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circRNA_0006470 promotes the proliferation and migration of gastric cancer cells by functioning as a sponge of miR-27b-3p

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Cancer pathogenesis is influenced by epigenetic alterations mediated by circular RNAs (circRNAs). In this study, we aimed to investigate the regulatory mechanisms and cytological function of hsa_circ_0006470/miR-27b-3p in gastric cancer (GC). circRNA and microRNA expressions in cancer cells were measured by the qRT-PCR method. A dual-luciferase reporter assay was performed to validate the binding of hsa_circ_0006470 with miR-27b-3p. hsa_circ_0006470 was silenced in AGS cells, and proliferation, migration, and invasion were tested via the CCK-8 assay and Transwell system, respectively. The autophagy in GC cells was assessed by marker protein detection and transmission electron microscope. The results showed that hsa_circ_0006470 expression was significantly elevated in GC cells, which was mainly distributed in cytoplasmic components and could directly bind with miR-27b-3p in GC cells. Silencing of hsa_circ_0006470 repressed cell proliferation, migration, and invasion, which may be through regulating miR-27b-3p/Receptor tyrosine kinase-like orphan receptor 1 (ROR1). Silencing of hsa_circ_0006470 also elevated LC3II and Beclin-1 and suppressed p62 protein abundances, which subsequently induced autophagy in AGS cells. Furthermore, we found that hsa_circ_0006470 promotes phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA) expressing by sponging miR-27b-3p. In conclusion, hsa_circ_0006470 promoted GC cell proliferation and migration through targeting miR-27b-3p and suppressing autophagy machinery.

Key words: hsa_circ_0006470, gastric cancer, proliferation, migration, autophagy

Gastric cancer (GC) is one of the major human malignant disorders mainly originating from the mucus-producing cells among the inside lining of stomach and featured with very high mortality caused by late diagnosis and lack of effective treatments. GC is regarded as the fifth most common cancer and the third cause of cancer-related deaths worldwide. The average five-year survival rates of GC patients were reported to be less than 20% and more than 700,000 deaths per year have been due to GC all over the world [1, 2]. It has been well-established that the development of GC was mainly attributed to the infection of *Helicobacter pylori*, as well as other risk factors like Epstein–Barr virus (EBV) infection and cigarette smoking [1, 3]. Recently, surgical resections and chemotherapy have been applied as the main therapeutic modalities for GC patients during the past decade [2]. Genetic mutations and abnormal alterations in various signaling pathways substantially contribute to the pathogenesis of GC, such as the Hedgehog signaling, Notch signaling, and nuclear factor-kB (NF-kB) signaling pathways [1, 2]. Therefore, targeted therapy was suggested as promising alternatives for GC treatment [4, 5] but its applications in clinical management greatly depend on the full elucidation of GC pathogenic mechanisms.

Autophagy refers to intracellular degradation processes of cellular materials mediated by the lysosomes and is responsible for providing energy and forming macromolecular precursors through basal turnover of cellular components [6]. The formation of autophagosomes, one double-membraned intracellular vesicles encapsulating cellular organelles or proteins, and its fusion with lysosomes controlled by a number of signaling events serve as key steps during autophagy machinery [6, 7]. Also, autophagy is an evolutionarily conserved cellular process closely implicated in many basal biological processes such as development, metabolism, and responses to extracellular stresses and stimuli, as well

as various pathogenic conditions such as cancer development [8–10]. Importantly, the autophagy-mediated degradation of damaged cellular organelles and long-lived proteins were closely involved in cell fate determination, epithelial-to-mesenchymal transition (EMT), degradation of extracellular matrix, microenvironment maintenance, angiogenesis, and metastasis during GC initiation and progression [11]. For instance, the inhibition of the autophagy process could promote GC metastasis through regulating reactive oxygen species (ROS) production and glycolysis [12]. Moreover, the modulation of autophagy processes has also been shown to mediate the repression and inhibition of GC induced by many therapeutic reagents such as paclitaxel, resveratrol, and celecoxib [13–15]. However, the regulation of autophagy machinery associated with GC development and treatment still remains poorly understood.

Non-coding RNAs have been characterized as key regulators of cancer development and progression due to their widespread involvement in the epigenetic landscape [16]. Circular RNAs (circRNAs) are a large set of non-coding RNAs with covalently closed-loop structures, which are commonly generated through the pre-mRNA back-splicing biogenesis process [17]. Importantly, circular RNAs possess several unique characteristics including high stability and abundance, tissue-specific expressional pattern, and evolutionarily conservation between species, which were closely associated with GC pathogenesis [17, 18]. For instance, the circPSMC3, one circRNA formed through back-splicing of PSMC3 (proteasome 26S subunit ATPase 3) gene, repressed GC cell proliferation and metastasis through sponging the miR-296-5p as one competitive endogenous RNA (ceRNA) [19]. Furthermore, many functional aspects of circRNAs were mediated by their potent regulation of the autophagy process during cancer development [20]. A recent report showed that the circ-DNMT1 promoted proliferation and survival of breast cancer cells through activation of the autophagy machinery [20]. In GC cells, the circ_0032821 has been characterized as a new oncogene due to its regulation of cell proliferation and metastasis via autophagy processes and mitogen-activated protein kinase 1 (MEK1) signaling [21]. Recent progress revealed the great potential of circRNAs targeting autophagy process as potential targets for GC diagnosis and treatments, which deserves further investigation.

