 3'-UTR of *HMGA2* (Fig. 5C). Then, a wild-type *HMGA2* luciferase reporter (*HMGA2*-WT) and mutant *HMGA2* luciferase reporter (*HMGA2*-MUT) were generated and transfected into HEK-293T cells to identify the regulatory relationship between *MIR-376B-3P* and *HMGA2*. Ectopic *MIR-376B-3P* markedly decreased the fluorescence intensity of the wild-type vector but not that of the mutant one (Fig. 5D), which substantiates the possibility of a regulatory relationship between *MIR-376B-3P* and *HMGA2*.

Accordingly, we further investigated the *MEG3/MIR-376B-3P/HMGA2* axis. First, data showed that these two ncRNAs were negative regulators of *HMGA2* in both cells and clinical samples (Fig. 5E–G). Next, *MIR-376B-3P* mimics and inhibitor were introduced into PDFS-*MEG3* cells, respectively. Interestingly, *MIR-376B-3P* inhibition weakened the negative regulation of *MEG3* on *HMGA2*, whereas enhanced *MIR-376B-3P* expression exerted an opposite effect (Fig. 5H and I). Moreover, Western blot analysis (Fig. 5J) and cyto-immunofluorescence (Fig. 5K) indicated that the presence of *MEG3* and *MIR-376B-3P* could suppress *HMGA2* expression. These data indicated that *MEG3* can negatively regulate *HMGA2* by enhancing *MIR-376B-3P* expression.

***MEG3* and *MIR-376B-3P*, Tumorigenesis, and *HMGA2* In Vivo**

To further explore the mechanisms of the *MEG3/MIR-376B-3P/HMGA2* axis in CNFPAs, the tumor formation experiment was performed to study their biofunctional role. Six groups of nude mice were established by hypodermic injection of cell suspension. Throughout tumor growth, mice injected with *MEG3*- and *MIR-376B-3P*-transfected cells developed smaller tumor lumps than those from the negative control group (Fig. 6A). After 4 weeks, subcutaneous masses were resected carefully. As expected, we found that xenograft tumors from the *MEG3* and *MIR-376B-3P* groups were smaller and lighter than NC tumors (Fig. 6B and C). Moreover, *MEG3* upregulated *MIR-376B-3P* expression, and downregulated *HMGA2* at both transcriptional and translational levels (Fig. 6D and E). Furthermore, immunohistochemical analysis also showed that the Ki-67 index, which is a cellular marker for proliferation, was strongly reduced in the tumor tissues generated from *MEG3*- and *MIR-376B-3P*-transfected cells (Fig. 6F). Collectively, we concluded that upregulated

MEG3 and *MIR-376B-3P* expression could inhibit tumor growth in vivo.

Discussion

Over the past decades, ncRNA has been an emerging hotspot in research on PAs.^{10,32,38} *MEG3*, the ortholog of mouse *Gtl2*,²⁶ has been identified as a candidate anti-oncogene in human CNFPAs.³⁷ We discovered that low *MEG3* expression had a connection with tumor invasiveness. Functional analyses confirmed the antitumor role of *MEG3* on PDFS cells. We further revealed that *MEG3* contributed to negative downregulation of *HMGA2* via increasing the expression of *MIR-376B-3P*. Therefore, the current research reveals a new pathway, namely the *MEG3/MIR-376B-3P/HMGA2* axis, which could shed light on the treatment of this difficult disease.

Mechanisms contributing to the regulatory crosstalk between gene clusters and their upstream host genes might be notable for uncovering CNFPA tumorigenesis. DNA methylation suppresses the expression of *MIR-770* and *MEG3*, a host gene of the former, during neoplasm development.¹³ In another study, the host gene *MIR155HG* served as a progression marker for patients with glioblastoma through interacting with *MIR-155*.³¹ Furthermore, miRNAs can also target their upstream genes via binding with matching sequences. Instead of the regular 3'-UTR, *MIR-932* regulated the transcription of its host gene *DNLG2*, via base pairing to the coding sequence region.²³ Interestingly, inconsistency between intronic miRNAs and their host genes was found to be quite common in hepatocellular carcinoma.²⁸ The above studies show that the interactions between up- and downstream genes are important in tumor formation. Therefore, we hypothesized that the regulatory network between *MEG3* and *MIR-376B-3P* might also exhibit a vital part in CNFPA development. We observed a low transcriptional level of *MIR-376B-3P*, which was related to invasiveness, in pituitary tumor tissues. After performing functional experiments, we discovered that *MIR-376B-3P* exerted an antitumor effect similar to that of *MEG3*. Moreover, our results supported the idea that *MEG3* overexpression induced *MIR-376B-3P* transcription.

