

miR-27a-3p attenuates induce autophagy-related cell death by suppressing Inhibitor of growth family 5 in cholangiocarcinoma

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Research

Keywords: microRNAs, autophagy, cholangiocarcinoma, Inhibitor of growth family

Posted Date: May 13th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-27745/v1>

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Abstract

Background MicroRNAs (miRNAs) are abnormally expressed in human tumors, including cholangiocarcinoma (CCA). miR-27a-3p was observed up-regulated in CCA, but its functions in CCA are largely unknown.

Methods CCK8 assay, Colony formation assays and Ki-67 staining was employed to detect the cell growth. The autophagy and proliferation relative-protein analyzed by western blot. The immunofluorescence staining was applied to analyze the expression level of LC3 I/II. Tumor xenografts was used to test the role of miR-27a-3p. Luciferase reporter assay, western bolt and qRT-PCR showed the relationship between miR-27a-3p and ING5.

Results miR-27a-3p expression was increased in human CCA tissues. Inhibition of miR-27a-3p suppressed the proliferative capacity of CCA cells, silencing of miR-27a-3p dramatically induced cell death and suppressed tumor growth in *vivo*. The proteins, such as Beclin-1, p62, p21, p-p53, CDK4 and CDK6, were decreased upon miR-27a-3p inhibitor transfection. Western blot assay and immunofluorescence analysis were showed the induced-autophagy after transfecting with miR-27a-3p or inhibitor of growth family 5 (ING5) in RBE. ING5 as a direct miR-27a-3p target in CCA. Co-transfect of miR-27a-3p and ING5 can reverse CCA cell death which induced by miR-27a-3p inhibitor alone.

Conclusions miR-27a-3p promotes oncogenesis of CCA by triggering autophagy-related cell death by interacting with ING5 directly.

Introduction

Cholangiocarcinoma (CCA) is a serious cancer disease, affecting human health[1].This disease is a lethal form of cancer that develops as a result of the neoplastic transformation of biliary epithelial cells (BEC). Cholangiocarcinoma is associated with poor prognosis and its incidence rate has continued to increase globally [2]. Because of its high malignancy and low 5-year survival rate (10%), it has become an important factor that seriously affects the quality of life[3]. At present, the main treatment methods for CCA are surgery, chemotherapy and radiotherapy, but the 5-year survival rate is still very low[4]. For the reason above, a new biomarker and therapeutic method for CCA is largely needed.

MicroRNAs (miRNAs) are a widely regulated human disease that exists in a wide range of diseases, such as cardiovascular disease, liver cancer, hypertension, brain diseases, and so on. However, the study of miRNA in cholangiocarcinoma is still largely unknown. A previous study implicated miR-27a-3p in the development of cardiovascular, kidney, and other diseases. Also, a study suggested that miR-27a could promote ineffective lysosomal clearance in the hippocampi of rats after chronic brain hypoperfusion. However, there are few relevant reports on cholangiocarcinoma.

Previous studies have showed that autophagy is a genera role in many diseases, such as ischemic myocardial injury[5], osteoarthritic [6] and CCA [7]. Lv P et al. showed miR-373 inhibits autophagy and

promotes apoptosis of CCA cells by ULK1[8]. Ma et al suggest that miR-124 induces autophagy-related cell death in CCA cells by direct targeting EZH2 [9]. However, the role of autophagy in the progression of miR-27a-3p in CCA is not fully understood, and the down- or up- stream factors that control autophagy require to better understood.

Inhibitor of growth family 5 (ING5) is a member of the ING protein family, ING5 regulates many diseases such as cardiovascular disease, especially cancers. ING5 contains a motif known as PHD-finger; the motif is commonly found in proteins that are involved in chromatin remodeling [10].The ING5 protein binds to p53 and is part of the p53-dependent regulatory pathway[11]. Also, downregulation or loss of ING5 expression is observed frequently in various types of cancer. Cui et al showed that ING5 is a tumor suppresser gene that functions in the progression of breast cancer mediated by miR-24 [12]. Zhao QY et al demonstrated that ING5 inhibits EMT in breast cancer by suppressing PI3K/Akt pathway [13] .Despite this, the role of ING5 and miR-27a-3p in CC remain unclear.

Herein, we show that miR-27a-3p had an oncogenic function in cholangiocarcinoma. ING5 has been proved to have an anti-cancer effect in multiple carcinomas. We demonstrate that miR-27a-3p direct targets ING5 expression in CCA. Through suppressing ING5, miR-27a-3p enhances autophagy signaling pathway to facilitate the progression of CCA. Our newly revealed mechanism could offer a novel potential treatment target for CCA.

Materials And Methods

Patient information

The Clinical Research Ethics Committee of Harbin Medical University approved the current study and animal experiments were performed as per the Guide for the Care and Use of Laboratory Animals. A total of 30 samples and 31 CCA specimens from patients admitted at the Second Affiliated Hospital of Harbin Medical University (Harbin, China). Specimens were collected from 2013 to 2018. Histopathological analysis was conducted on the specimens to confirm the diagnosis. Each patient provided informed consent. Patients who had undergone cancer treatment before were not allowed in the present study.

Cell culture and Reagents

Rabbit monoclonal anti-p53, p21, Beclin-1, p62, CDK4, CDK6, and anti-GAPDH-actin were procured from Abcam (Cambridge, UK). The HIBEC, REB, HCCC-9810, and SSP25 were procured from the Chinese Academy of Sciences (Shanghai, China). Cells were grown (conditions 37 °C, 5% CO₂) in RPMI-1640/DMEM augmented with FBS (10%) and streptomycin/penicillin (50 mg/mL/100 U/mL, respectively). Less than 6-month old cultures were used to conduct the experiments.