In this study, we aimed to investigate the roles of one new circular hsa_circ_0006470 in GC cell proliferation and migration, as well as its effects on autophagy processes in GC cells. These analyses would provide new insights into the pathogenic mechanism of GC mediated by circular RNAs, which could serve as a basis for non-coding RNA-mediated GC diagnosis and treatment.

Table 1. Primer pairs used for quantitative RT-PCR analysis.

Primer name	Sequence (5'–3')
U6. F	CTCGCTTCGGCAGCACA
U6. R	AACGCTTCACGAATTTGCGT
GAPDH. F	TGTTCGTCATGGGTGTGAAC
GAPDH. R	ATGGCATGGACTGTGGTCAT
hsa_circ_0138960. F	TCCTTGCCGACATTACAGATA
hsa_circ_0138960. R	GTGGCAGGTCTATGCTACTTC
hsa_circ_0001895. F	CATCGTGATAGTACCCAAGGAC
hsa_circ_0001895. R	CTCCCGATCTGCCTCTTTG
hsa_circ_0006470. F	ACTCATCATGGACTCCCTGC
hsa_circ_0006470. R	TGAGCACCTCCTTAGCAGACA
hsa-miR-27b-3p. RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGGCAGAACT
hsa-miR-27b-3p. F	ACACTCCAGCTGGGTTCACAGTGGCTAAGT
hsa-miR-27b-3p. R	CTCAACTGGTGTCGTGGA
hsa_circ_0006470X. F	GGGACCGCATCTTCTTTGT
hsa_circ_0006470X. R	GTGCTGCTCAAACTTGGTCTT

Abbreviations: F-forward primer; R-reverse primer; RT-reverse transcription primer

Material and methods

Cell culture and transfection. Human gastric mucosa epithelial cell line GES-1 and four GC cell lines AGS, HGC-27, Hs 746.T, and SNU-1 were purchased from Procell (Wuhan, China). Cells were cultured in DMEM medium (Thermo Fisher Scientific) supplemented with 10% bovine serum albumin (BSA; Sigma Aldrich) and 50 U/ml penicillin and streptomycin at 37 °C in a humidified atmosphere with a supply of 5% CO_2.

RT-PCR and quantitative RT-PCR. The total RNA samples from GC cells or the control group were prepared using the TRIzol Universal Total RNA Extraction Reagent (#DP424; TianGen Biotech, Beijing, China) following the manufacturer's instructions. Concentrations of RNAs samples were determined by measuring on a NanoDrop microvolume spectrophotometer and applied for the synthesis of the cDNA library using the FastQuant RT Super Mix (#KR108; Tiangen Biotech, Beijing, China) according to the protocol by the manufacturer. Subsequently, the quantitative PCR method was performed using the Talent Fluorescence Quantitative Detection Kit (SYBR Green) (#FP209; Tiangen Biotech, Beijing, China) following steps suggested by the manufacturer. Relative expressions of circRNA or microRNAs were finally calculated by the $2^{-\Delta\Delta Ct}$ method through normalization to the expression of U6 or GAPDH. The existence of circular RNAs in cancer cells was validated by the RT-PCR method using opposite-directed and divergent primer pairs targeting linear mRNAs and circular RNAs, respectively. Primers used for quantitation are listed in Table 1.

Fluorescence in situ hybridization (FISH). The subcellular distribution of hsa_circ_0006470 in AGS cells was detected through the fluorescence in situ hybridization (FISH) method, using the RNA FISH probe kit A (#F03301; GenePharma, Shanghai, China) following the protocol provided by the producer. Briefly, GC cells grown on slides were first washed with PBS solution two times, fixed with

4% paraformaldehyde for 12 min at room temperature, and incubated overnight in the dark with circular RNA probes, which were synthesized by the GenePharma Company (Shanghai, China). After being washed again with PBS solution for 8 min at room temperature, the GC cell slides were then counter-stained with DAPI for 8 min at room temperature to visualize the cell nuclei and finally analyzed and photographed under a confocal laser scanning microscope.