Furthermore, it has been shown that miRNAs serve as suppressors by targeting mRNA in various tumors. In this study, online database analysis showed that *MIR-376B-3P* shares common binding sites with *HMGA2* mRNA. Existing evidence has revealed that *HMGA2* is regarded as an oncogene in many cancers. With respect to PAs, regulatory networks including *HMGA2* may offer new insights for the exploration of treatments for precision medicine.^{6,20,22}

FIG. 3. *MIR-376B-3P* exhibits an inhibitory role similar to that of *MEG3* in the CNFPA cell line. **A:** The relative expression level of *MIR-376B-3P* in PDFS cells transfected with miRNA mimics. Data are presented as the fold change of PDFS-MIR-376B-3P to PDFS-MIR-NC cells (Student t-test, $p < 0.0001$). **FIG. 3.** (continued)→

FIG. 3. B: Cell proliferation assay of PDFS cells was performed after transfection of *MIR-376B-3P* mimics using CCK-8 (Student t-test, 24 hours, $p = 0.0009$; 48 hours, $p = 0.0005$; 72 hours, $p = 0.0011$). **C:** Flow cytometry was applied to examine the apoptotic rate of PDFS-*MIR-376B-3P*. **D:** TUNEL assay was used to detect apoptotic cells. DAPI was used to locate the nuclei; red cells indicate TUNEL-positive apoptotic cells (original magnification $\times 100$). Quantitative results of TUNEL assay are shown on the graph (Student t-test, $p < 0.0001$). **E:** The protein levels of BAX, BCL2, and C-MYC in PDFS-*MIR-376B-3P* were detected by Western blot. **F:** Ectopic expression of *MIR-376B-3P* suppressed PDFS cell colony formation. The quantities of relative colony formation are shown on the graph (Student t-test, $p = 0.0013$). **G:** Wound healing assays were performed after transfection (original magnification $\times 100$). **H:** Transwell invasion assay indicated that *MIR-376B-3P* reduced cell invasiveness (crystal violet stain, original magnification $\times 100$). The quantitative result is shown on the graph (Student t-test, $p = 0.0001$). ** $p < 0.01$, *** $p < 0.001$. Figure is available in color online only.

Based on our findings, a hypothesis of whether the *MEG3*/*MIR-376B-3P*/*HMGA2* axis was involved in phenotypic regulation of CNFPAs had been proposed. We first validated that the presence of *MEG3* could enhance the efficiency of *MIR-376B-3P* on the transcriptional regulation

of *HMGA2*. Next, enriching or silencing *MIR-376B-3P* expression could block or boost the expression of *HMGA2*, which is regulated by the upstream gene *MEG3*. Finally, we verified our analysis of this regulatory axis via in vivo experiments. In summary, our study affirmed that lncRNA

FIG. 4. *MEG3* is involved in the expression of *MIR-376B-3P*. **A:** The relative expression level of *MIR-376B-3P* in PDFS cells over-expressing *MEG3* (Student t-test, $p = 0.032$). **B:** After 5-Aza-CdR treatment, the transcriptional levels of *MEG3* (Student t-test, $p = 0.0012$) and *MIR-376B-3P* (Student t-test, $p = 0.0378$) were detected by qRT-PCR, and both showed similar tendencies following demethylation. **C:** The *MEG3* expression level was analyzed by qRT-PCR after transfecting *MIR-376B-3P* mimics (Student t-test, $p = 0.3805$) or inhibitor (Student t-test, $p = 0.8991$), both of which did not statistically change the transcriptional status of *MEG3*. * $p < 0.05$, ** $p < 0.01$, n.s. = no significance.

FIG. 5. *MEG3* negatively regulates the transcriptional activity of *HMGA2* by promoting *MIR-376B-3P* expression. **A:** The expression level of *HMGA2* between noninvasive tumors and invasive neoplasms was detected via qRT-PCR (Student t-test, $p < 0.0001$). **B:** Immunohistochemical analysis of *HMGA2* regarding different biological characteristics of tumors (immunoperoxidase stain, original magnification $\times 200$ and $\times 400$ [inset]). **C:** The predicted binding sites of *MIR-376B-3P* on *HMGA2* and the corresponding mutant sequence are shown. **D:** Luciferase reporter assay showed that *MIR-376B-3P* overexpression decreased the fluorescence activity in HEK-293T cells transfected with the *HMGA2*-WT (wild-type) vector (Student t-test, $p = 0.0017$). Data are presented as the relative ratio of firefly luciferase activity to *Renilla* luciferase activity. MUT = mutant. **E:** The *HMGA2* expression level was negatively correlated with *MEG3* and *MIR-376B-3P* (Student t-test; left, center, and right: $p = 0.0111$, $p = 0.0025$, and $p = 0.0028$, respectively). **F:** Pearson correlation analysis showed a significant negative correlation between *MEG3* and *HMGA2* (Pearson $r = -0.5214$; $p = 0.0031$, $n = 30$). The line represents the linear regression of the data ($y = -1.994x + 0.1883$; $r^2 = 0.2719$). **G:** Pearson correlation analysis showed a significant negative correlation between *MIR-376B-3P* and *HMGA2* (Pearson $r = -0.506$; $p = 0.0043$, $n = 30$). The line represents the linear regression of the data ($y = -80.88x + 0.2343$; $r^2 = 0.256$). **H:** Inhibition of *MIR-376B-3P* reversed the negative effect of *MEG3* on *HMGA2* (Student t-test; from left to right: $p = 0.0001$, $p < 0.0001$, $p < 0.0001$). **I:** Enrichment of *MIR-376B-3P* enhanced the suppressive effect of *MEG3* on *HMGA2* (Student t-test; from left to right: $p = 0.0001$, $p < 0.0001$, $p < 0.0001$). **J:** Immunoblot analysis of *HMGA2* protein in PDFS cells under different treatments. Quantitative analysis of *HMGA2* protein is shown on the graph (Student t-test; from left to right: $p = 0.008$, $p = 0.0005$, $p = 0.0035$, $p = 0.0009$). **K:** Immunofluorescence photomicrographs of *HMGA2* immunostain-positive cells after different treatments. TRITC (red) indicates positive cells and DAPI (blue) shows nuclear counterstaining (original magnification $\times 100$ and $\times 200$ [inset]). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, N.S. = no significance. Figure is available in color online only.

