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ORIGINAL RESEARCH

Circ_0058124 Aggravates the Progression of Papillary Thyroid Carcinoma by Activating LMO4 Expression via Targeting miR-370-3p

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Background: Thyroid cancer is the most common malignant tumor in the endocrine system. Papillary thyroid carcinoma (PTC) accounts for the vast majority of cases in this cancer. Recently, the vital role of circular RNA (circRNA) has been acknowledged in various cancers, and this study aimed to investigate the role of circ_0058124 and related mechanism of its action in PTC.

Materials and Methods: The expression of circ_0058124, miR-370-3p and LIM domain only (*LMO4*) was detected by qRT-PCR in tissue samples (PTC tissues or normal tissues, n=20) and cell lines (non-cancer cell line, Nthy-ori 3–1, and PTC cell lines, IHH-4 and TPC-1). For functional analysis, cell proliferation was investigated using CCK-8 assay and colony formation assay. Cell migration and invasion were determined using transwell assay, and cell migration was also assessed by wound healing assay. Cell apoptosis was monitored by flow cytometry assay. For mechanism analysis, the interaction between miR-370-3p and circ_0058124 or *LMO4* predicted by the bioinformatics analysis was validated by dual-luciferase reporter assay or RIP assay. The effect of circ_0058124 on tumor growth in vivo was identified by establishing the Xenograft model.

Results: The expression of circ_0058124 was enhanced in PTC tissues and cells. Circ_0058124 knockdown inhibited viability, colony formation, migration and invasion and promoted apoptosis of PTC cells. Besides, circ_0058124 knockdown also blocked tumor growth in vivo. miR-370-3p was a target of circ_0058124, and circ_0058124 regulated the expression of *LMO4*, a target of miR-370-3p, by targeting miR-370-3p. Rescue experiments presented that miR-370-3p inhibition reversed the inhibitory effects of circ_0058124 knockdown on PTC development, and *LMO4* overexpression reversed the effect of miR-370-3p restoration on PTC development.

Conclusion: Circ_0058124 promoted the development of PTC by mediating the miR-370-3p/*LMO4* axis, and circ_0058124, functioned as an oncogene in PTC, might be used as a promising biomarker for PTC diagnosis and treatment.

Keywords: circ_0058124, miR-370-3p, LMO4, papillary thyroid carcinoma

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Introduction

Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumor and accounts for about 80% of all thyroid cancer cases.[1] Recently, the incidence of PTC has been on the rise, but the reason is unclear.[2] PTC is usually detected in people with an average age of 40 years, and women seem to be more likely to develop this cancer than men.[3] Fortunately, PTC is well differentiated and

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considered to be a low degree of malignancy.[4] Therefore, it is of great significance to actively look for effective molecular therapeutic targets and control the biological behavior of malignant tumors to further improve the prognosis of patients with PTC and improve the life quality of patients.

All along, researchers have made great efforts to study the pathogenesis of PTC and have identified series of promising biomarkers for the diagnosis and treatment of this cancer, including different types of non-coding RNAs (ncRNAs).[5–7] Circular RNA (circRNA) is a novel class of ncRNAs, characterized by a closed-loop structure formed by a covalent linkage at the 3′ and 5′ ends.[8] With the boom of sequencing technology, numerous circRNAs differently expressed in PTC tissues and normal tissues have been identified, revealing that circRNA dysregulation might play a part in PTC progression and pathogenesis.[9] Recently, the diverse functions and the potential mechanisms of several circRNAs have been investigated in PTC, which provides valuable references and unique perspectives to understand PTC pathogenesis.[10,11] Circ_0058124 is a novel circRNA, which is derived from a precursor mRNA, fibronectin 1 (FN1). It was previously identified to be abundantly expressed in PTC tissues.[12] In general, the functional role of circ_0058124 in cancer progression is still lacking and needs to be further explored.

MicroRNA (miRNA) is characterized by ~22 nucleotides in length and governs multiple biological processes, including tumorigenesis and tumor development.[13] Increasing evidence has revealed that circRNAs are implicated in various regulatory activities by sponging miRNAs, and miRNAs degrade downstream mRNA expression by binding to mRNA 3′ untranslated regions (3′UTRs).[13,14] MiR-370-3p was previously reported to be a target of circRNA-NEK6 and involved in the development of thyroid cancer.[15] However, the associated mechanisms of miR-370-3p action in PTC are not fully addressed, and the association between miR-370-3p and circ_0058124 is not identified as yet.

LIM domain only (*LMO4*) is a member of the LIM-only family and is confirmed to play a crucial role in cancer aggravation.[16,17] *LMO4* was reported to be a target of several miRNAs, such as miR-150 and miR-139-5p, and *LMO4* overexpression abolished the regulatory effects of these miRNAs, thus promoting the aggravation of burkitt lymphoma and PTC.[18,19] Similar functional mechanisms of *LMO4* in PTC are insufficient, and the potential interaction between *LMO4* and miR-370-3p is not explored.

Currently, we investigated the expression and function of circ_0058124 in PTC. Mechanically, the competing endogenous RNA (ceRNA) theory was applied in this study to expound a potential mechanism of circ_0058124 action in PTC, associated with miR-370-3p and *LMO4*. The objective of our study was to provide evidence for the involvement of circ_0058124 in PTC pathogenesis and the possibility of circ_0058124 as a biomarker in PTC diagnosis or treatment.

Materials and Methods

Clinical Samples

Clinical samples were collected from tumor tissues and non-tumor tissues of 20 patients with PTC from Tianjin Third Central Hospital. The use of the tissues was approved by the patients, and all of them had signed informed consent. These tissues were sealed in liquid nitrogen as soon as they are isolated and then stored at −80°C. The Ethics Committee of Tianjin Third Central Hospital had approved this study.

Cell Lines

Human PTC cell lines (IHH-4 and TPC-1) and normal human thyroid cells (Nthy-ori 3–1) were purchased from Cobioer Co., Ltd (Nanjing, China). According to the specification, IHH-4, TPC-1 and Nthy-ori 3–1 cells were cultured in 90% RPMI1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FBS at 37°C incubators containing 5% CO_2.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using a Total RNA Extractor (Sangon Biotech, Shanghai, China). The subsequent synthesis of cDNA was performed using a reverse transcription kit (Takara, Dalian, China) for circRNA and mRNA, or using a Mir-X miRNA 1st-Strand Synthesis Kit (Takara) for miRNA. The relative expression was then detected by qRT-PCR using SYBR Green PCR Kit (Takara) on a PCR System (Bio-Rad, Hercules, CA, USA), with GAPDH or U6 as an internal reference. The data were summarized using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were listed as below:

circ_0058124, 5′-AACAGACAACCAGCAACACC-3′ (F) and 5′-GCTCATCTCCCTCCTCACTC-3′ (R); GA

PDH, 5′-TGGAAGGACTCATGACCACA-3′ (F) and 5′-TTCAGCTCAGGGATGACCTT-3′ (R); miR-370-3p, 5′-GCCTGCTGGGGTGGAACCTGGT-3′ (F) and 5′-CCA GTGCAGGGTCCGAGG-3′ (R); U6, 5′-CTCGCTTCGG CAGCACA-3′ (F) and 5′-AACGCTTCACGAATTTGCGT -3′ (R); *LMO4*, 5′-GGACCG CTTTCTGCTCTATG-3′ (F) and 5′-AAGCACCGCTATTCCCAAAT-3′ (R).

