

CircNFIX promotes progression of pituitary adenoma via CCNB1 by sponging miR-34a -5p

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Research

Keywords: CircNFIX, MiR-34a-5p, CCNB1, Progression, Pituitary adenoma

Posted Date: September 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-73445/v1>

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Version of Record: A version of this preprint was published at Molecular and Cellular Endocrinology on April 1st, 2021. See the published version at <https://doi.org/10.1016/j.mce.2020.111140>.

Abstract

Background: Previous studies have shown that CCNB1 affects the invasiveness of pituitary adenomas, and it is of great significance to find the upstream mechanism of regulating CCNB1.

Methods: RT-qPCR was used to measure the expression of circNFIX and miR-34a-5p in pituitary adenoma tissues. Western blotting was used to detect the expression of CCNB1 in pituitary adenomas. The relationship between circNFIX and miR-34a-5p was determined using dual-luciferase reporter assays. Pituitary adenoma cell invasion, migration and proliferation were analyzed using transwell, colony formation and CCK-8 assays, respectively. Additionally, xenograft experiments were performed to determine the effect of circNFIX silencing on tumor growth.

Results: In pituitary adenoma tissues, the expression of circNFIX (has-circ_0005660) and CCNB1 were upregulated, while miR-34a-5p expression was downregulated. The silencing of circNFIX or overexpression of miR-34a-5p inhibited cell invasion, migration and proliferation. Inhibition of miR-34a-5p expression reversed the inhibitory effect of circNFIX silencing on the progression of pituitary adenoma.

Conclusions: CircNFIX affects cell invasion, migration, and proliferation in pituitary adenomas by sponging miR-34a-5p through CCNB1. Therefore, circNFIX is expected to serve as a potential target for the treatment of pituitary adenomas.

Background

Pituitary adenomas account for about 17% of all intracranial tumors [1]. In recent years, the detection rate of pituitary adenomas has increased [2]. Histologically, pituitary adenomas are benign, but 34–60% of the pituitary adenomas show malignant invasive growth. Invasive pituitary adenomas can invade the dura mater and cavernous sinus, wrap the internal carotid artery, and destroy the skull base bone [3]. The invasive and proliferative abilities of pituitary adenomas often lead to adverse consequences, such as, difficult surgical treatment, high probability of recurrence, multiple operations, and poor prognosis, which seriously threaten the physical and mental health of the patients [4].

Tumorigenesis is a disease with abnormal cell cycle [5]. Abnormal cell cycle regulation plays a key role in the occurrence and development of tumors [6, 7]. Cyclin is not expressed or low expressed in normal tissues but abnormally expressed in tumor tissues. The abnormal expression of cyclin in tissue leads to the acceleration of cell cycle and excessive cell proliferation, which further leads to tumorigenesis [8, 9]. CyclinB1 (CCNB1) is a member of the cyclin family, after binding to CDK1, it initiates cells from the G1/S phase to the G2/M phase to promote mitosis [10]. CCNB1 is reported to be closely related to the pathogenesis of pituitary adenomas [11]. Previous research has shown that CCNB1 is overexpressed in invasive pituitary adenomas as compared to non-invasive pituitary adenomas [12]. In addition, CCNB1 affects the invasive and migratory abilities of pituitary adenoma cells [13]. Overexpression of CCNB1 is related to the invasion and migration behavior and poor prognosis of pituitary adenomas. Our previous research also found that the expression of miR-34a-5p in invasive pituitary adenomas was significantly

lower than that in non-invasive pituitary adenomas and it affects the biological characteristics of pituitary adenomas by regulating CCNB1 expression [14]. However, the mechanism underlying the influence of miR-34a-5p on the biological characteristics of pituitary adenomas through CCNB1 is still unclear. Therefore, further investigation of the mechanism of abnormal upregulation of CCNB1 in pituitary adenomas might provide novel strategies for invasive pituitary adenomas.

Circular RNA (circRNA) is a group of noncoding RNAs formed by reverse splicing of exons in the process of reverse transcription [15, 16]. It has covalent ends and plays a significant role in post-transcriptional regulation of gene expression [17]. MicroRNA sponge is a ubiquitous regulator of miRNA activity in many eukaryotes [18]. In tumor cells, circRNAs competitively bind to targeted microRNAs to promote or inhibit the functions of the corresponding microRNAs and play an important role in tumorigenesis by affecting the invasive and migratory abilities of cancer cells [19, 20]. This competitive binding of miRNA is called miRNA sponge action [21]. Researches have shown that circular RNA MAPK4 (circMAPK4) inhibits the apoptosis of glioma cells by sponging miR-125a-3p [1]. Circular RNA SLC26A4 promotes the invasiveness of cervical cancer cells by sponging miR-1287-5p [22]. Hsa_circ_RNA0000066 and hsa_circ_RNA0069707 are closely related to the prognosis of patients with nonfunctional pituitary adenomas, but their involvement in the invasiveness of pituitary adenomas has not been reported [23]. Therefore, it is important to explore the role of circRNAs in invasion, migration and proliferation of pituitary adenomas.

Previous research has shown that miR-34a-5p affected the biological characteristics of pituitary adenomas by regulating CCNB1. In the following research, we selected and verified the circRNA that may modulate CCNB1 and miR-34a-5p. And then, the expression and correlation of circNFIX and miR-34a-5p in pituitary adenomas were determined. Finally, the effects of circNFIX/miR-34a-5p on the invasion, migration and proliferation of pituitary adenomas were further verified. In summary, we aimed to provide a new mechanism to regulate the invasion, migration and proliferation of pituitary adenomas through the circNFIX/miR-34a-5p/CCNB1 axis.

Materials And Methods

Clinical materials and patient tissue specimens

A total of 22 invasive pituitary adenomas and 22 non-invasive pituitary adenomas were collected between January 2008 and January 2018 from Beijing Tiantan Hospital, Capital Medical University. This experiment was approved by the Ethics Committee of Beijing Tiantan Hospital. All patients underwent preoperative examination, especially contrast-enhanced magnetic resonance imaging (MRI). The invasion of pituitary adenomas was determined according to the Knosp grade. Pituitary adenoma tissues were preserved by quickly transferring to liquid nitrogen within 30 min after surgical resection. Detailed information is presented in Table 1.