Cell viability assay

The cholecystokinin-8 (CCK8) kit (Dojindo, Kumamoto, Japan) was applied to examine the viability of the cells. The cells (8×10^3) were seeded onto 96-well plates 24 hours before transfection was performed.

Next, we incubated the cells with miR-27a-3p mimic/inhibitor for 48 hours using Lipofectamine™ 2000 as per the methods described by the manufacturer. These experiments were repeated thrice.

Colony formation assays

CCA cells were transfected with miR-27a-3p inhibitor or NC group. 48 hours after transfection, 800 cells were counted and seeded into 3.5-cm dishes. After 14 days, colonies were stained with 0.1% crystal violet in 20% methanol for 15 minutes. The dishes were photographed and the numbers of visible colonies were counted.

Western blotting analysis

After protein extraction, the proteins were resolved in 10% SDS-PAGE. Following blocking, the membranes were incubated together with p-p53, p-21 and GAPDH (1:1000 dilution), Beclin-1, CDK4, CDK6 and p62 (1:500 dilution) antibodies incubated overnight at 4 °C. The bands from the assay were imaged and their intensities were evaluated by Odyssey 3.0 software. β -actin was used as the reference protein.

Extraction of total RNA and quantitative real-time PCR analysis

RNAiso Plus reagent (Takara Biotechnology, Dalian, China) was applied in the extraction of total RNA from the cells. After the extraction, reverse transcription was performed using a reverse transcription kit (Takara). Subsequently, RT-PCR was conducted using the primers:

ING5: 5'-AAACGAACCCACGTACTGC-3' (sense) and 5'-TTGCGACACGAATGAAGG-3' (antisense);

GAPDH: 5'-CATTGCCGACAGGATGCA-3' (sense) and

5'-CATCTGCTGGAAGGTGGACAG-3' (antisense). The thermocycler conditions were: initial denaturation for 10 min at 95 °C; then 40 cycles of denaturation for 30 s at 95 °C, annealing for 15 s at 55 °C, and extension for 20 s at 72 °C; a final extension was run for 15 s at 65 °C. The $2^{-\Delta\Delta CT}$ method was employed to evaluate expression levels. And GAPDH was used as internal control.

Tumor xenografts in mice

The athymic BALB/c nude mice used in the present study were procured from the SLRC Laboratory-Animals (Shanghai, China). And the lentiviral constructs of NC and miR-27a-3p inhibitors were designed and constructed by the Biowit Technology (Shenzhen, China). Seven days after cancer xenografts, each mouse was infected with vectors. Briefly, 10^6 REB cells were intraperitoneally injected into four-week-old female mice. Subsequently, the mice were raised in aseptic conditions for 28 days after which they were sacrificed. For the purpose of immunohistochemical (ki67) staining, the xenograft cancer tissues were extracted from the mice and weighed then fixed using formalin. After fixation, the tissues were embedded in paraffin. The residual cancer tissues were preserved at - 80 °C for further analysis of the protein and RNA levels.

Immunofluorescence staining and Immunohistochemistry

In this procedure, the cells were first put on coverslips then fixed for 20 minutes with 4% paraformaldehyde. Next, the cells were blocked for 1 hour in BSA (10%) then maintained at 4 °C overnight at together with the ki67 antibody. After that, the cells were maintained at 37 °C for 1 hour together with the secondary antibody. The cells were assessed by confocal microscopy to determine autophagy. A total of 10 visual fields were selected randomly. Image-Pro Plus 6.0. was used to measure the optical density.

Statistical analysis

Each experiment was repeated three to six times and presented as the mean \pm standard deviation. Unpaired Student's t-test was used for the parametric data analysis. Values with $P < 0.05$ were considered statistically significant.

Results

The role of miR-27a-3p in CCA

To examine the primary role of miR-27a-3p in CCA development, we assessed the miR-27a-3p level in CCA patients by TCGA. The miR-27a-3p level was remarkably overexpressed in CCA tissues relative to adjacent normal tissues (Fig. 1A), and this was correlated with low survival (Fig. 1B). To examine the function of miR-27a-3p in CCA cells, we assessed its level of expression in REB, SSP25 and REB cells which all belong to CCA. It was found the miR-27a-3p was remarkably higher in all CCA cell lines than in the HIBEC (a normal human intrahepatic biliary epithelial cell) (Fig. 1C). For our result of Fig. 1C, we found there is no different among HCCC-9810, SSP25 and REB cells, for further study we use REB cells only.

miR-27a-3p knockdown reduces CCA cells growth

The effect of miR-27a-3p on CCA tumorigenicity was assessed by treating REB cells with NC/miR-27a-3p inhibitors. The colony formation and CCK-8 revealed that knockdown of miR-27a-3p inhibited REB cell growth (Fig. 2A-C). Furthermore, immunofluorescence staining showed a significantly lower Ki-67 signal in the miR-27a-3p knockdown group relative to the NC group (Fig. 2D). Consistently, the levels of CDK4 and CDK6 also decreased whereas p-p53 and p21 levels were increased due to miR-27a-3p knockdown than those in the NC group (Fig. 2E, F).

To assess the miR-27a-3p effect on CCA in vivo, we constructed a nude mouse xenograft cancer model by subcutaneous inoculation of REB transfected with NC/ miR-27a-3p inhibitors. As expected, the size and the weight of the xenograft tumor in the miR-27a-3p knockdown group were considerably smaller relative to that of the NC group (Fig. 2G-I). In addition, a much lower Ki-67 level in xenograft specimens was observed in the miR-27a-3p inhibitor transfected REB group relative to the NC group (Fig. 2G).