CircRNA Stability Detection

The stability of circ_0058124 was evaluated using RNase R and actinomycin D. In brief, 2 μg isolated total RNA was incubated with 0.2 μL RNase R (20 U/μL; Epicentre, Madison, WI, USA) at 37°C for 15 min. Then, qRT-PCR was performed as mentioned above to examine the abundance of linear mRNA (GAPDH) and circ_0058124.

IHH-4 and TPC-1 cells were treated with 2 μg/mL actinomycin D (Sigma, St. Louis, MO, USA) for 0, 6, 12, 18, or 24 h. Then, cells at different points were collected to isolate total RNA for further qRT-PCR analysis as mentioned above.

Cell Transfection

Small interference RNA (siRNA) was used to mediate circ_0058124 knockdown (si-circ_0058124), which was synthesized by Genomeditech (Shanghai, China), with siRNA negative control (si-NC) as a control. For miR-370-3p restoration or inhibition, miR-370-3p mimic and inhibitor were assembled by Ribobio (Guangzhou, China), with miRNA NC or inhibitor NC as the corresponding control. For *LMO4* overexpression, fusion plasmid pcDNA3.1-*LMO4* (pc-*LMO4*) was constructed by Sangon Biotech, with an empty pcDNA vector (pc-NC) as a control. These oligonucleotides or plasmids were individually transfected or cotransfected into IHH-4 and TPC-1 cells for functional analyses using Lipofectamine 3000 (Invitrogen).

Cell Counting Kit-8 (CCK-8) Assay

Transfected IHH-4 and TPC-1 cells were collected and plated into a 96-well plate at a density of 2000 cells/well. After 48 h, 10 μL CCK-8 reagent (Sangon Biotech) was pipetted into all wells, maintaining for another 2 h. The absorbance at 450 nm was measured using the iMark microplate reader (Bio-Rad).

Colony Formation Assay

Transfected IHH-4 and TPC-1 cells were placed into a 6-well plate (200 cells/well). Afterwards, cells in the 6-well plate were cultured at 37°C conditions containing 5% CO_2. The status of colony formation was observed every three days. After 12 days, colonies were fixed with paraformaldehyde (Beyotime, Shanghai, China) and stained with 0.1% crystal violet (Beyotime), followed by photograph and count.

Transwell Assay

To monitor cell invasion, transwell chambers (BD Bioscience, San Jose, CA, USA) needed to be pre-coated with Matrigel (BD Bioscience). Transfected IHH-4 and TPC-1 cells were collected, resuspended in 100 μL fresh RPMI1640 (1×10^5 cells) and then placed into the top of chambers pre-coated with or without Matrigel. Meanwhile, fresh RPMI1640 containing 10% FBS was added into the bottom of chambers to induce cell migration and invasion. After 24 h, cells migrated or invaded into the lower surface were treated with paraformaldehyde and 0.1% crystal violet, followed by observation by a microscope (100×; Olympus, Tokyo, Japan).

Wound Healing Assay

Transfected cells were seeded into a 6-well plate (5×10^5 cells/well). The confluent monolayers were scratched using a 1 mL pipette tip and rinsed using PBS to remove detached cells. After 24 h, cell migrated to the wound area was monitored using an optical microscope (Olympus). Quantification of monolayer closure was distinguished using the Image J software (National Institutes of Health, Bethesda, MA, USA), and the results were shown as a percentage of wound closure relative to the control monolayer.

Flow Cytometry Assay

Cell apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (Beyotime). In brief, transfected cells were collected, trypsinized and washed with pre-cooled PBS. Then, a total of 5×10^4 cells were resuspended in 195 μL Annexin V-FITC binding buffer. Subsequently, the reaction system was orderly probed with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) at room temperature for 15 min avoiding light. Finally, the apoptotic cells were analyzed by a flow cytometer (BD Biosciences).

Target Prediction

The target miRNAs of circ_0058124 were predicted by one bioinformatics tool Circular RNA Interactome (https://circinteractome.nia.nih.gov/). The target mRNAs of miR-370-3p

were predicted by another bioinformatics tool starbase (http://starbase.sysu.edu.cn/).

Dual-Luciferase Reporter Assay

Sequence fragment of mutations at miR-370-3p binding sites for circ_0058124 and *LMO4* 3′UTR were designed based on their predicted wild-type targeted binding sites. Then, these wild-type and mutant sequence fragments of circ_0058124 and *LMO4* were cloned into the PGL4 luciferase reporter plasmid, and the fusion reporter plasmids were named as WT-circ_0058124, MUT-circ_0058124, WT-*LMO4*-3′UTR and MUT-*LMO4*-3′UTR. IHH-4 and TPC-1 cells were cotransfected with miR-370-3p mimic or miRNA NC and above-mentioned fusion plasmid using Lipofectamine 3000 (Invitrogen). After 48 h, luciferase activity was examined using the dual-luciferase assay system (Promega, Madison, WI, USA).

RNA Binding Protein Immunoprecipitation (RIP) Assay

IHH-4 and TPC-1 cells were lysed in RIP lysis buffer using the Magna RIP Kit (Millipore, Bedford, MA, USA). Cell lysates were incubated with magnetic beads conjugated with Argonaute 2 (Ago2; Millipore) antibody or Immunoglobulin G (IgG; control; Millipore) antibody. The complexes were eluted to extract RNA, and qRT-PCR analysis was then implemented to monitor the expression of circ_0058124 and miR-370-3p.

Western Blot

Total proteins extracted using a total protein extraction kit (Sangon Biotech) were quantified using a BCA protein assay kit (Sangon Biotech). Equal amount of protein was separated and transferred into PVDF membrane (Bio-Rad). The membranes containing the isolated proteins were blocked in 5% skim milk, exposed to the primary antibodies targeting LMO4 (ab229226; Abcam, Cambridge, MA, USA) and GAPDH (ab8245; Abcam) at 4°C overnight, and next reacted with the secondary antibody (ab205718; Abcam) for 2 h at room temperature. The indicated protein signals were presented using the enhanced chemiluminescence kit (Sangon Biotech) and quantified using the Image J software.

Xenograft Model

Lentiviral vector-packaged short hairpin RNA (shRNA) targeting circ_0058124 (sh-circ_0058124) and corresponding control (sh-NC) were constructed by Genomeditech. TPC-1 cells (4×10^6 cells) stably infected with sh-circ_0058124 or sh-NC were subcutaneously inoculated into BALB/c nude mice (Female, 6–8 weeks-old) purchased from JunKeBiological Co., Ltd (Nanjing, China). The mice were assigned into two groups in accordance with the infection: sh-circ_0058124 group and sh-NC group (n=5 per group). Tumor volume was calculated once a week according to a formula: 0.5 × length × width^2. Twenty-eight days later, all mice were sacrificed to excise tumor nodes. The removed tissues were used for weighting and expression detection. This study was approved by the Committee Ethics of Animal Center of Tianjin Third Central Hospital and implemented following the Guide for the Care and Use of Laboratory Animals (GB/T 35,892–2018).