Table 1
Sequences of the primers used for RT-qPCR

Gene	Forward primer	Reverse primer
CCNB1 (human)	TGTTGGTTTCTGCTGGGTGT	TGCCATGTTGATCTTCGCCT
CCNB1 (mmu)	GCACTTCTCCGTAGAGCATCT	GCACCATGTCGTAGTCCAGCAT
CircNFIX(human)	TACAAGTCGCCTCAGTGCTC	TCGATGAACGGGTGGAAGTCTC
CircNFIX(mmu)	CTCTTCGGCTTCTCCATTGAA	CGCTGTGAAAGTGAAAGTTTAGACT
NFIX(rat)	TGCCTGTGCTTGCTGGAGTCA	ATGGTGGTGGTGGTAGCGGATG
CircDICER1 (human)	TTCCATCAGTGGGAACCTACCTG	GCCTTCTAGGCTGTTGCTCT
CircLDHA (human)	TTCCGGATCTCATTGCCACG	GAACCCACGTGTGAGTCGG
CircXBP1 (human)	AGTGTAGCTTCTGAAAGGTGC	CCATAGCTCCAGACTACGCA
CircACTB (human)	CGTCTTCCCCTCCATCGTG	ATCATCCATGGTGAGCTGGC
CircTFG (human)	AGGAGCTCAGACTCAAGCA	CGCCGAATATCCTCCCCAAG
CircPARP1 (human)	GGAGGACGACAAGGAAAACAG	CGCACCTGGCCCTTTTCTAT
CircALDOA(human)	GAGCTGTCTGACATCGCTCA	CAATCGTCCGAGGGACCG
GAPDH (human)	AATGGGCAGCCGTTAGGAAA	GCCCAATACGACCAAATCAGAG
GAPDH (mmu)	GAAGGGCATCTTGGGCTACAC	GTTGTCATTGAGAGCAATGCCA

Cell culture

In order to study the biological characteristics of pituitary tumor cells, we used GT1-1 and GH3 cell lines, which are the most commonly used pituitary tumor cell lines[24–26]. The mouse pituitary adenoma cell line GT1-1 (ATCC, USA) was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, USA) and the rat pituitary tumor cell line GH3 (ATCC, USA) were cultured in 12K Medium (F-12K) supplemented with 15% horse serum and 2.5% FBS. HEK-293T cells were maintained in DMEM containing 10% fetal bovine serum. All cells were cultured at 37 °C in a 5% CO₂ humidified incubator.

Cell Transfection

MiR-34a-5p mimics and miR-34a-5p inhibitor was synthesized by Genesee (Guangzhou, China). si-circNFIX was synthesized by the Beijing Genomics institution (Beijing, China). The sequences are as follows:

si-circNFIX, CUCCGGGAUGAGUCCACCTT; GH3 and GT1-1 cells were inoculated in 6-well plates at a concentration of 1×10^5 cells per well. After 24 h of incubation at 37 °C in a 5% CO₂ humidified

incubator, the cell density reached 50–70% and each group was transfected with miR-34a-5p mimics, miR-34a-5p inhibitor or si-circNFIX using lipo3000. The cells were collected after 24 h of incubation at 37 °C under 5% CO₂ atmosphere.

RNA extraction and RT-qPCR

Trizol reagent (Invitrogen, USA) and RNeasy Mini Kit (Invitrogen, USA) were used to extract total RNA from the tissues and cells. MiRNeasy Mini Kit was used to extract microRNA. The purity and concentration of the extracted RNA was determined using NanoDrop 2000 (Thermo, Wilmington, Delaware, USA). RNA extraction and RT-qPCR 's experimental method was described in previous studies[27]. U6 was used as a control for miRNA expression and GAPDH was used as a control for mRNA expression. Primers for miR-34a-5p (CD202-0036, Mouse; CD201-0034, Human) were obtained from Tiangen (Beijing, China). The sequences of primers and siRNAs used are listed in Table 1.

Protein extraction and western blotting

The Westen Blot experimental procedure is described in the previous article [13].

The following antibodies were used in the experiment: primary antibody (Anti-Cyclin B1 antibody, No. ab2949, Abcam, Britain) and secondary antibody (Anti-Rabbit antibody, NO. ZB-2301, ZSGB-BIO China). β -actin was used as an internal control.

Dual-luciferase reporter assays

The binding sites between circNFIX or CCNB1 and miR-34a-5p were predicted by the biological prediction websites miRanda and RNAhybrid, respectively. In the dual-luciferase reporter assay, the corresponding circNFIX and CCNB1 wild-type and mutant sequences of miR-34a-5p were constructed and fused with the luciferase vector pmiR-RB-Report™ (Ribobio, Guangzhou, China). The wild-type and mutant luciferase reporter plasmids of circNFIX and CCNB1 were co-transfected with miR-34a-5p mimics and mimic NC, respectively, into HEK-293T cells. After 48 h of transfection, the cells were harvested and lysed, and luciferase activity was detected using a dual-luciferase report analysis system (Promega, USA).

Cell invasion and migration assays

The migration and invasion ability of pituitary tumor cells were detected by Transwell method. Transwell plates pre-coated with Matrigel were used to determine the invasiveness of the cells. Cell migration was measured using Transwell chambers with 8- μ m holes in a 24-well culture plate (Corning, USA).

Transfected cells (GT1-1 and GH3) were incubated at 37 °C under 5% CO₂ atmosphere for 24 h, and the cells invaded the submembrane surface. After 24 h of incubation, cells on the surface of the membrane filter were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The cell numbers were determined by counting a randomly selected 10-fold mirror visual field.

Cell viability and proliferation assays

After trypsin digestion, GT1-1 and GH3 cells transfected with si-circNFIX were inoculated into a 96-well plate (1×10^4 cells per well). Cell viability was determined using the CCK-8 method (APExBio, USA) at

24 h, 48 h, 72 h, and 96 h. At each time point, 10 μ L CCK-8 solution was added to each well and incubated at 37 °C for 3 h. The absorbance was measured at 450 nm with the spectrophotometer (Tecan, Austria).

For cell proliferation assay, the transfected GT1-1 and GH3 cells were incubated in 6-well plates at a concentration of 1×10^3 cells per well. After two weeks of incubation, the cells were fixed with 4% paraformaldehyde for 30 min, stained with 0.5% crystal violet (Solarbio, China) for 30 min, and counted under a microscope.

Xenograft experiments and immunohistochemistry

BALB/c male nude mice (Charles River, Beijing, China), aged 6–8 weeks, were used for in vivo experiments. A total of 12 nude mice were randomly divided into two groups. GT1-1 and GH3 cells (2×10^6) stably transfected with sh-circNFIX or sh-circNFIX-NC were suspended in 100 μ L serum-free medium and transplanted subcutaneously into the right upper back of nude mice. The mice were sacrificed after 4 weeks, and the volume and weight of the tumor were measured. All the specimens were stained with immunohistochemistry (IHC), and the detailed method was as described in the previous article[13].