The findings presented here implied that depletion of miR-27a-3p reduces CCA cell proliferation capability.

miR-27a-3p knockdown enhances autophagy induction in CCA cells

Autophagy, an intracellular catabolic process, provide energy and macromolecular precursors for cell growth in many diseases[14]. Herein, transmission electron microscope (TEM) was used to test the autophagy corpuscle in REB cells after treat with miR-27 inhibitor. Our data showed silence of miR-27a-3p induce autophagy in REB cells (Fig. 3A). Then, we test the protein level of beclin-1 and p62 after transfect with miR-27a-3p inhibitor. Our results showed miR-27a-3p knockdown remarkably induced Beclin-1 level and accumulated p62 level in REB cells compared to NC groups indicating that miR-27a-3p mediated autophagy in CCA cells (Fig. 3B). Further, it was observed that miR-27a-3p depletion promoted the conversion of LC3-II/LC3-I (Fig. 3C). To strength our data, the autophagic flux was examined by the tandem-tagged LC3 construct mRFP-GFP-LC3. REB cells with miR-27a-3p silencing tended to be red. However, inhibitor-NC transfected cells predominantly in yellow. These observations demonstrated that miR-27a-3p knockdown promotes autophagy induction in CCA cells.

ING5 directly target miR-27a-3p

miRNA is involved in the pathophysiological process in many diseases by negative regulation of target genes[15]. To found the potential target gene of miR-27a-3p in CCA, we used two independent algorithms, TargetScan (www.targetscan.org/), and mirbase (www.mirbase.org/), and found that ING5 could be a potential gene of miR-27a-3p target (Fig. 4A). We next determined the expression protein and mRNA levels of ING5 after transfection with miR-27a-3p mimics. Results indicated that mRNA level of ING5 was lower than the NC group after miR-27a-3p-mimic transfection, and western blot assay indicated a similar pattern of ING5 protein level. MiR-27a-3p inhibitor remarkably up-regulated ING5 in mRNA as well as protein levels (Fig. 4B-C) [$P < 0.05$]. Based on the above results, we think ING5 maybe a possible miR-27a-3p target.

Luciferase reporter assay was conducted to determine if miR-27a-3p directly targets ING5. The miR-27a-3p mimics suppressed luciferase activity in the ING5-WT group rather than the ING5-Mut group (Fig. 4D). Hence, we believed that miR-27a-3p direct targeted ING5 in CCA.

Over-expression of ING5 enhances autophagy induction in CCA cells

A prior study showed ING5 induces autophagy in gastric cancer cells [16]. However, there is no evidence showed ING5 can regulate autophagy in CCA. We first test the expression of ING5 in CCA cells after transfect with ING5 overexpression. We found the expression of ING5 is up-regulate in CCA cells (Figure S1). The TEM was employed to test the autophagy corpuscle in CCA cells after treat with ING5 over-expression (Fig. 5A). And then we test the expression of autophagy-relative proteins. Our data showed

that overexpression of ING5 can remarkably induced Beclin-1 level and accumulated p62 level in REB cells compared to NC groups indicating that ING5 mediated autophagy in CCA cells (Fig. 5B). Further, it was observed that ING5 overexpression promoted the conversion of LC3-II/LC3-I (Fig. 5C). Our data demonstrated that up-regulate ING5 promotes autophagy induction in REB cells.

The effects of miR-27a-3p were eliminated by ING5

To further demonstrate that ING5 mediated miR-27a-3p to enhance tumor growth, we evaluated the effect of miR-27a-3p on the ability of REB cells to proliferate by ING5-siRNA. The results of the CCK8 assay indicated that the proliferation of the cells inhibited by miR-27a-3p inhibitor relative to the inhibitor NC group. But, the co-transfection of ING5 siRNA at least partially reversed growth inhibition caused by miR-27a-3p knockdown (Figure. 6A). And also, a similar phenotype was observed by colony formation assay and KI67 staining (Figure. 6B-C). Further, we found that the co-transfection of ING5 siRNA restored the cell apoptosis induced by miR-27a-3p inhibitor (Fig. 6D). The findings of the current study suggested that miR-27a-3p could partially enhance the proliferation of REB cells by direct targeting ING5.

Discussion

CCA is the second most prevalent primary hepatobiliary malignancy worldwide[17]. Despite several medical studies on the condition, CCA treatment/management still poses a serious challenge to public health[18]. The latest findings have suggested that miRNAs could play an essential role in CCA development[19]. These studies laid the foundation for exploring the molecular mechanisms underlying the CCA development. Herein, it was revealed that miR-27a-3p silencing could inhibit the growth and induce autophagy of CCA cells. miR-27a-3p exerted its partial oncogenic effect by interacting with the autophagy signaling pathway, thus suppressing the ING5 gene expression.

miRNA is able to target direct mRNA and modulate down-stream gene expression post-transcriptionally, can regulated in many diseases, such as miR-27a-3p [20, 21]. The miRNA promotes the progression of gastric cancer by BTG2 regulation [22]. miR-27a-3p has been identified as marker of the recurrence of clear cell renal cell carcinoma[23]. According to the results herein, high miR-27a-3p mRNA levels was observed in CCA tissues and in cell lines (Figure 1). MiR-27a-3p silencing inhibited CCA cell proliferation and induce apoptosis (Figure 2). Our results were consistent with other studies which also verified that miR-27a-3p possesses excellent oncogenic effects in CCA.