Statistical Analysis

All experiments were repeated at least three times. Statistical analysis was conducted using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The comparison of differences between two groups was evaluated by Student's *t*-test, and the comparison of differences among ≥3 groups were performed by analyses of variance with Tukey post-test. The data were eventually shown as mean ± standard deviation. *P*-value less than 0.05 was deemed as statistically significant.

Results

Circ_0058124 Was Significantly Upregulated in PTC Tissues and Cells

The expression pattern of circ_0058124 was examined in PTC clinical tissues and cell lines. The result showed that the expression of circ_0058124 was notably increased in PTC tissues (n=20) compared to normal tissues (n=20) (Figure 1A). Meanwhile, the data presented that the expression of circ_0058124 was also remarkably enhanced in IHH-4 and TPC-1 cells compared with that in Nthy-ori 3–1 cells (Figure 1B). Moreover, the expression of circ_0058124 was not affected by RNase R treatment relative to Mock treatment, while the expression of GAPDH was strikingly weakened by RNase R (Figure 1C and D). Similarly, actinomycin D treatment hardly affected circ_0058124 expression but significantly reduced GAPDH expression relative to Mock treatment (Figure 1E and F). The data hinted that circ_0058124 might regulate PTC development.

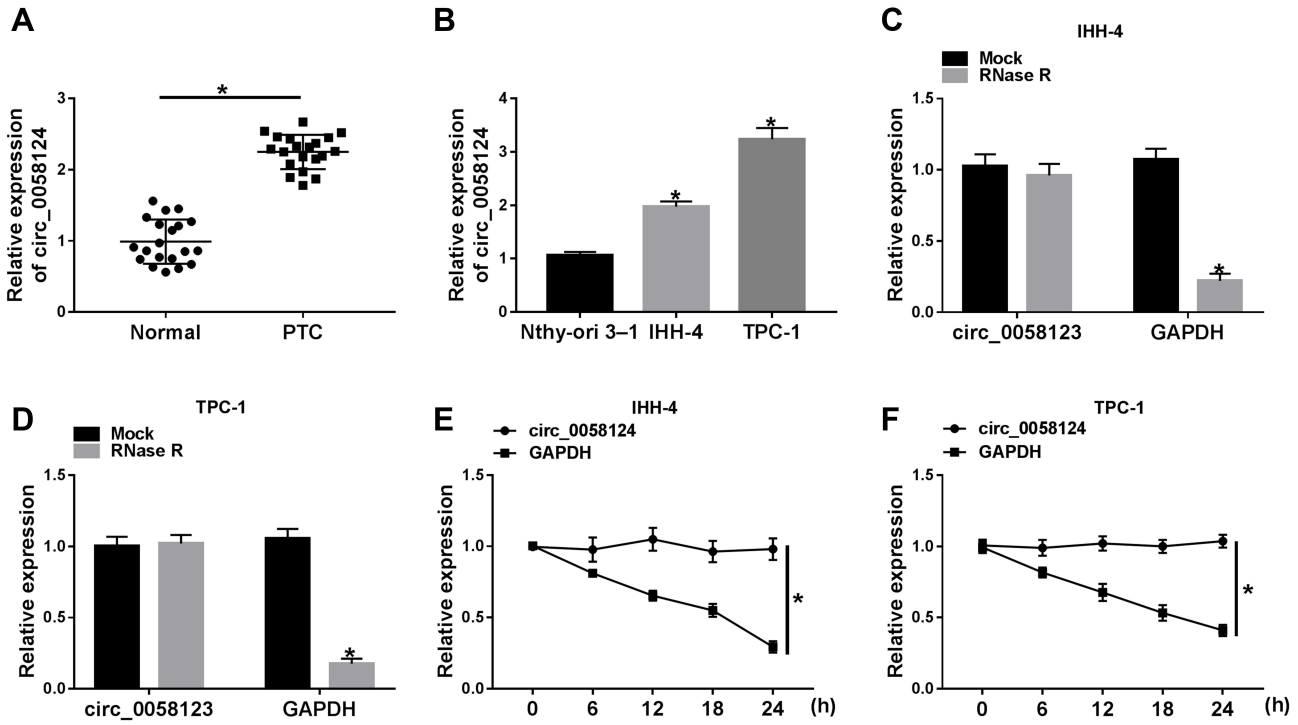

Figure 1 Circ_0058124 was overexpressed in PTC tissues and cells. (**A**) The expression of circ_0058124 in PTC tissues (n=20) and paired normal tissues (n=20) was detected by qRT-PCR. (**B**) The expression of circ_0058124 in PTC cells (IHH-4 and TPC1) and normal cells (Nthy-ori 3–1) was detected by qRT-PCR. (**C** and **D**) The stability of circ_0058124 was tested using RNase R treatment. (**E** and **F**) The stability of circ_0058124 was examined using actinomycin D. *P<0.05.

Circ_0058124 Knockdown Inhibited Cell Viability, Colony Formation, Migration and Invasion and Promoted Apoptosis in IHH-4 and TPC-1 Cells

Considering that the expression of circ_0058124 was increased in PTC cells, siRNA was utilized to mediate circ_0058124 downregulation in IHH-4 and TPC-1 cells to explore circ_0058124 function. The expression from qRT-PCR displayed that the expression of circ_0058124 was significantly declined in IHH-4 and TPC-1 cells transfected with si-circ_0058124 compared with that in si-NC-transfected cells and untransfected cells (Figure 2A). In function, cell viability was notably declined in si-circ _0058124-transfected cells compared with that in si-NC-transfected cells and untransfected cells (Figure 2B). Circ_0058124 downregulation also impaired the number of colonies in IHH-4 and TPC-1 cells (Figure 2C). Moreover, transwell assay presented that circ_0058124 knockdown notably suppressed cell migration and invasion (Figure 2D and E), and the similar effect on migration by circ_0058124 knockdown was also verified by wound healing assay (Figure 2F). As expected, the rate of cell apoptosis was significantly promoted by si-circ_0058124 transfection compared to si-NC or Control (Figure 2G). These functional analyses suggested that circ_0058124 knockdown blocked PTC cell malignant behaviors.