Statistical analysis

SPSS25.0 statistical software was used for t-test and analysis of variance, and all the results were summarized and expressed by mean \pm standard deviation (SD). A P-value ≤ 0.05 is considered to be statistically significant (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). No samples were excluded from the analysis.

Results

Tumor characteristics and the expression of CCNB1 in pituitary adenomas

Tissue samples from 44 patients with pituitary adenomas were included in this study (Table 2). According to the Knosp grading system for predicting invasion of the cavernous sinus and internal carotid, the pituitary adenomas were classified as non-invasive (Knosp grades 0–II) and invasive pituitary adenomas (Knosp grades III–IV) (Fig. 1A). To confirm the expression of CCNB1 in pituitary adenomas, western blot assay (WB) was performed in invasive pituitary adenomas and non-invasive pituitary adenomas. WB results indicated that CCNB1 was overexpressed in invasive pituitary adenomas as compared to non-invasive pituitary adenomas (Fig. 1B and 1C).

Table 2
Correlations between circNFIIX expression and clinical characteristics in pituitary adenoma patients

Parameters	Group	Cases	Circ-NFIX expression		p-value
			Low	High	
Gender	Male	19	11	9	0.979
	Female	25	11	13	
Age (years)	≥ 45	25	12	14	0.965
	< 45	19	10	6	
Tumor size	≤10 mm	2	2	0	0.047 *
	10 mm-30 mm	34	20	14	
	>30 mm	8	0	8	
(KnoSp grade)	I-II	22	22	0	0.043 *
	III-IV	22	0	22	

Kaplan-Meier survival curve showed the effects of high expression of CCNB1 and low expression of CCNB1 on of patients with progression-free survival (n = 22, p < 0.01) (Fig. 1D). This suggests that, like previous studies[13], CCNB1 is related to the invasiveness of pituitary adenomas and affects the prognosis of patients.

MiR-34a-5p inhibits the progression of pituitary adenomas by targeting CCNB1

Our previous research demonstrated that miR-34a-5p negatively regulates CCNB1 [28]. According to RNAhybrid prediction, CCNB1 targets miR-34a-5p. Dual-luciferase reporter assays confirmed that miR-34a-5p downregulated the activity of CCNB1 promoter but had little inhibitory effect on the mutant CCNB1 promoter (Fig. 2A and 2B). MiR-34a-5p expression was detected in pituitary adenoma tissues, and it was found that the expression was significantly lower in invasive pituitary adenomas than that in non-invasive pituitary adenomas (Fig. 2C). To explore the function of miR-34a-5p, we transfected miR-34a-5p mimics into GT1-1 and GH3 pituitary tumor cell lines. The results of the RT-qPCR assay confirmed that the expression of miR-34a-5p was significantly increased in GT1-1 and GH3 cells (Fig. 2D). The results of RT-qPCR and western blot experiments demonstrated that overexpression of miR-34a-5p decreased the expression of CCNB1 (Fig. 2E-G). Transwell analysis showed that overexpression of miR-34a-5p significantly inhibited the invasive and migratory abilities of GT1-1 and GH3 cells (Fig. 2H-K). Colony formation and CCK-8 experiments showed that overexpression of miR-34a-5p inhibited cell proliferation (Fig. 2L-O). Overall, the above experimental results demonstrated that miR-34a-5p inhibits cell invasion, migration and proliferation in pituitary adenomas by targeting CCNB1.

CircNFIX is highly expressed in invasive pituitary adenomas and acts as a sponge of miR-34a-5p

circRNAs can sponge miRNAs to regulate the expression of downstream genes and consequently affect tumor progression [18]. To identify circRNAs which can bind to miR-34a-5p, we used bioinformatics analysis (TargetScan, Starbase, RNA Hybrid, and Circbase) and identified circRNAs which share a complementary sequence with miR-34a-5p (Fig. 3A). According to the number of binding sites between circRNA and miR-34a-5p, we screened the top 10 circRNA and verified their expression in pituitary adenomas. RT-qPCR was used to detect the expression of 10 circRNAs in invasive and non-invasive pituitary adenomas. Figure 3B shows that in 22 pairs of invasive and non-invasive pituitary adenomas, circ-TFG, circ-LDHA and circ-NFIX are significantly up-regulated in tumor tissues (Fig. 3B), of which circNFIX is the most up-regulated ($p < 0.01$) (Fig. 3C). Similarly, dual-luciferase reporter assays confirmed the binding relationship between circNFIX and miR-34a-5p (Fig. 3D and 3E). Taken together, our results suggest that circNFIX is the target molecule of miR-34a-5p. Furthermore, there was a significant negative correlation between the circNFIX expression and miR-34a-5p expression in pituitary adenoma tissues (Fig. 3F, $p < 0.001$, $R^2 = 0.5468$).

Silencing of circNFIX inhibits pituitary adenoma cell invasion, migration and proliferation

To study the biological functions of circNFIX, we designed circNFIX siRNA (si-circNFIX) for the reverse splicing sequence of circNFIX. Transfection of GT1-1 and GH3 cells with circNFIX siRNA successfully silenced the mRNA expression of circNFIX (Fig. 3G). The RT-qPCR analysis demonstrated that circNFIX knockout increased the expression of miR-34a-5p (Fig. 3H). Transwell analysis revealed that the invasive and migratory abilities of pituitary adenoma cells were significantly inhibited by circNFIX silencing (Fig. 3I-L). Colony formation and CCK-8 assays were used to detect cell proliferation and viability, respectively. Compared to the FC (full blank control) and NC (negative control) group cells, circNFIX silencing dramatically reduced cell proliferation and viability (Fig. 3M-P). Altogether, the results indicate that circNFIX silencing inhibits cell invasion, migration and proliferation in pituitary adenomas.

Inhibition of miR-34a-5p reverses the effect of circNFIX silencing on pituitary adenoma cells

Furthermore, we sought to determine whether miR-34a-5p can reverse the effect of circNFIX silencing on the function of pituitary adenoma cells. GT1-1 and GH3 cells were co-transfected with si-circNFIX and miR-34a-5p inhibitor to assess whether the effect of si-circNFIX could be rescued by miR-34a-5p inhibition. The results indicated that the inhibitory effect of circNFIX silencing on CCNB1 could be rescued by miR-34a-5p inhibition (Fig. 4A,4B). Similarly, transwell, CCK-8, and colony formation assays showed that, compared to NC group cells, circNFIX silencing dramatically reduced cell invasion, migration and proliferation in pituitary adenomas, which was reversed by inhibition of miR-34a-5p (Fig. 4C-J). These results indicate that the inhibitory effect of circNFIX silencing on pituitary adenoma cells could be rescued by inhibition of miR-34a-5p.