Autophagy has a crucial function in the formation and development of several diseases, such as CCA[9, 24, 25]. Autophagy occurs to maintain cell balance [26] . The process occurs to ensure the survival of cells amid environmental stress. But in the process, autophagy seems to enhance cell morbidity and death [27] .Evidence shows that abnormal autophagy signaling pathway activation contributes to cancer development. Ma et al showed that miR-124 could trigger autophagy-associated apoptosis in CCA cells by regulating the EZH2-STAT3 signaling axis.[9] But studies are yet to reveal whether miR-27a-3p exercises its oncogenic function by autophagy signaling pathway regulation in CCA. Herein, we show that miR-27a-3p inhibition promotes the ratio of LC3 II and LC3 I, up-regulate the autophagy relative protein

beclin-1 and p62 expression. Immunofluorescence examination revealed that cells in which miR-27a-3p was silenced exhibited lower-expression relative to the inhibitor-NC group, suggesting that miR-27a-3p knockdown promoted autophagy induction in CCA cells.

ING5 has been identified as a tumor inhibitor in numerous human cancer diseases [28][29]. Besides, ING5 is downregulated in several cancers, thus suppressing its function of inhibiting the autophagy signaling pathway[30].Herein, miR-27a-3p inhibition can induce autophagy. Over-expression ING5 also promote autophagy in REB cells. These data suggesting that miR-27a-3p probably activates the autophagy signaling pathway through suppressing ING5. Thus, the ING5 function in CCA might not be restricted to autophagy signaling pathway inhibition. Also, MiR-27a-3p might suppress the transcriptional role of autophagy through ING5.

In summary, we revealed that miR-27a-3p suppression triggered apoptosis and suppressed cell proliferation by regulating cell autophagy in human CCA cells. Thus, the partial oncogenic role of the gene in CCA cells could be through its regulation of the autophagy signaling pathway via directly targeting ING5. Our findings may provide a novel insight into miR-27a-3p in CCA progression, and the gene could be a possible CCA treatment target.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable

Authors' contributions

MW and YF C: Conceptualization, Methodology, Software.

FM Z, PC K and XM J: Original draft preparation.

All authors read and approved the final manuscript.

Acknowledgements

Not applicable

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Figures

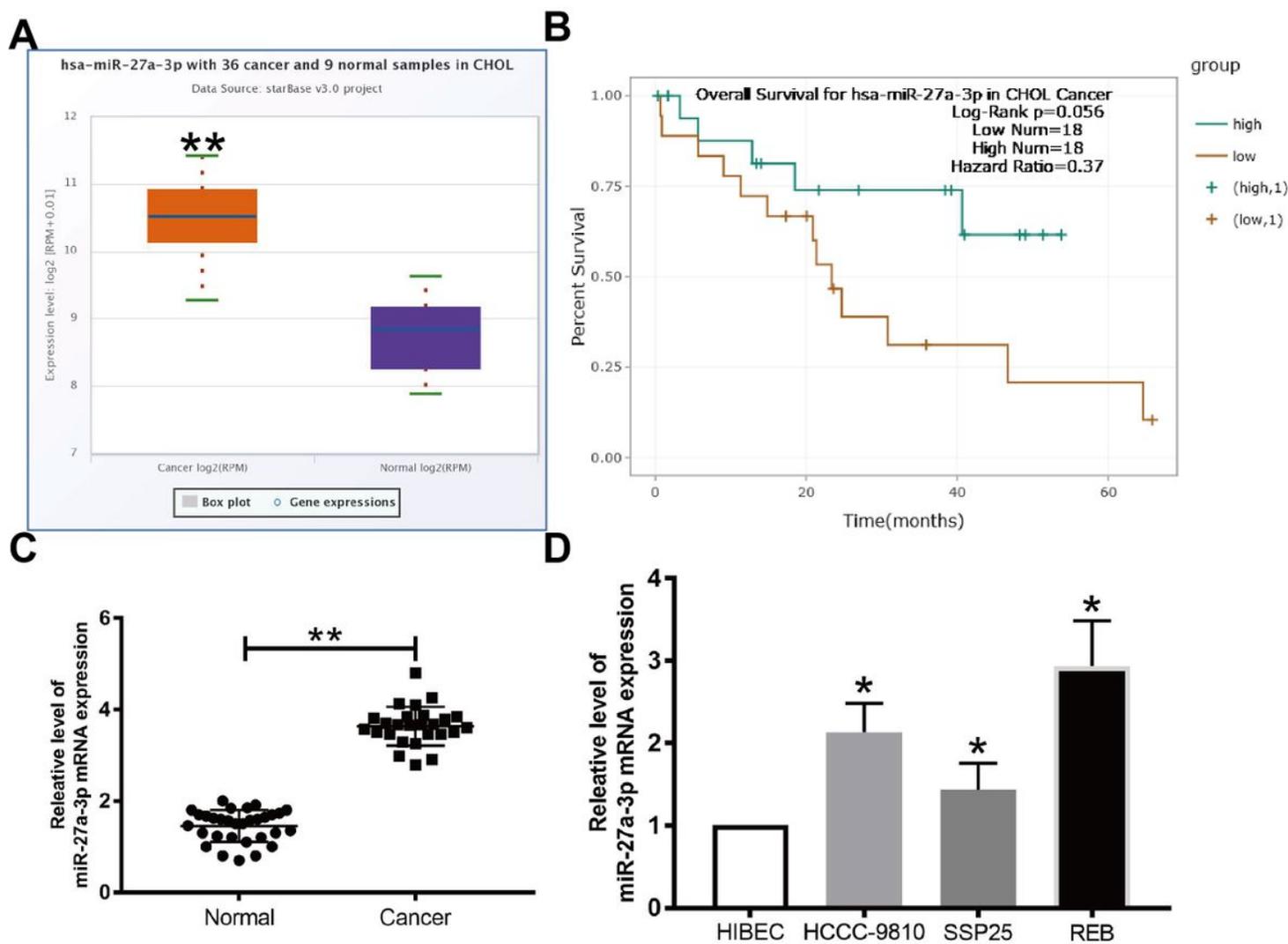


Figure 1

The expression of miR-27a-3p in CCA. GEO dataset showed the expression of miR-27a-3p in human CCA tissues and their matched noncancerous tissues(A). (B)The survival of miR-27a 3p in CCA patients. (C-D) Relative miR-27a-3p mRNA expression levels in CCA tissue and HIBE and CCA cell lines. * $P < 0.05$ versus normal group or HIBE group.