MiR-370-3p Was Identified to Be a Target of circ_0058124

Next, we explored the potential mechanism of circ_0058124 action through the ceRNA mechanism. The online bioinformatics tool Circular RNA Interactome predicted that miR-370-3p was one of the targets of circ_0058124, and there was special binding site between circ_0058124 and miR-370-3p fragment (Figure 3A). In addition, this relationship that circ_0058124 targeted miR-370-3p was further verified by dual-luciferase reporter assay. The results showed that the luciferase activity was strikingly declined in IHH-4 and TPC-1 cells transfected with miR-370-3p mimic and WT-circ _0058124, while the luciferase activity was hardly changed in IHH-4 and TPC-1 cells transfected with miR-370-3p mimic and MUT-circ_0058124 compared to miRNA NC (Figure 3B and C). RIP assay presented that both circ_0058124 and miR-370-3p were abundantly enriched in Ago2 antibody-associated complex but not in the control IgG group, implying that miR-370-3p was a circ_0058124-targeting miRNA (Figure 3D and E). For expression analysis,

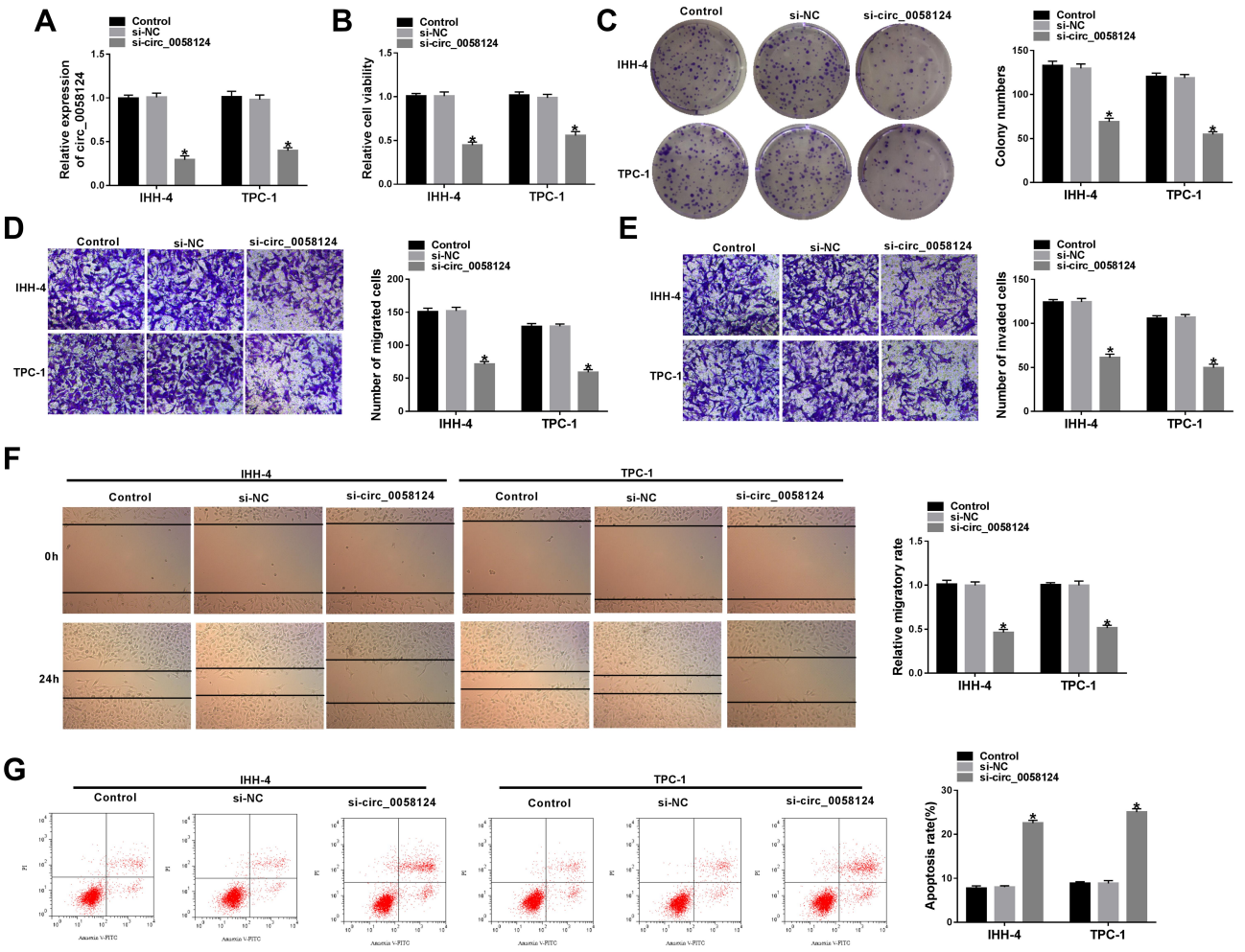

Figure 2 Circ_0058124 knockdown inhibited viability, colony formation, migration and invasion but induced apoptosis of PTC cells. In IHH-4 and TPC-1 cells with siRNA-mediated circ_0058124 knockdown, (**A**) the efficiency of siRNA was checked by qRT-PCR. (**B**) Cell viability was assessed using CCK-8 assay. (**C**) Colony formation assay was performed to observe colony growth. (**D** and **E**) Cell migration and cell invasion were monitored using transwell assay. (**F**) Cell migration was also explored using wound healing assay. (**G**) Cell apoptosis was examined using flow cytometry assay. *$P<0.05$.

we noticed that the expression of miR-370-3p was noticeably decreased in PTC tissues (n=20) compared with that in normal tissues (n=20) (Figure 3F). The expression of miR-370-3p was also declined in IHH-4 and TPC-1 cells compared with that in Nthy-ori 3–1 cells (Figure 3G). The above evidence verified that miR-370-3p was a target of circ_0058124.

MiR-370-3p Deficiency Reversed the Inhibitory PTC Cell Malignant Behaviors Caused by circ_0058124 Knockdown

We subsequently addressed whether circ_0058124 played functions by targeting miR-370-3p in PTC cells. The data from qRT-PCR showed that miR-370-3p inhibitor significantly lessened the expression of circ_0058124 in IHH-4 and TPC-1 cells (Figure 4A). Then, IHH-4 and TPC-1 cells were transfected with si-circ_0058124 or si-circ_0058124 +miR-370-3p inhibitor, with si-NC or si-circ_0058124 +inhibitor NC as the control. The expression of miR-370-3p promoted in si-circ_0058124-transfected cells was largely impaired in si-circ_0058124+miR-370-3p inhibitor-transfected cells (Figure 4B). In function, combined circ_0058124 knockdown with miR-370-3p deficiency strikingly strengthened IHH-4 and TPC-1 cell viability and colony formation that were suppressed by circ_0058124 knockdown alone (Figure 4C and D). Besides, cell migration and invasion blocked by si-circ_0058124 transfection alone were substantially recovered by si-circ_0058124+miR-370-3p inhibitor transfection in IHH-4 and TPC-1 cells (Figure 4E–G). Circ_0058124 knockdown-induced cell apoptosis was largely repressed