MEG3 enriched downstream miRNA *MIR-376B-3P* expression and thus negatively regulated *HMGA2*.

Nevertheless, limitations of this study must also be acknowledged. In the latest classification of endocrine tu-

mors, the pedigree of PAs has been emphasized. However, some commercially available antibodies are imperfect and exhibit unstable performance. Therefore, the patients enrolled in this study did not align with the classification

FIG. 6. *MEG3* and *MIR-376B-3P* inhibit tumorigenesis by targeting *HMGA2* in vivo. **A:** A tumor growth curve of experimental nude mouse models was generated following PDFS cell injection. Tumor volumes were calculated weekly (Student t-test; from top to bottom: $p = 0.002$, $p = 0.0007$, $p = 0.0002$). **B:** Mouse tumor sizes at the end of 28 days. **C:** Tumor weights are represented as means $\pm$ SEM (Student t-test; from left to right: $p = 0.0007$, $p = 0.0001$, $p = 0.0011$). **D and E:** The transcriptional (Student t-test; from left to right: $p < 0.0001$, $p < 0.0001$, $p = 0.0009$) and translational (Student t-test; from left to right: $p = 0.0008$, $p = 0.0005$, $p = 0.0006$) levels of *HMGA2* were measured after dissociating the obtained tumor samples. **F:** Immunohistochemistry assay of Ki-67 was conducted to detect the proliferative activity of different subcutaneous tumors (Student t-test; from left to right: $p = 0.0003$, $p < 0.0001$, $p = 0.0463$; immunoperoxidase stain, original magnification $\times 200$ and $\times 400$ [inset]). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Figure is available in color online only.

criteria. Although we discovered that *MEG3* enriched *MIR-376B-3P* expression, the specific underlying mechanisms are yet to be identified. It has been confirmed that lncRNA could be engaged in processing primary miRNA transcripts by sponging *MIR-361* to relieve the inhibitory effect on pri-*MIR-484* and promote Drosha-mediated processing into pre-*MIR-484*.³⁰ Another study reported a similar effect of lncRNA on pri-miRNA processing.¹⁵ Hence, a complex network involving the processing of primary transcripts between *MEG3* and *MIR-376B-3P* is conceptually plausible. Transcriptional factors regulated by *MEG3* might also interact with the enhancer region of *MIR-376B-*

3P. The specific regulatory network between *MEG3* and *MIR-376B-3P* needs to be comprehensively analyzed in future studies.

Conclusions

We present the first report on a novel mechanism of lncRNA *MEG3* as a negative regulator in *HMGA2* by upregulating the expression of *MIR-376B-3P* to regulate invasiveness in CNFPAs (Fig. 7). Targeting the interaction of *MEG3/MIR-376B-3P/HMGA2* will aid in designing new strategies for therapeutic intervention in PAs.

FIG. 7. Model for the relationship between the *MEG3/MIR-376B-3P/HMGA2* axis and tumor invasiveness. DNA methylation negatively regulates *MEG3*, which induces the *MIR-376B-3P* mRNA level. *MIR-376B-3P* inhibits the *HMGA2* expression level, thereby affecting pituitary tumor invasiveness. Figure is available in color online only.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: H Wang, Y Zhu. Acquisition of data: D Zhu, Xiao. Analysis and interpretation of data: D Zhu, Xiao, Gao. Drafting the article: D Zhu, Xiao. Critically revising the article: H Wang, Y Zhu. Reviewed submitted version of manuscript: H Wang, Z Wang, Y Zhu. Approved the final version of the manuscript on behalf of all authors: H Wang. Statistical analysis: Z Wang, Hu. Administrative/technical/material support: Hu, Duan, Z Zhu, Gao. Study supervision: H Wang, Z Zhu, Y Zhu.

Supplemental Information

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