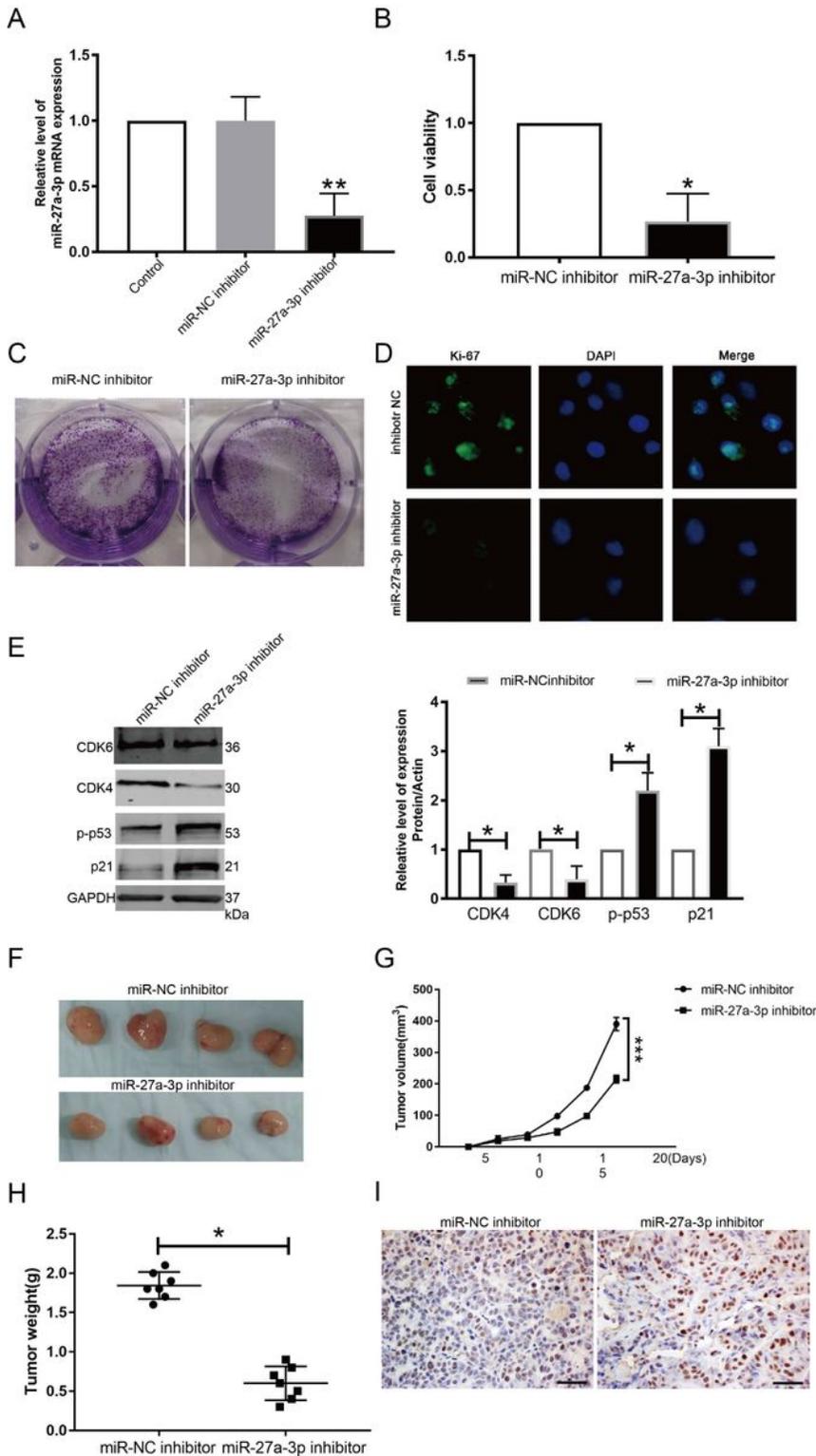


Figure 2

miR-27a-3p knockdown reduces CCA cells growth (A) The expression of miR-27a-3p after transfect with miR-27a-3p inhibitor. (B) Cell variability was tested by CCK-8 after treatment with miR-27a-3p inhibitor or miR-inhibitor NC in REB cells. (C-D) Colony formation and Ki67 assay showed cell proliferation after the treatment of miR-27a-3p inhibitor or miR-inhibitor NC. (E) The protein level of CDK4, CDK6, p-p53 and p21 were measured by western blotting following transfect with miR-27a-3p inhibitor. (F-I) The tumor growth

measured in the nude mouse xenograft tumor model after transfection with miR-27a-3p. Error bars show the mean \pm SD of three experimental repeats. * P <0.05 vs. miR-inhibitor NC group.