Silencing circNFIX inhibits tumor formation in vivo

To confirm the effect of circNFIX on subcutaneous tumorigenesis, xenograft models were established using BALB/c nude mice. Each mouse was injected subcutaneously with sh-circNFIX or sh-circNFIX-NC. It was observed that tumor formation in the sh-circNFIX group was suppressed as compared to the control

group (Fig. 5A-C). Western blot and IHC analysis further revealed that the expression of CCNB1 in the sh-circNFIX group was significantly lower as compared to the NC group (Fig. 5D-5F). Taken together, the results indicate that circNFIX silencing reduces CCNB1 expression and inhibits tumor formation in nude mice.

Discussion

Invasive adenomas are generally pituitary macroadenomas, which often infiltrate the surrounding dura mater, cavernous sinus, brain, and bone tissue [29]. The cavernous sinus invasion rate has been reported to be between 30% and 63% [30]. Studies have shown that invasion of the cavernous sinus and internal carotid artery is an important factor leading to poor prognosis of pituitary adenomas [31, 32]. Invasion of the cavernous sinus and other important tissues poses great challenges to surgical resection and increases the potential risk of cerebrospinal fluid leakage and internal carotid artery injury [33]. The invasiveness of pituitary adenomas is related to the poor prognosis of the patients. Therefore, the study of the progression of invasive pituitary adenomas has important clinical significance to improve the prognosis of patients with pituitary adenomas.

CCNB1 is a member of the cyclin family [34]. CCNB1 can affect the pathogenesis and progression of tumors by affecting the cell cycle [12, 35, 36]. It has been reported that the expression of CCNB1 is upregulated in glioma tissues; overexpression of CCNB1 affects tumor cell invasion, migration, and proliferation, and is associated with poor prognosis [37]. Zhang et al. (2016) reported that CCNB1 was associated with the pathogenesis of pituitary adenomas [11]. In previous research, we demonstrated that CCNB1 affects cavernous sinus invasion in pituitary adenomas through epithelial-mesenchymal transition (EMT) [13]. In this study, we also confirmed that CCNB1 is overexpressed in invasive pituitary adenomas and further affects the ability of invasion and migration of pituitary adenomas.

Previous studies have shown that miR-34a-5p expression exhibits marked changes in various tumors, such as gastric cancer [38], uterine leiomyosarcoma [39], lung cancer [40], hepatocellular carcinoma [41]. Besides, the research also found miR-34a-5p can inhibit EMT in lung cancer, consequently affecting tumor progression [42]. Similarly, in pituitary adenomas, miR-34a-5p can inhibit cell proliferation and promote apoptosis [43]. Furthermore, previous research has revealed that miR-34a-5p affects the biological characteristics of pituitary adenomas through CCNB1, therefore, we speculated that there might be a relationship between miR-34a-5p and CCNB1 [28]. Through the gene prediction websites TargetScan and miRanda, we found that circNFIX (hsa_circ_0005660) had the binding relationship of miR-34a-5p, which was confirmed by the dual-luciferase reporter assays. Therefore, we found a new targeting relationship between miR-34a-5p and circNFIX.

A circRNA is a closed-loop formed by the splicing of precursor RNA, which is mainly formed from exons of protein-coding genes and has a highly conservative ring structure [44]. Recent studies have found that circRNA plays an important role in a variety of biological processes, including cell cycle, apoptosis, vascularization, and invasion [45, 46]. circRNAs can play a wide range of roles including acting as a

miRNA sponge to regulate the expression of protein-coding genes [47]. Therefore, circRNA can be used as a potential biomarker for many human diseases [46]. Studies have shown that a circRNA can bind to more than one miRNA [48]. CircNFIX (hsa_circ_0049658) has been reported to affect the progression of gliomas by regulating the miR-378e/RPN2 axis [49]. It has also been reported that circNFIX (hsa_circ_0109102) promotes the proliferation of glioma cells by regulating the expression of miR-34a-5p [50]. However, the function of circNFIX in pituitary adenomas is unknown. Therefore, this study explains the effect of circNFIX (hsa_circ_0005660) on the invasive biological characteristics of pituitary adenomas.

In this study, we explored the role of circNFIX in regulating the expressions of miR-34a-5p and CCNB1 in the development and progression of pituitary adenomas *in vivo* and *in vitro*. The roles of circNFIX in pituitary adenomas were summarized in Fig. 6. The results indicated that circNFIX was highly expressed in invasive pituitary adenomas. However, the expression of miR-34a-5p was nearly the opposite. We also revealed that circNFIX silencing or miR-34a-5p overexpression inhibited pituitary adenoma cell invasion, migration, and proliferation. To detect the detailed regulation mechanism between circNFIX and miR-34a-5p, bioinformatics analysis showed there were complementary binding sites between circNFIX and miR-34a-5p. Through dual-luciferase reporter assays and RT-qPCR assays, we confirmed that circNFIX reduced the expression of miR-34a-5p by acting as ceRNA. Further analysis showed that the high expression of circNFIX in pituitary adenomas was related to high Knosp grade and poor prognosis. This evidence suggests that circNFIX is a potential biomarker of pituitary adenomas. Furthermore, our data showed that silencing of circNFIX upregulated miR-34a-5p expression, which in turn downregulated the expression of CCNB1, consequently inhibiting pituitary adenoma cell invasion, migration, and proliferation. All these results demonstrate that circNFIX can sponge miR-34a-5p and competitively suppress its function. Besides, we demonstrated that the inhibitory effect of circNFIX silencing on pituitary adenomas was partially rescued by miR-34a-5p inhibitor. Animal experiments revealed that circNFIX silencing inhibited tumor formation *in vivo*.

Conclusions

This study is the first to reveal that circNFIX affects the progression of pituitary adenomas and provides a new strategy for the treatment of invasive pituitary adenomas. We confirmed that circNFIX sponges with miR-34a-5p affect the invasive, migratory and proliferative abilities of pituitary adenoma cells by regulating CCNB1. In addition, we hypothesized that circNFIX through affecting EMT to affect invasion and migration of pituitary adenomas; however, further research is needed to prove this hypothesis.