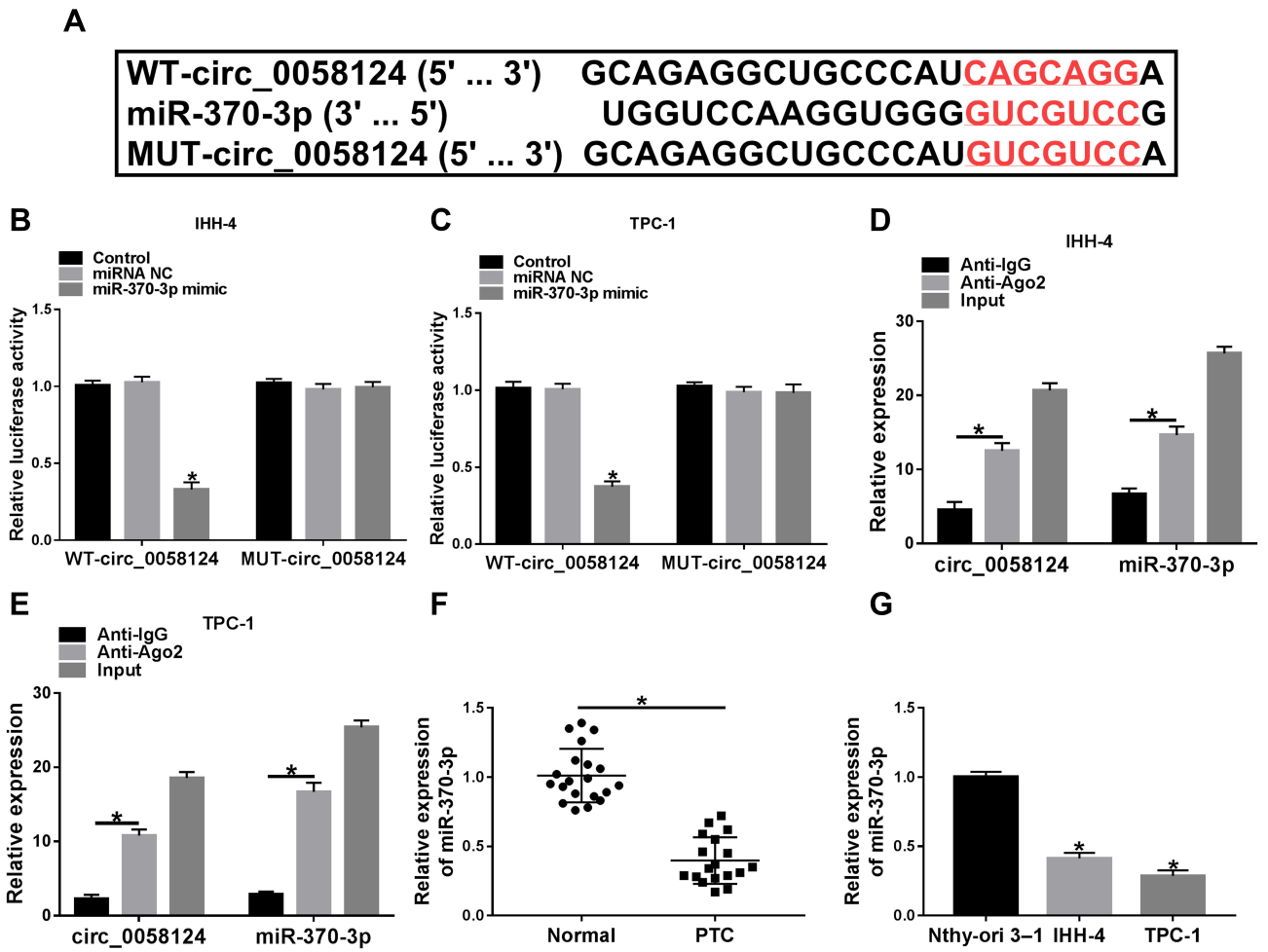

Figure 3 MiR-370-3p was a target of circ_0058124. (**A**) The target relationship between circ_0058124 and miR-370-3p was predicted by the bioinformatics tool Circular RNA Interactome, and then a new sequence fragment of circ_0058124 mutated at miR-370-3p binding site was designed for the following analysis. (**B** and **C**) The target relationship between circ_0058124 and miR-370-3p was validated by dual-luciferase reporter assay. (**D** and **E**) The target relationship between circ_0058124 and miR-370-3p was also validated by RIP assay. (**F**) The expression of miR-370-3p in PTC tissues (n=20) and paired normal tissues (n=20) was detected by qRT-PCR. (**G**) The expression of miR-370-3p in Nthy-ori 3–1, IHH-4 and TPC-1 cells was detected by qRT-PCR. *P<0.05.

in IHH-4 and TPC-1 cells transfected with si-circ_0058124 +miR-370-3p inhibitor (Figure 4H). The data highlighted that circ_0058124 knockdown suppressed PTC cell malignant activities by increasing miR-370-3p expression.

LMO4 Was a miR-370-3p-Targeted Gene, and circ_0058124 Regulated LMO4 Expression by Targeting miR-370-3p

Furthermore, we identified the potential mRNAs that were targeted by miR-370-3p. Bioinformatics tool starbase displayed that miR-370-3p bound to *LMO4* 3′UTR through a special binding site (Figure 5A). Dual-luciferase reporter assay showed that the luciferase activity was notably weakened in IHH-4 and TPC-1 cells transfected with miR-370-3p mimic and WT-*LMO4*-3′UTR but not MUT-*LMO4* -3′UTR (Figure 5B and C). The expression of *LMO4* was significantly elevated in PTC tissues (n=20) compared with that in normal tissues (n=20) (Figure 5D), and the expression of *LMO4* was also elevated in IHH-4 and TPC-1 cells compared with that in Nthy-ori 3–1 cells (Figure 5E). IHH-4 and TPC-1 cells transfected with pc-*LMO4* exhibited a higher expression level of *LMO4* (Figure 5F). In addition, the expression of *LMO4* was suppressed in IHH-4 and TPC-1 cells transfected with miR-370-3p mimic but partly recovered in cells transfected with miR-370-3p mimic+pc-*LMO4* (Figure 5G), suggesting that miR-370-3p bound to *LMO4* and suppressed *LMO4* expression. Additionally, the expression of *LMO4* was declined in IHH-4 and TPC-1 cells transfected with si-circ_0058124 but restored in cells transfected with si-circ_0058124+miR-370-3p inhibitor