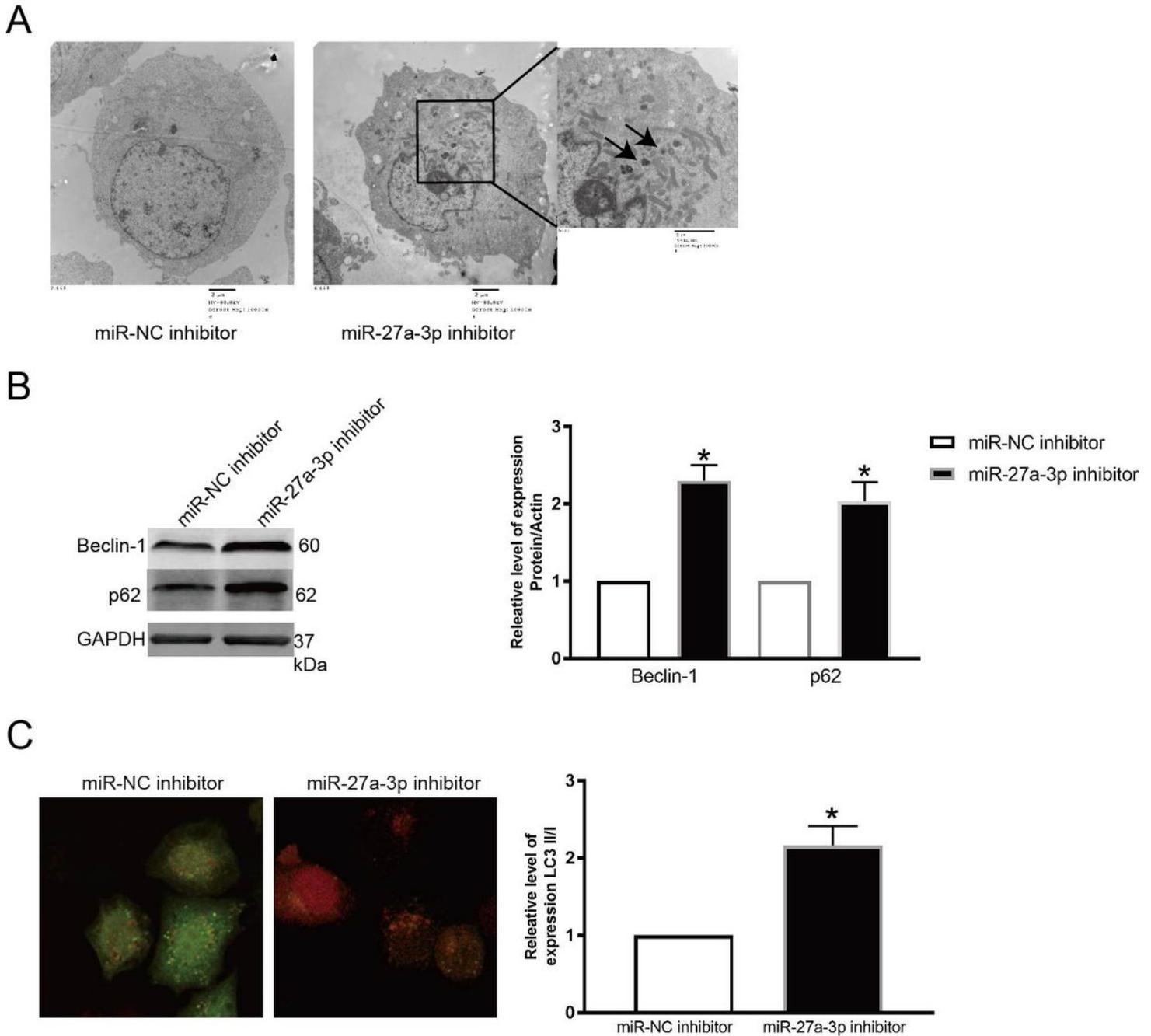


Figure 3

miR-27a-3p knockdown promotes autophagy induction in CCA cells. Notes: (A) Autophagy corpuscle was tested by TEM after transfects with miR-27a-3p inhibitor. (B) Protein level of Beclin-1 and p62 were test by western blotting after treatment with miR-27a-3p inhibitor/ miR-inhibitor NC in REB cells. (C) LC3 II/I assay showed the cell autophagy. Error bars show the mean \pm SD of 3 experimental repeats. * P <0.05 vs. miR-inhibitor NC group.