Abbreviations

CCNB1: CyclinB1; circRNAs: Circular RNAs; MRI: magnetic resonance imaging; RT-PCR: Reverse Transcription Polymerase Chain Reaction; WB: Western blot; IHC: immunohistochemistry; CCK-8: Cell Counting Kit-8; siRNA: small interfering RNA; HEK, human embryonic Kidney; EMT: mesenchymal transition; FC: full blank control; NC: negative control

Declarations

Acknowledgments

None

Author contributions

PZ and JC conceived and designed the study. JC, BL and DN conducted the experiments and data analysis. CL, SG, and ZZ performed sample collection and analysis. JC and PZ wrote the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the Natural Science Foundation of Beijing Municipality (CN) (7162034) and Beijing Neurosurgical Institute Innovation Exploration Foundation (201721).

Availability of data and materials

All relevant data and materials are available.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Beijing Tiantan Hospital, Capital Medical University (Beijing, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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Figures

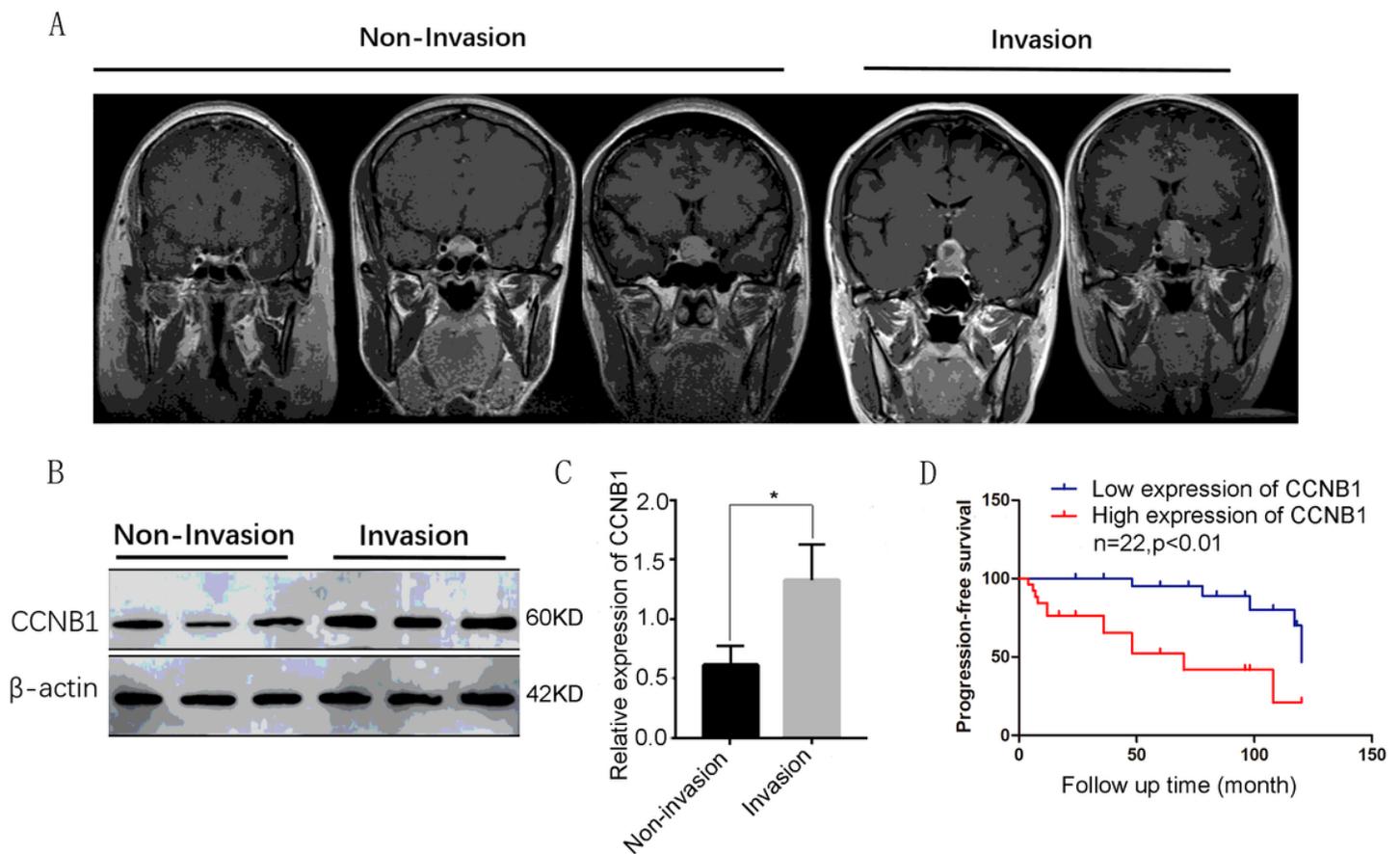


Figure 1

Tumor characteristics of non-invasive and invasive pituitary adenomas and CCNB1 expression. (A) MRI performance of non-invasive and invasive pituitary adenomas. (B, C) The expression of CCNB1 in pituitary adenomas was detected by western blotting. (D) Kaplan-Meier survival curve showed progression-free survival in patients with different expression of CCNB1. *p < 0.05