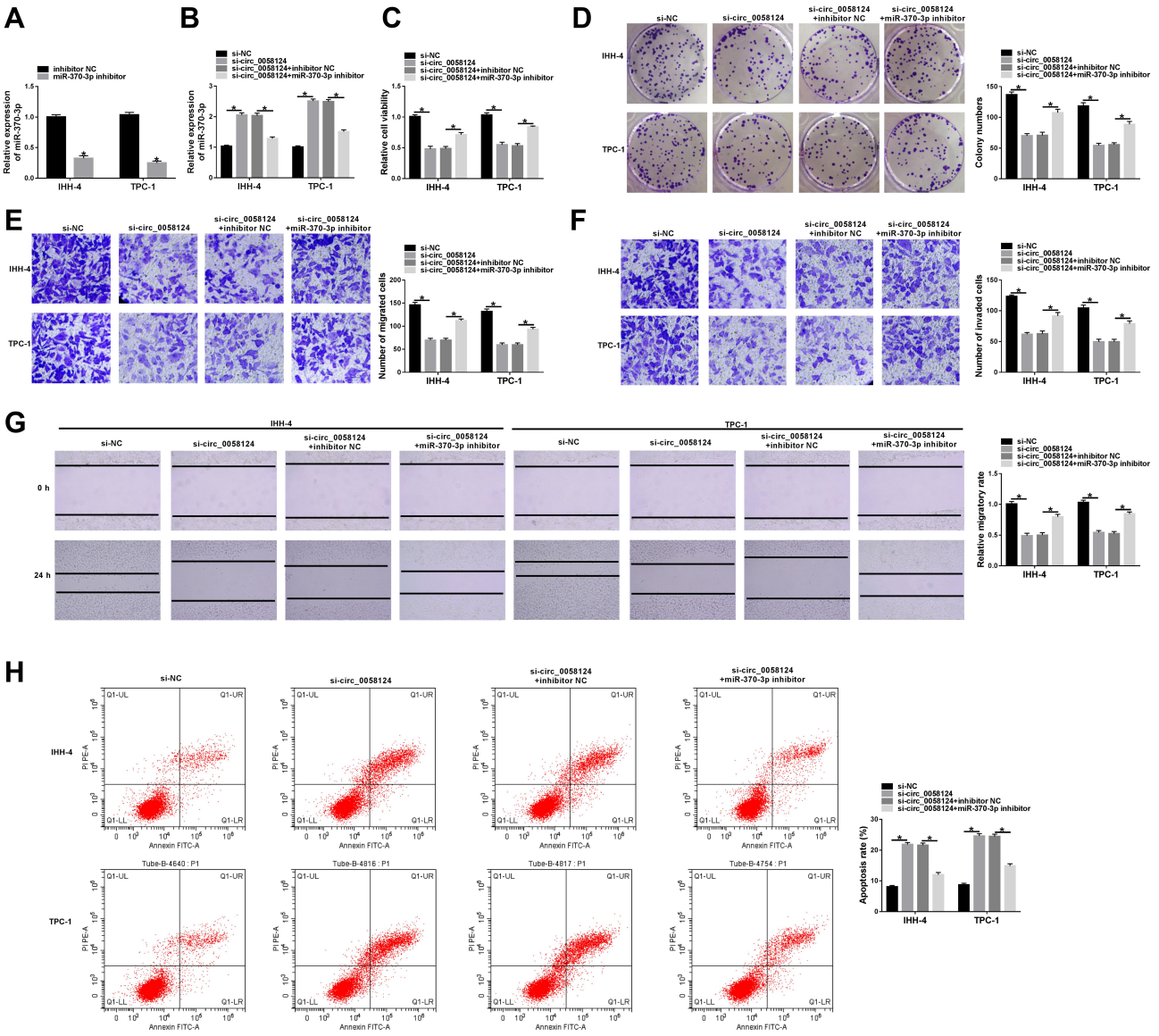

Figure 4 MiR-370-3p inhibition reversed the effects of circ_0058124 knockdown. (**A**) The efficiency of miR-370-3p inhibitor was tested using qRT-PCR. IHH-4 and TPC-1 cells were transfected with si-circ_0058124, si-NC, si-circ_0058124+miR-370-3p inhibitor or si-circ_0058124+inhibitor NC. (**B**) The expression of miR-370-3p in these transfected cells was detected by qRT-PCR. (**C**) Cell viability in these indicated cells was examined using CCK-8 assay. (**D**) Colony formation ability was assessed using colony formation assay. (**E** and **F**) Cell migration and invasion is these indicated cells was evaluated by transwell assay. (**G**) Cell migration was also determined using wound healing assay. (**H**) Cell apoptosis was monitored by flow cytometry assay. *P<0.05.

(Figure 5H), suggesting that circ_0058124 positively regulated *LMO4* expression by targeting miR-370-3p.

LMO4 Overexpression Abolished the Inhibitory PTC Cell Malignant Behaviors Caused by miR-370-3p Restoration

To ascertain the hypothesis that miR-370-3p functioned in PTC by degrading *LMO4*, IHH-4 and TPC-1 cells were challenged with miR-370-3p mimic transfection or miR-370-3p mimic+pc-*LMO4* transfection, with miRNA NC or miR-370-3p mimic+pc-NC as the control. In function, cell viability and colony formation were significantly weakened in IHH-4 and TPC-1 cells transfected with miR-370-3p mimic but largely rescued in cells transfected with miR-370-3p mimic+pc-*LMO4* (Figure 6A and B). The capacities of cell migration and cell invasion were also markedly impaired in cells transfected with miR-370-3p mimic but partly recovered in cells transfected with miR-370-3p mimic+pc-LMO4 (Figure 6C–E). MiR-370-3p restoration pronouncedly induced cell apoptosis, which was inhibited by the reintroduction of LMO4 (Figure 6F). These data