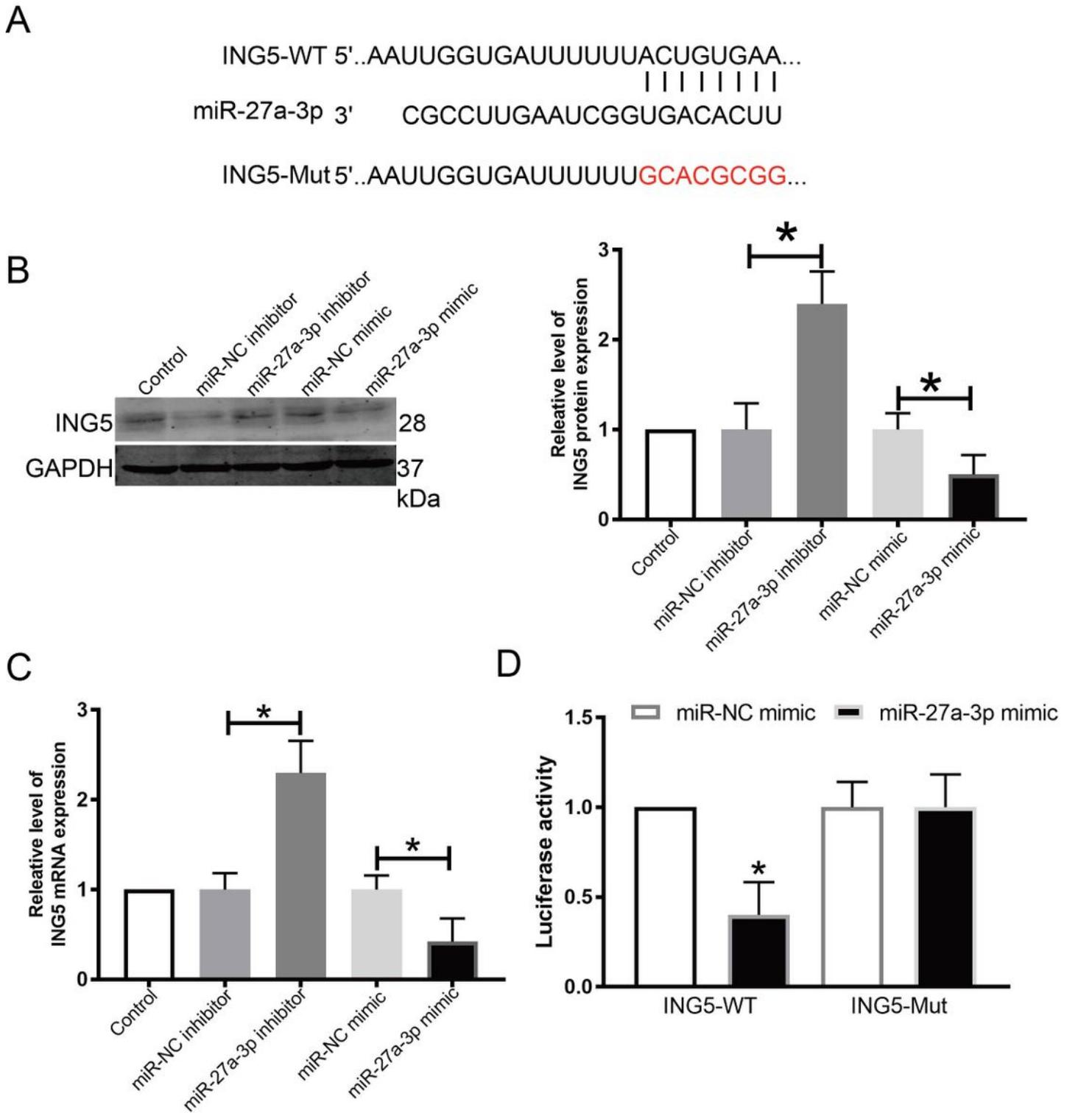


Figure 4

ING5 is a direct miR-27a-3p target (A) Binding site in miR-27a-3p and the 3'-UTR of the ING5 gene. (B) Relative ING5 mRNA expression after treatment with miR-27a-3p mimic, miR-mimic NC or miR-27a-3p inhibitor, miR-inhibitor NC by qRT-PCR. (C) The expression of ING5 after treatment of miR-27a-3p mimic, miR-mimic NC or miR-27a-3p inhibitor, miR-inhibitor NC by western blotting. (D) The luciferase activity of

ING5 3'-UTR mutant construct regulated by miR-27-3p. (Error bars represent means \pm SEM, n = 3 for every group. *P<0.05 vs. miR-mimic NC group or miR-inhibitor NC group.