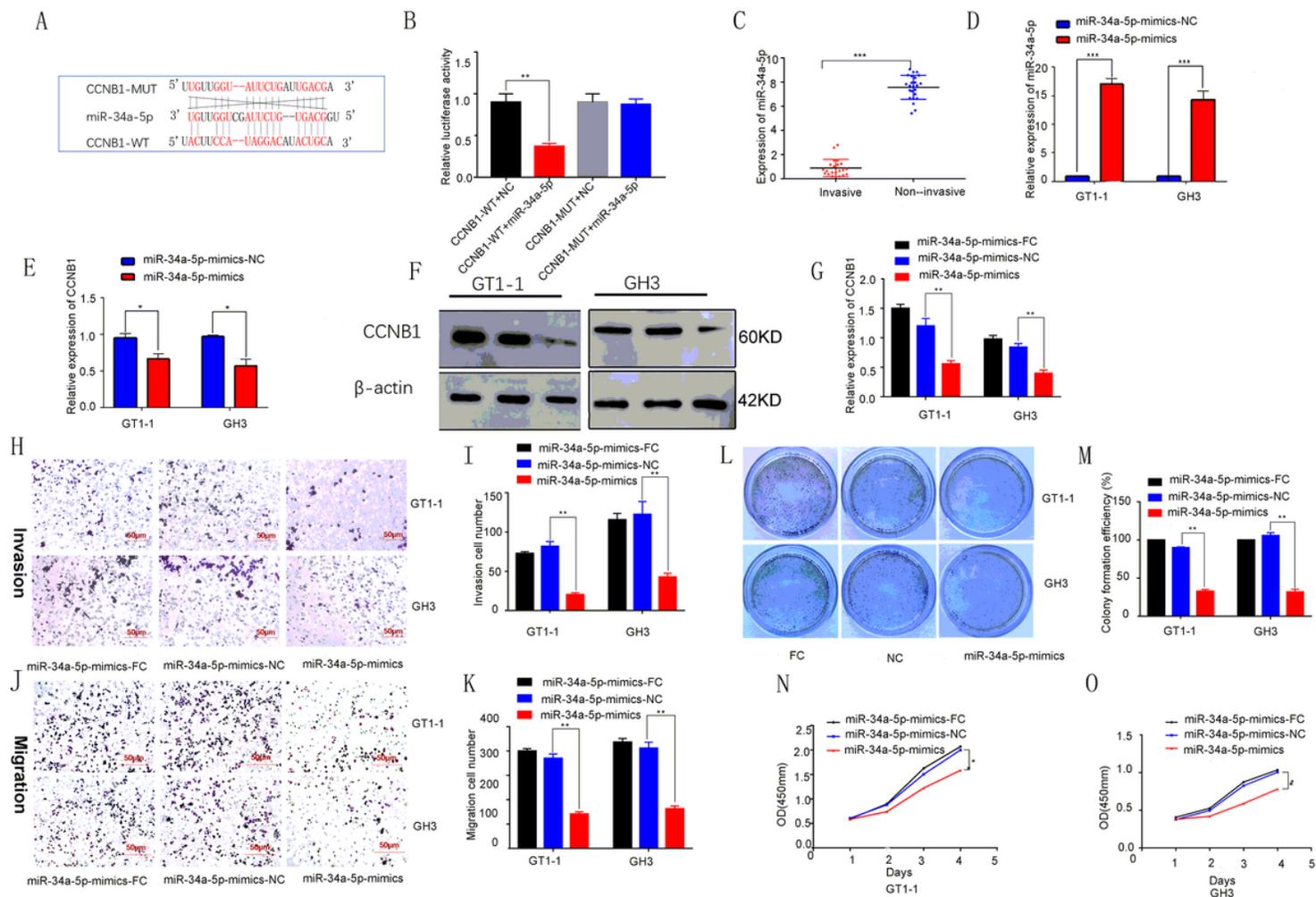


Figure 2

MiR-34a-5p targets CCNB1 and affects cell invasion, migration, and proliferation in pituitary adenomas. (A, B) Dual-luciferase reporter assays proved the binding relationship between miR-34a-5p and CCNB1. (C) MiR-34a-5p expression-NC in 22 non-invasive and 22 invasive pituitary adenomas was detected by RT-qPCR. (D) RT-qPCR was used to detect the overexpression of GT1-1 and GH3 cells transfected with miR-34a-5p mimics. E-G RT-qPCR and western blot assays were used to detect the expression of CCNB1 after overexpression of miR-34a-5p in GT1-1 and GH3 cells. (H-K) Transwell, colony formation, and CCK-8 assays were used to detect GT1-1 and GH3 cell invasion, migration, and proliferation, respectively, after overexpression of miR-34a-5p. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ FC: full blank control; NC: negative control