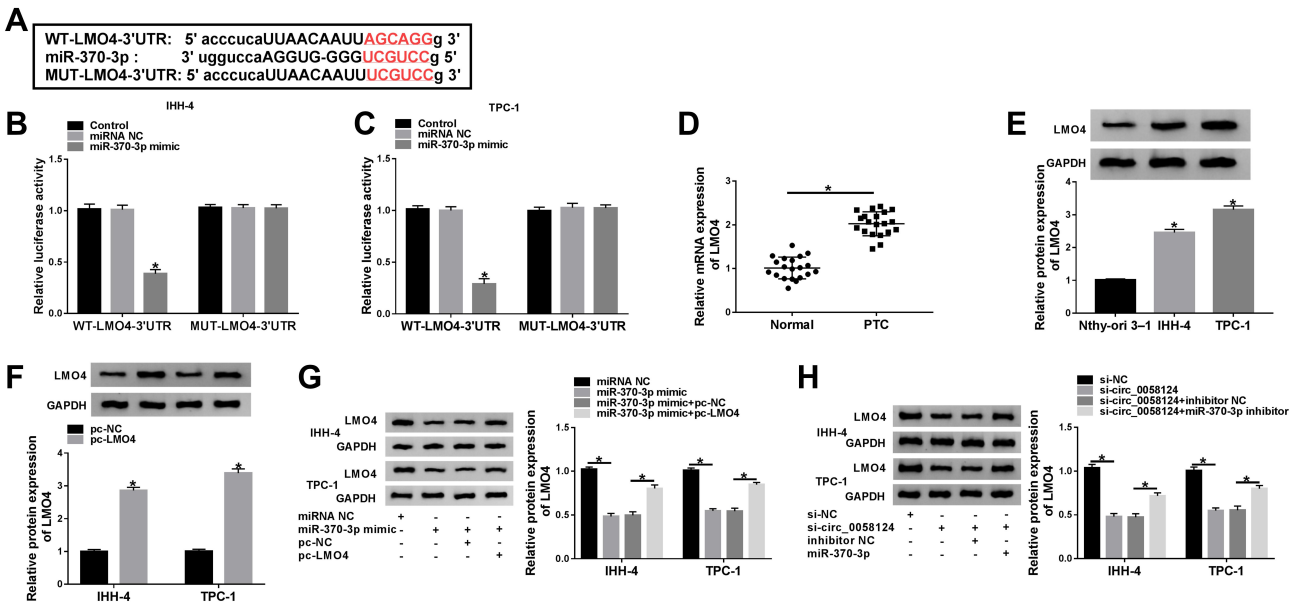

Figure 5 MiR-370-3p bound to *LMO4* 3′UTR and suppressed *LMO4* expression. (**A**) *LMO4* was a putative target of miR-370-3p, which was predicted by the online tool starbase. (**B** and **C**) The association between miR-370-3p and *LMO4* was verified by dual-luciferase reporter assay. (**D**) The expression of *LMO4* in PTC tissues (n=20) and normal tissues (n=20) was detected by qRT-PCR. (**E**) The expression of LMO4 in Nthy-ori 3–1, IHH-4 and TPC-1 cells was measured by Western blot. (**F**) The efficiency of LMO4 overexpression was detected by Western blot. (**G**) The expression of LMO4 in IHH-4 and TPC-1 cells transfected with miR-370-3p mimic, miRNA NC, miR-370-3p mimic+pc-LMO4 or miR-370-3p mimic+pc-NC was detected by Western blot. (**H**) The expression of LMO4 in IHH-4 and TPC-1 cells transfected with si-circ_0058124, si-NC, si-circ_0058124+miR-370-3p inhibitor or si-circ_0058124+inhibitor NC was quantified by Western blot. *P<0.05.

confirmed that miR-370-3p inhibited PTC cell aggressive behaviors by suppressing *LMO4*.

Circ_0058124 Knockdown Inhibited Tumor Growth by Regulating miR-370-3p and LMO4 in Mice

The function of circ_0058124 was additionally investigated in vivo. As a result, circ_0058124 stable knockdown significantly suppressed tumor growth, including tumor volume and tumor weight (Figure 7A and B). In the removed tumor node samples, the expression of circ_0058124 and LMO4 was notably decreased in the sh-circ_0058124 group, while the expression of miR-370-3p was notably promoted in the sh-circ_0058124 group compared with that in the sh-NC group (Figure 7C–E). The data suggested that stable knockdown of circ_0058124 effectively inhibited tumor in vivo growth by downregulating LMO4 via releasing miR-370-3p.

Discussion

Recently, circRNA-associated research has been carried out in a vigorous and orderly manner, and the functions of numerous novel circRNAs have been steadily identified in cancer progression. Besides, the characteristics of circRNAs, such as stability, abundant expression and wide distribution, make them more promising biomarkers for cancer detection and prognosis.[20] In PTC, circRNA-ITCH reintroduction blocked PTC cell proliferation and restrained tumor growth in vivo.[21] CircRNA-NUP214 downregulation inhibited PTC cell proliferation, migration/invasion and tumorigenesis in vivo.[22] CircRNA-RAPGEF5 knockdown suppressed the aggressive biological behaviors of PTC, such as proliferation and metastasis.[23]

In our study, we focused on circ_0058124, which was abundantly expressed in PTC tissues and cells, hinting that circ_0058124 might be involved in PTC development. Further analysis disclosed that circ_0058124 knockdown blocked PTC cell viability, colony formation, migration and invasion but accelerated apoptosis. Circ_0058124 was a significantly upregulated circRNA in PTC tissues by microarray analysis in a previous study, and high expression of circ_0058124 was closely related with poor prognosis of PTC.[12] Recently, circ_0058124 was also documented to deteriorate PTC progression by triggering the abilities of proliferation, metastasis and metabolism.[24] Besides, circ_0058124 expression was also elevated in lung cancer tissues and cells, and silencing circ_0058124 weakened malignant behaviors of lung cancer cells.[25] Sufficient evidence emphasized the carcinogenic effects of circ_0058124 in various cancers.

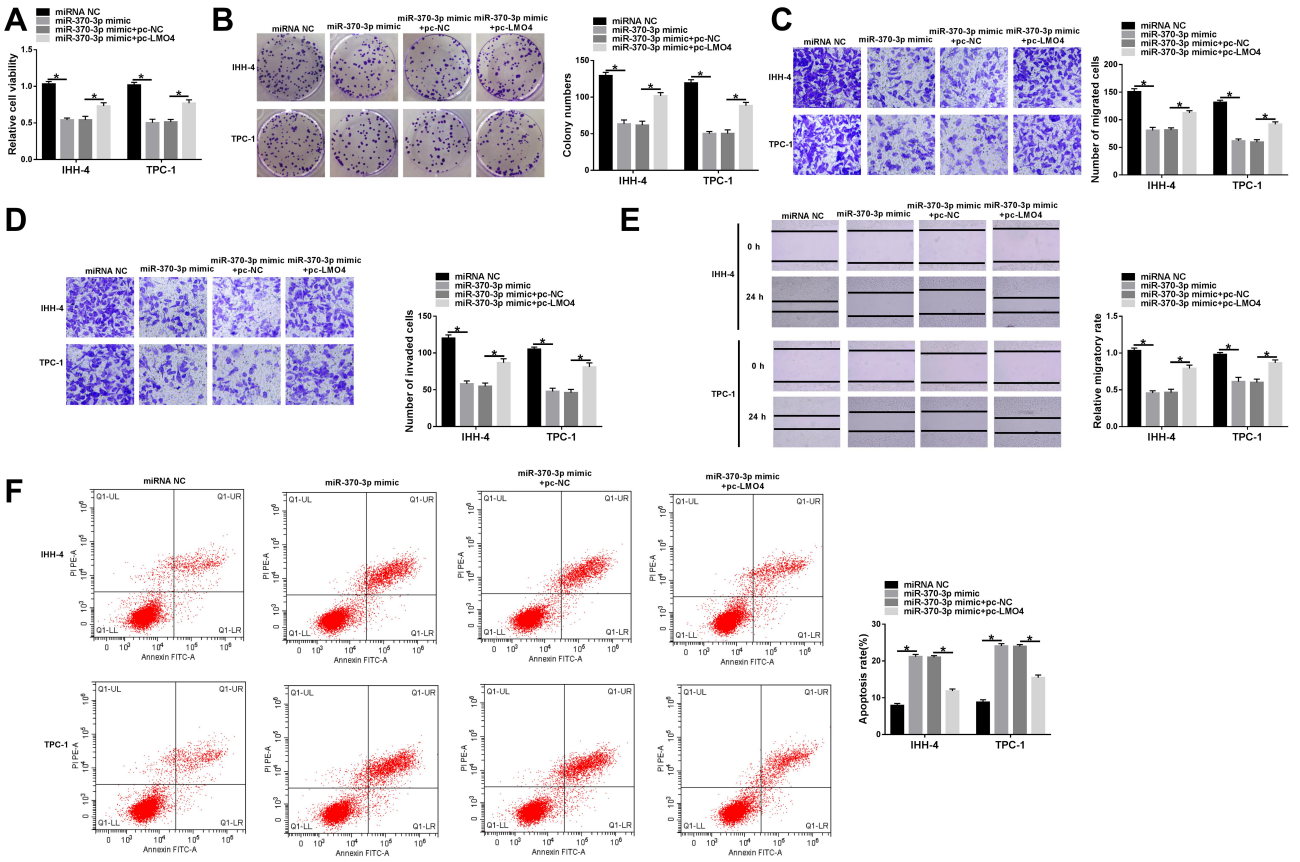

Figure 6 *LMO4* overexpression reversed the effects of miR-370-3p restoration. IHH-4 and TPC-1 cells were transfected with miR-370-3p mimic, miRNA NC, miR-370-3p mimic+pc-*LMO4* or miR-370-3p mimic+pc-NC. (**A**) Cell viability in these transfected cells was assessed by CCK-8 assay. (**B**) Colony formation ability in these cells was monitored by colony formation assay. (**C** and **D**) Cell migration and invasion in these indicated cells were checked by transwell assay. (**E**) Cell migration was also assessed using wound healing assay. (**F**) Cell apoptosis in these transfected cells was monitored by flow cytometry assay. **P*<0.05.