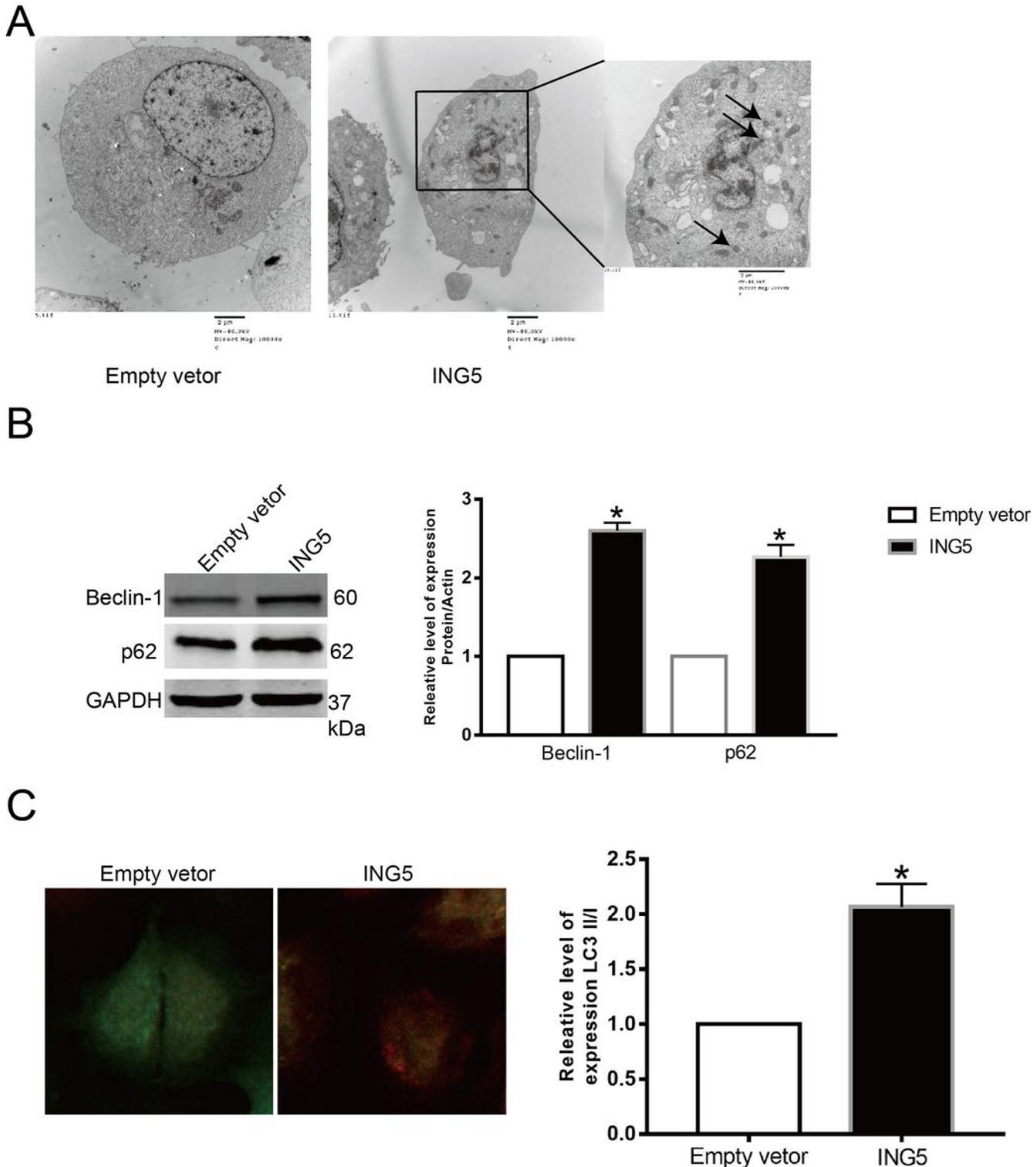


Figure 5

Over-expression ING5 promotes autophagy induction in REB cells. Notes: (A) Autophagy corpuscle was tested by TEM after transfects with ING5 overexpression. (B) Protein level of Beclin-1 and p62 were test by western blotting after treatment with ING5 overexpression / empty vector in REB cells. (C) LC3 II/I

assay showed the cell autophagy. Error bars show the mean \pm SD of 3-6 experimental repeats. *P<0.05 vs. empty vector group.

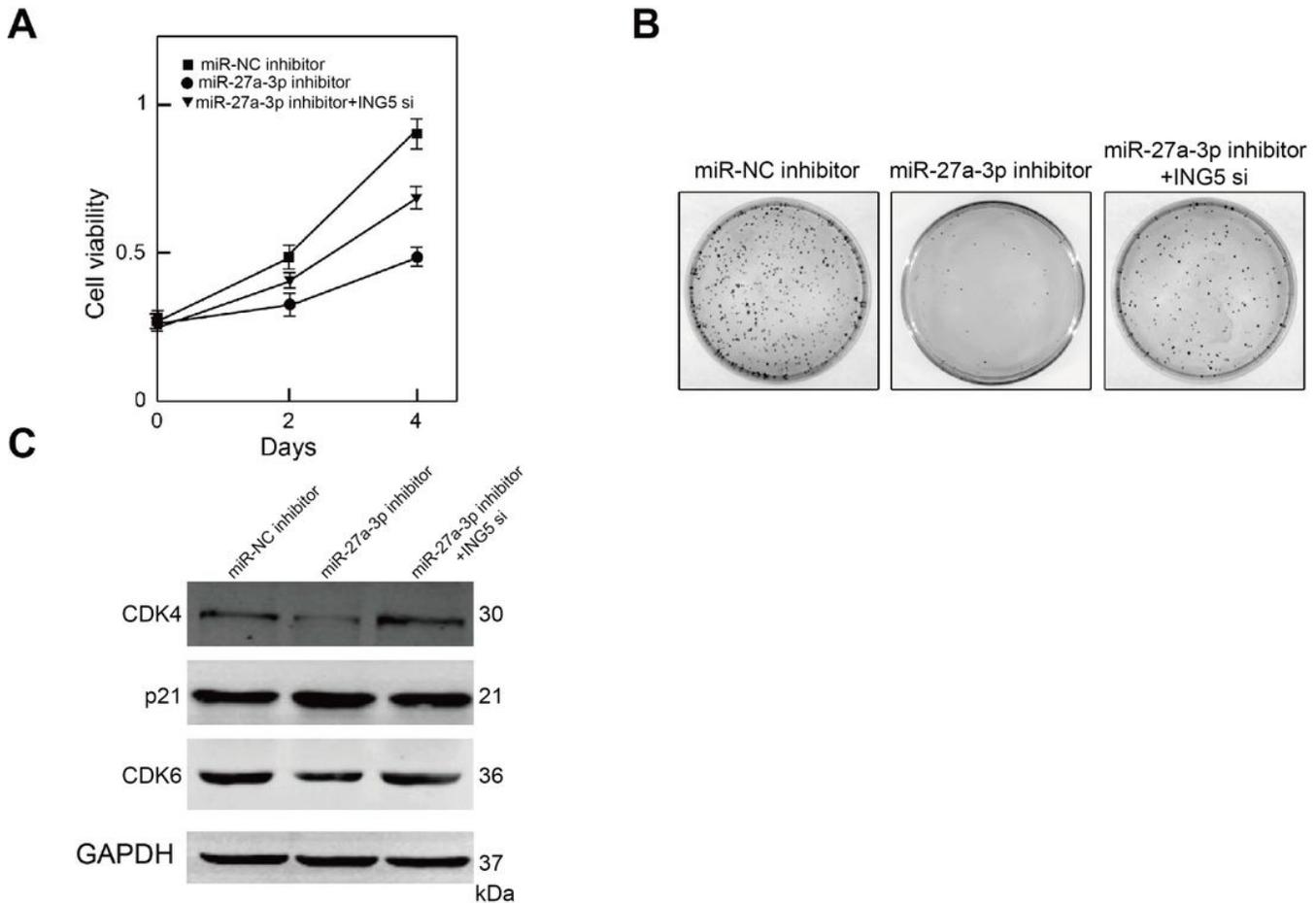


Figure 6

The effects of miR-27a-3p were eliminated by ING5. Notes: REB cells were treated with miR-27a-3p-inhibitor, miR-inhibitor NC or miR-27a-3p-inhibitor+si-ING5 for 48 hours. (A) Cell viability was test by CCK-8. (B) colony formation assay, (C) protein levels of CDK4, CDK6 and p21. All experiments were repeated three times. Each bar represents the mean \pm standard deviation of three independent experiments. *P<0.05 versus miR-mimic NC group

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1.jpg](#)