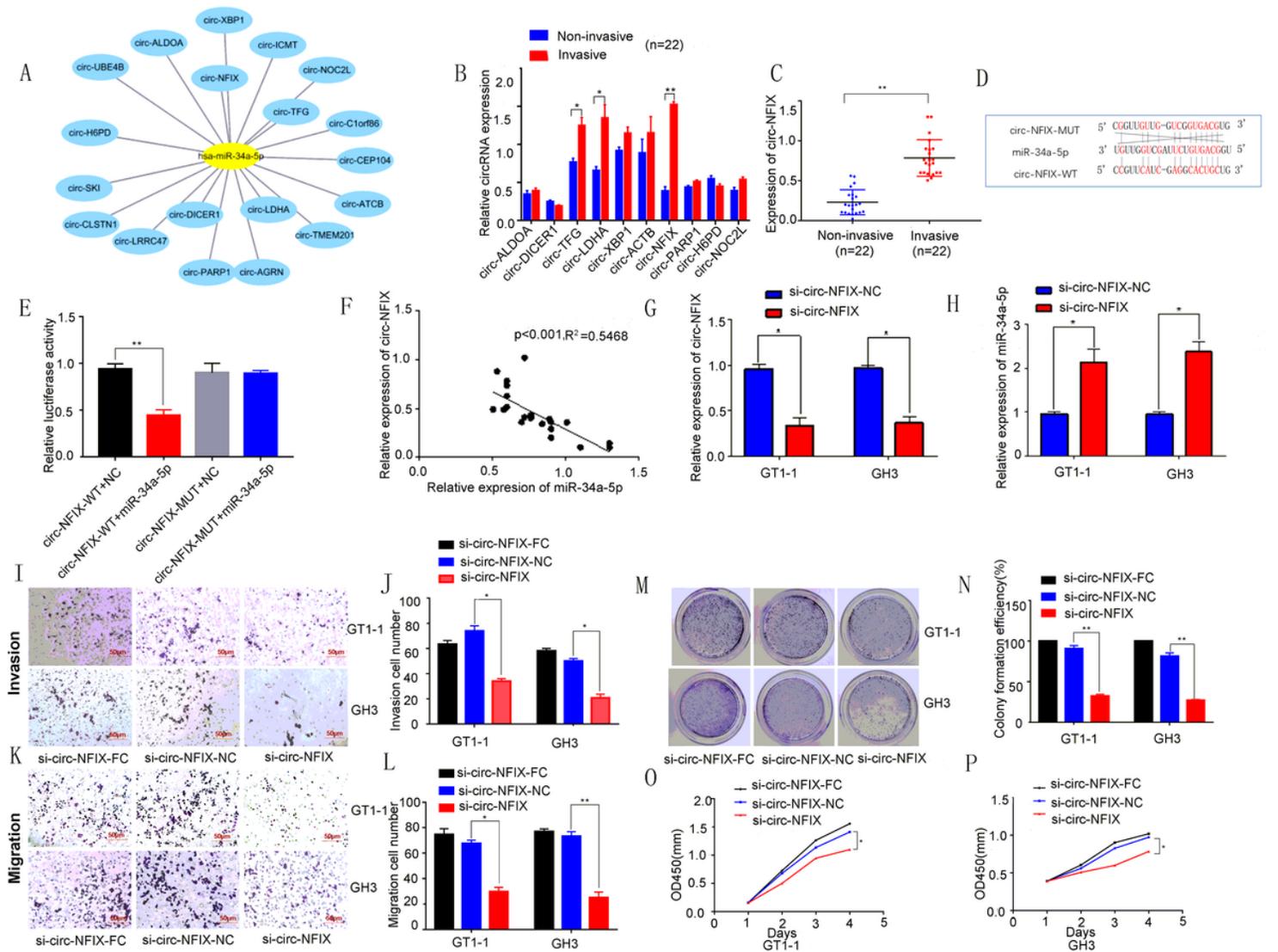


Figure 3

CircNFIX sponges miR-34a-5p and affects cell invasion, migration, and proliferation in pituitary adenomas. (A) Gene interaction network showing the relationship between circRNAs and miR-34a-5p. (B) Expression of circRNAs in non-invasive and invasive pituitary adenomas. (C) CircNFIX is upregulated in invasive pituitary adenoma as compared to non-invasive pituitary adenomas (p < 0.01) (D, E). Dual-luciferase reporter assays proved the binding relationship between circNFIX and miR-34a-5p. (F) Expression of circNFIX and miR-34a-5p inversely correlated in pituitary adenomas (p < 0.001, R² = 0.5468). (G) RT-q PCR was used to detect the silencing efficiency of circNFIX-targeted siRNA transfection into GT1-1 and GH3 cells. (H) RT-qPCR was used to measure the expression of miR-34a-5p after circNFIX silencing in GT1-1 and GH3 cells. (I-P) Transwell, colony formation, and CCK-8 assays were used to detect GT1-1 and GH3 cell invasion, migration, and proliferation, respectively, after circNFIX silencing. *p < 0.05, **p < 0.01. FC: full blank control; NC: negative control.

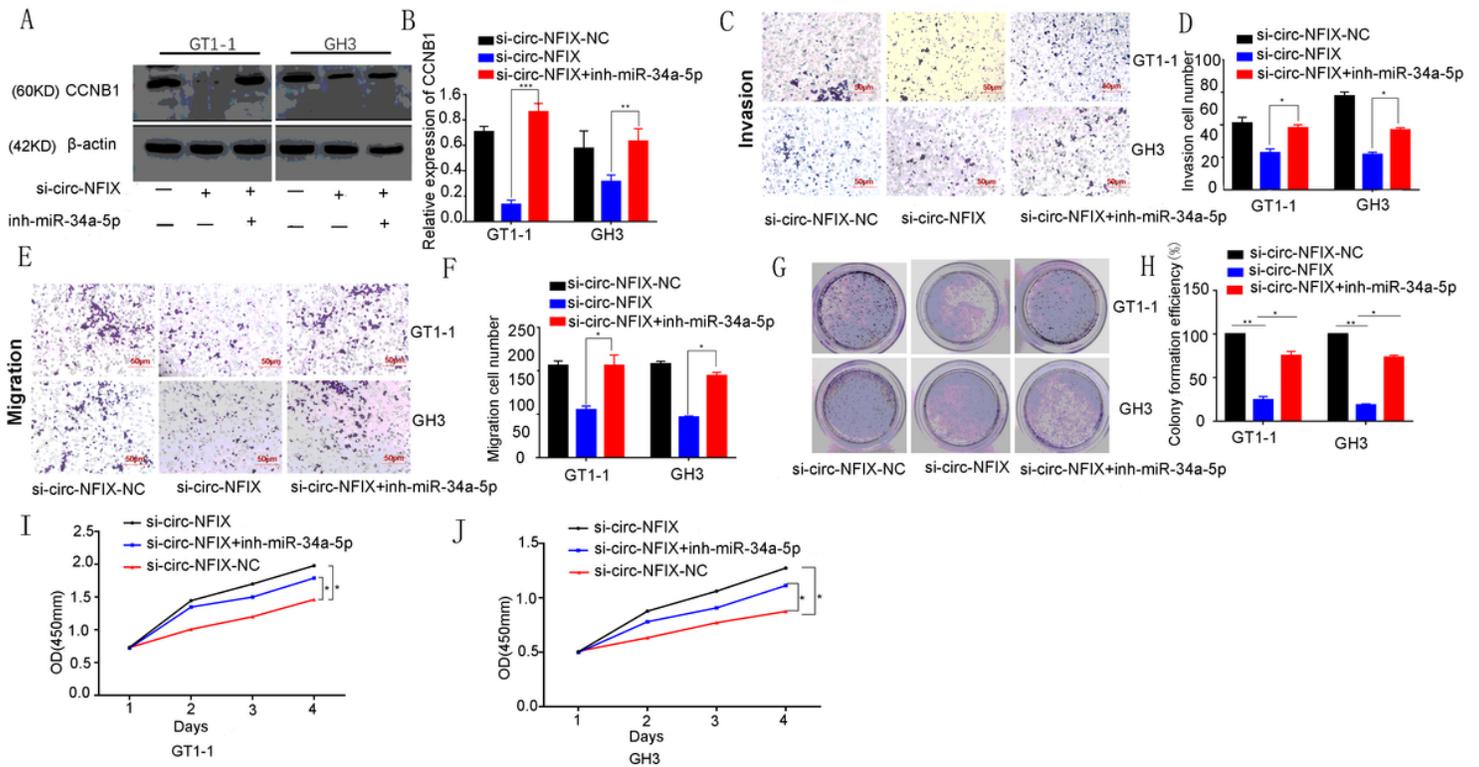


Figure 4

MiR-34a-5p inhibition reversed the effect of circNFIx silencing on pituitary adenoma cells (A,B) MiR-34a-5p inhibition rescued CCNB1 downregulation induced by circNFIx silencing.(C-J) MiR-34a-5p inhibition reversed the inhibitory effect of circNFIx silencing on pituitary adenoma cell invasion, migration, and proliferation, as shown by transwell, colony formation, and CCK-8 assays, respectively. *p < 0.05, **p < 0.01 FC: full blank control; NC: negative control