For mechanism exploration, we, for the first time, found that circ_0058124 could function as a molecular sponge to deplete the expression and function of miR-370-3p. MiR-370-3p was a well-recognized tumor suppressor in different types of cancers, including colorectal cancer, pituitary adenomas and glioma.[26–28] Likewise, the abundance of miR-370-3p was notably lower in thyroid cancer tissues relative to normal tissues, and miR-370-3p overexpression impaired viability and invasion of thyroid cancer cells.[15] Our study viewed that miR-370-3p expression was also declined in PTC tissues compared to normal tissues. The enrichment of miR-370-3p restricted PTC cell viability, colony formation, migration and invasion. Rescue experiments showed that miR-370-3p inhibition overturned the inhibitory effects of circ_0058124 deficiency on malignant PTC cell behaviors, indicating that circ_0058124 knockdown suppressed PTC cell malignant activities by increasing miR-370-3p expression.

Further, our data innovatively demonstrated that *LMO4* was a target of miR-370-3p. *LMO4* functioned as an oncogene in diverse cancers, and high expression of *LMO4* was linked to poor prognosis and low survival.[29,30] In mechanism, *LMO4* interacted with proteins involved, among else, in tumorigenesis, such as P53.[31] Besides, a previous study demonstrated that *LMO4* was targeted by miR-139-5p, and circRNA-BACH2 facilitated PTC deterioration by activating the expression of *LMO4* by targeting miR-139-5p.[19] Similarly, we discovered that the expression of *LMO4* was downregulated by circ_0058124 knockdown, while miR-370-3p inhibition recovered *LMO4* expression. Besides, *LMO4* reintroduction abrogated the inhibitory effects of miR-370-3p restoration on PTC cell viability, migration and invasion, confirming that miR-370-3p inhibited PTC cell aggressive behaviors by suppressing *LMO4*. These data hinted that circ_0058124 knockdown blocked PTC development by partly targeting the miR-370-3p/*LMO4* pathway, which was a new mechanism for the involvement of circ_0058124 in PTC progression.

Collectively, we identified that circ_0058124 expression was remarkably elevated in PTC tissues and cells. In function, circ_0058124 downregulation blocked PTC cell

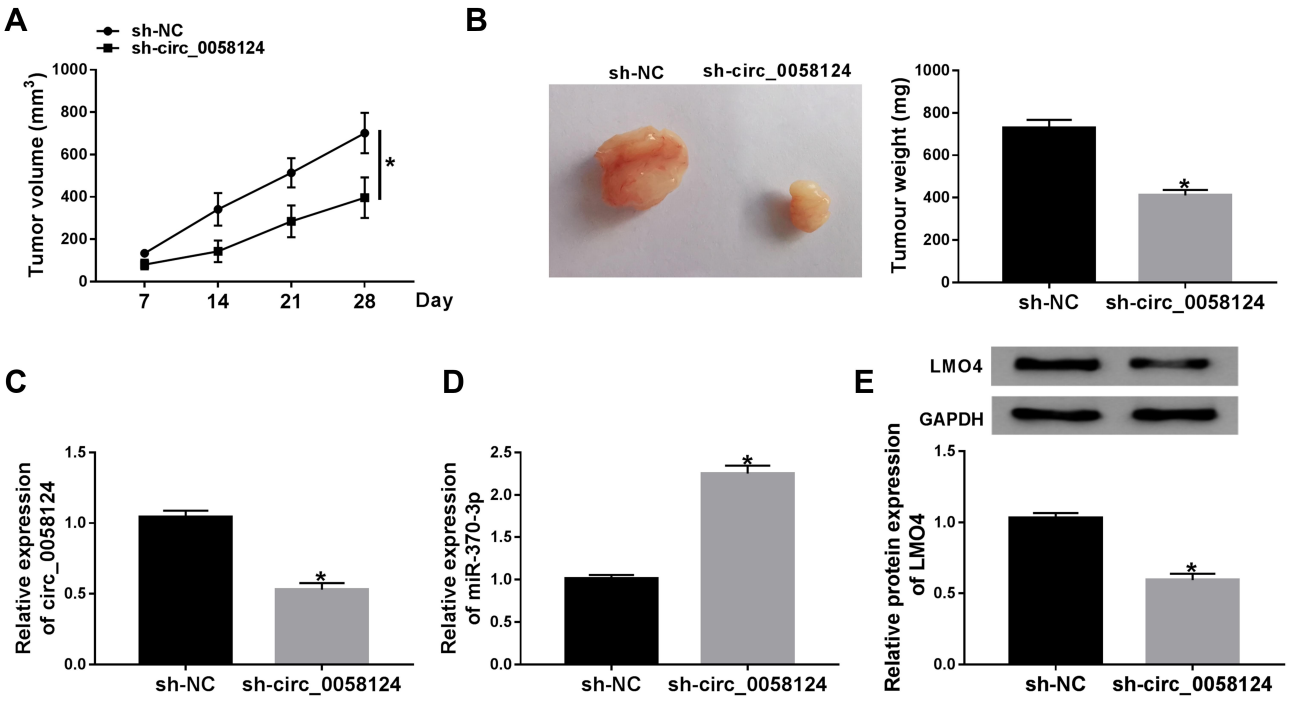

Figure 7 Circ_0058124 knockdown blocked tumor development in vivo. TPC-1 cells transfected with sh-circ_0058124 or sh-NC were inoculated into nude mice. (**A**) Tumor volume was recorded once a week after inoculation. (**B**) Tumor weight was measured in each group after 28 days when tumor tissues were excised. (**C** and **D**) The expression of circ_0058124 and miR-370-3p in the removed tissues was detected by qRT-PCR. (**E**) The expression of LMO4 at the protein level was detected by Western blot. *P<0.05.

viability, colony formation, invasion and migration in vitro, also inhibited tumor growth in vivo. In mechanism, we put forward that circ_0058124 promoted PTC development by upregulating *LMO4* via sponging miR-370-3p. Forceful evidence highlighted that circ_0058124 might be a vital regulator in PTC pathogenesis.

Abbreviations

PTC, papillary thyroid carcinoma; LMO4, LIM domain only; CCK-8, cell counting kit-8; FN1, fibronectin 1; pc-NC, pcDNA vector.

Ethics Approval and Consent Participate

Written informed consent was obtained from patients with approval by the Institutional Review Board in Tianjin Third Central Hospital.

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Disclosure of Interest

The authors declare that they have no financial or non-financial conflicts of interest for this work.

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