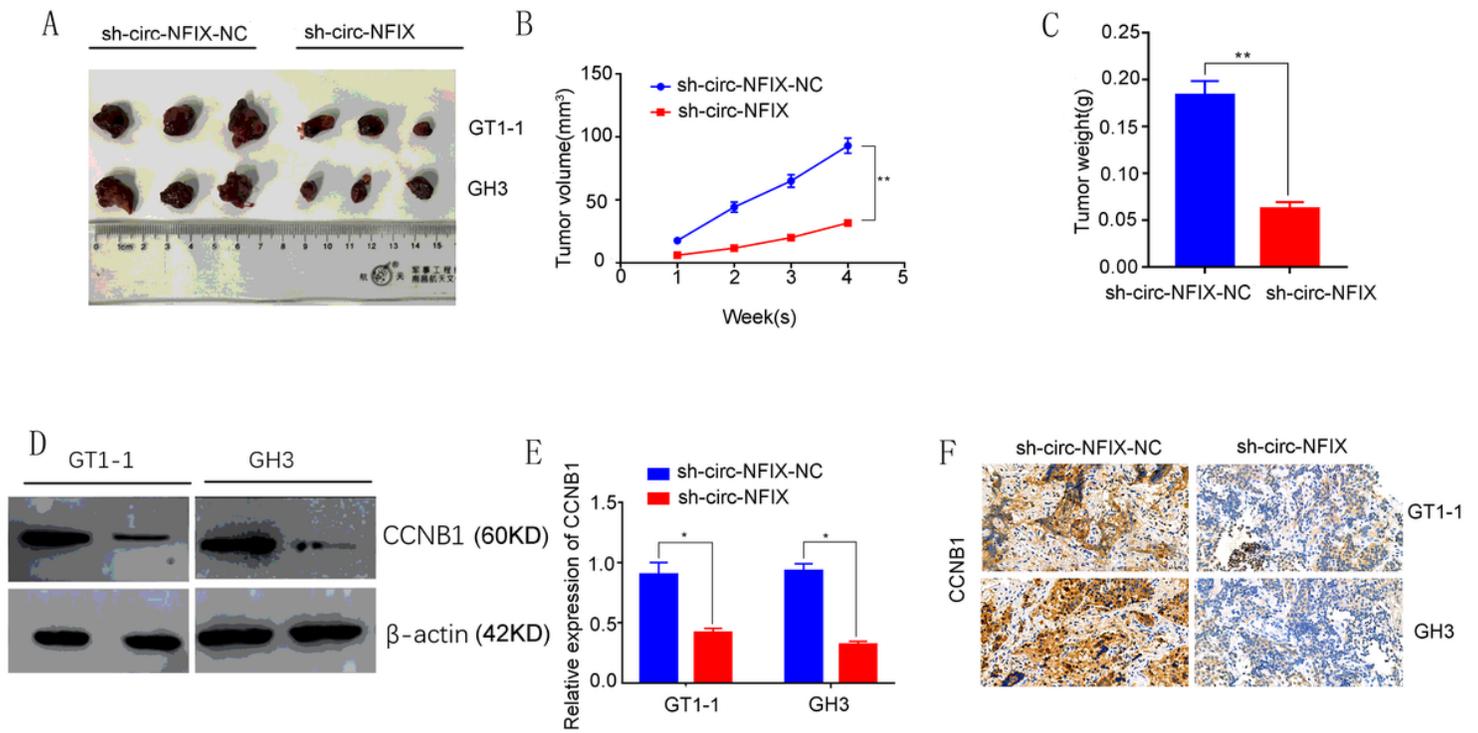


Figure 5

Silencing of circNFIX inhibits tumor formation in vivo (A) The tumors in the sh-circNFIX group were significantly smaller than that in the NC group after 4 weeks of injection. (B) The size of the xenograft tumor was measured every week. (C) Weight of xenograft tumors. (D, E) Expression of CCNB1 in xenograft tumors was measured by WB. (F) Expression of CCNB1 was measured by IHC. * $p < 0.05$, ** $p < 0.01$ FC: full blank control; NC: negative control

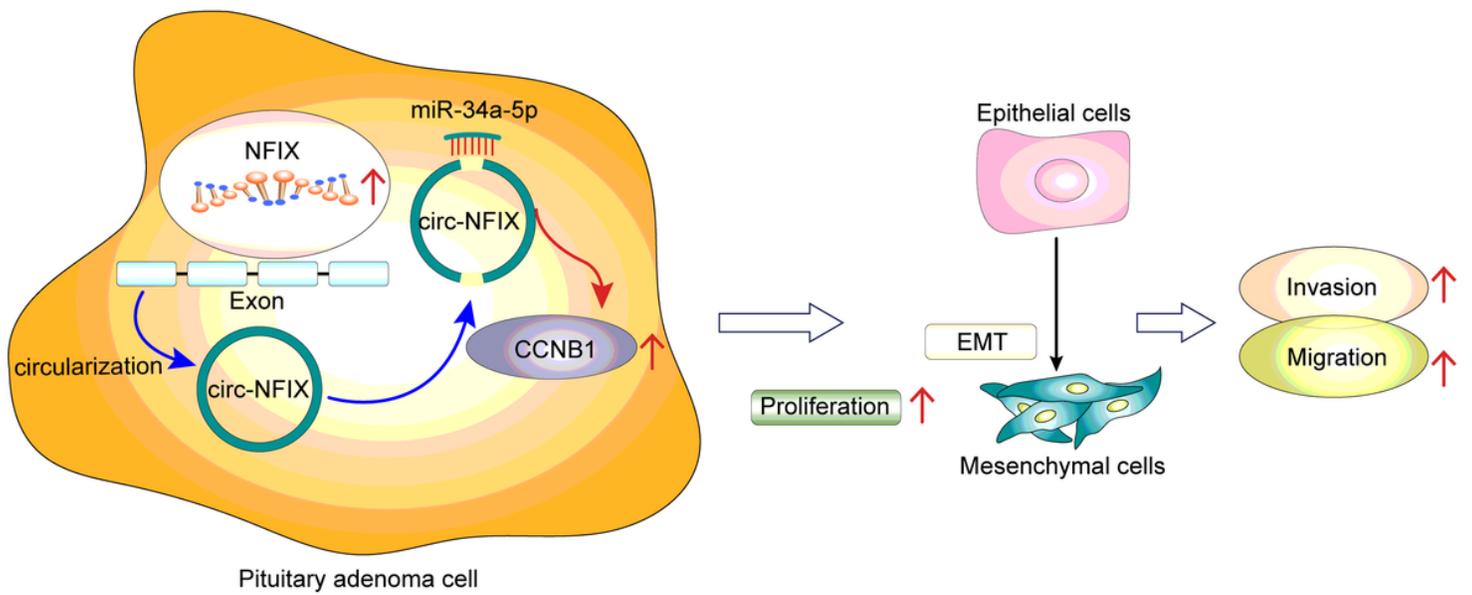


Figure 6

Schematic diagram of the endogenous competition mechanism In pituitary adenomas, the upregulation of circNFIX leads to the downregulation of miR-34a-5p expression. Furthermore, it upregulates the expression of CCNB1, thus promoting cell proliferation and EMT, in turn, it promotes the invasion and migration of pituitary adenomas.