Dual-luciferase reporter assay. The direct bindings of the hsa_circ_0006470 with miR-27b-3p and miR-27b-3p with the 3'UTR sequence of PIK3CA in GC cells were verified through the dual-luciferase reporter assay. Briefly, the wild-type (WT) or mutant (MUT) sequences of hsa_circ_0006470 and 3'UTR sequence of PIK3CA were first ligated with the pmirGLO Dual-Luciferase miRNA Target Expression Vector (#E1330; Promega) as instructed by the manufacturer. Also, the miR-27b-3p mimics (5'-UUCACAGUGGCUAAGUUCUGC-3') and its negative control (NC; 5'-UUUGUACUACACAAAAGUACUG-3') sequences were synthesized by the GenePharma Company (Shanghai, China). The above-mentioned pmirGLO-circ_0006470 WT or pmirGLO-circ_0006470 MUT vectors, and pmirGLO-PIK3CA-3'UTR WT or pmirGLO- PIK3CA-3'UTR MUT vectors, were transfected into the cultured AGS cells using the Lipofectamine 3000 reagent (Thermo Fisher Scientific) following the producer's instructions, together with miR-27b-3p mimics or NC sequences as specified. The luciferase activity in cells was finally measured by a luminometer for evaluation of the interaction between circRNA and microRNA.

Cell proliferation and migration. The proliferation of GC cells was measured by the CCK-8 method using the Cell Counting Kit-8 reagent (#CK04; Dojindo, Japan) according to the producer's instructions. Briefly, AGS cells were collected by centrifugation at 800×g for 5 min and seeded in 96-well plates (4,000 cells/well). After being cultured under normal culture conditions for 24 h, 48 h, and 72 h at 37 °C, cells were incubated with 10 μl CCK-8 reagent for another 3 h at 37 °C, and the absorbances at 450 nm (OD450) were finally measured on a plate reader. The proliferation of GC cells was assessed by the alterations of OD450 values. The migration of GC cells was analyzed using the Transwell System (Corning). Briefly, cells in serum-free DMEM were seeded in the upper chamber of the Transwell plates and cultured for 24 h at 37 °C. Cells that migrated into the lower chambers filled with DMEM containing 10% FBS were then stained with 1% crystal violet and observed under a microscope.

Western blotting. Total proteins were extracted from cultured GC cells using the RIPA Lysis and Extraction Buffer (#89900; Thermo Fisher Scientific) following the producer's instructions. Protein concentration was detected by the Pierce BCA Protein Assay Kit (#23227; Thermo Fisher Scientific) as instructed by the manufacturer. Subsequently, proteins were boiled at 100 °C for 5 min in protein loading buffer, separated by SDS-PAGE, and transferred onto PVDF membranes (Millipore). After being blocked with 5% lipid-free milk solution for 2 h at room temperature, proteins on PVDF membranes were then incubated with diluted primary antibodies overnight at 4 °C or for 2 h at room temperature, followed by the secondary antibody incubation for 1–2 h at room temperature and final development with the Pierce ECL Plus Western Blotting Substrate (#32134; Thermo Fisher Scientific). The GAPDH proteins were simultaneously detected as the internal standard. Antibodies used for protein quantitation include anti-LC3B (#ab51520; Abcam), anti-p62 (#88588; CST), anti-Beclin-1 (#ab210498; Abcam), ROR1 (#ab91187; Abcam), PIK3CA (#ab40776; Abcam), and anti-GAPDH (#ab181602; Abcam).

Transmission electron microscopy. The autophagy progression in AGS cells was evaluated through observation of autophagosome formation by transmission electron microscopy (TEM). Briefly, GC cells were grown on slides, which were then washed twice with PBS solution, fixed in 2.5% glutaraldehyde solution (dissolved in PBS solution) for 1.5 h at room temperature, and incubated with 1.2% osmium tetroxide solution for 20 min at room temperature. The formation of autophagosomes in GC cells was finally observed under a transmission electron microscope.

Statistical analysis. The SPSS 20.0 software was used in this study to analyze quantitative data presented as mean ± standard deviation. Differences among two groups or more groups were evaluated by the Student t-test or ANOVA (analysis of variance) method. The p-value < 0.05 was used for the definition of significant differences.

Results

Elevated hsa_circ_0006470 and decreased miR-27b-3p expression in GC cells. To study the potential pathogenic roles of non-coding RNAs in GC, we first detected the expressional alterations of three candidate circRNAs, including hsa_circ_0138960, hsa_circ_0001895, and hsa_circ_0006470 in GC cell lines. Compared with the human gastric mucosa epithelial cell line GES-1, we showed by the quantitative RT-PCR method that the expression of hsa_circ_0138960 was remarkably increased in AGS, HGC-27, and SNU-1 cells, while hsa_circ_0006470 and hsa_circ_0001895 were significantly elevated in all four GC cells (Figure 1A). As the biggest expression difference, the cyclic structure of hsa_circ_0006470 in AGS cells was further validated by the significant signaling during the RT-PCR method using divergent primer pairs other than the opposite-directed primers (Figure 1B). Also, through FISH assay we found that hsa_circ_0006470 was mainly distributed in the cytosol components of AGS cells (Figure 1C). Moreover, the binding site of hsa_circ_0006470 between a miRNA, miR-27b-3p, was found by the Starbase database (http://starbase.sysu.edu.cn/starbase2/) (Figure 1D). Meanwhile, the expression of miR-27b-3p was significantly decreased in

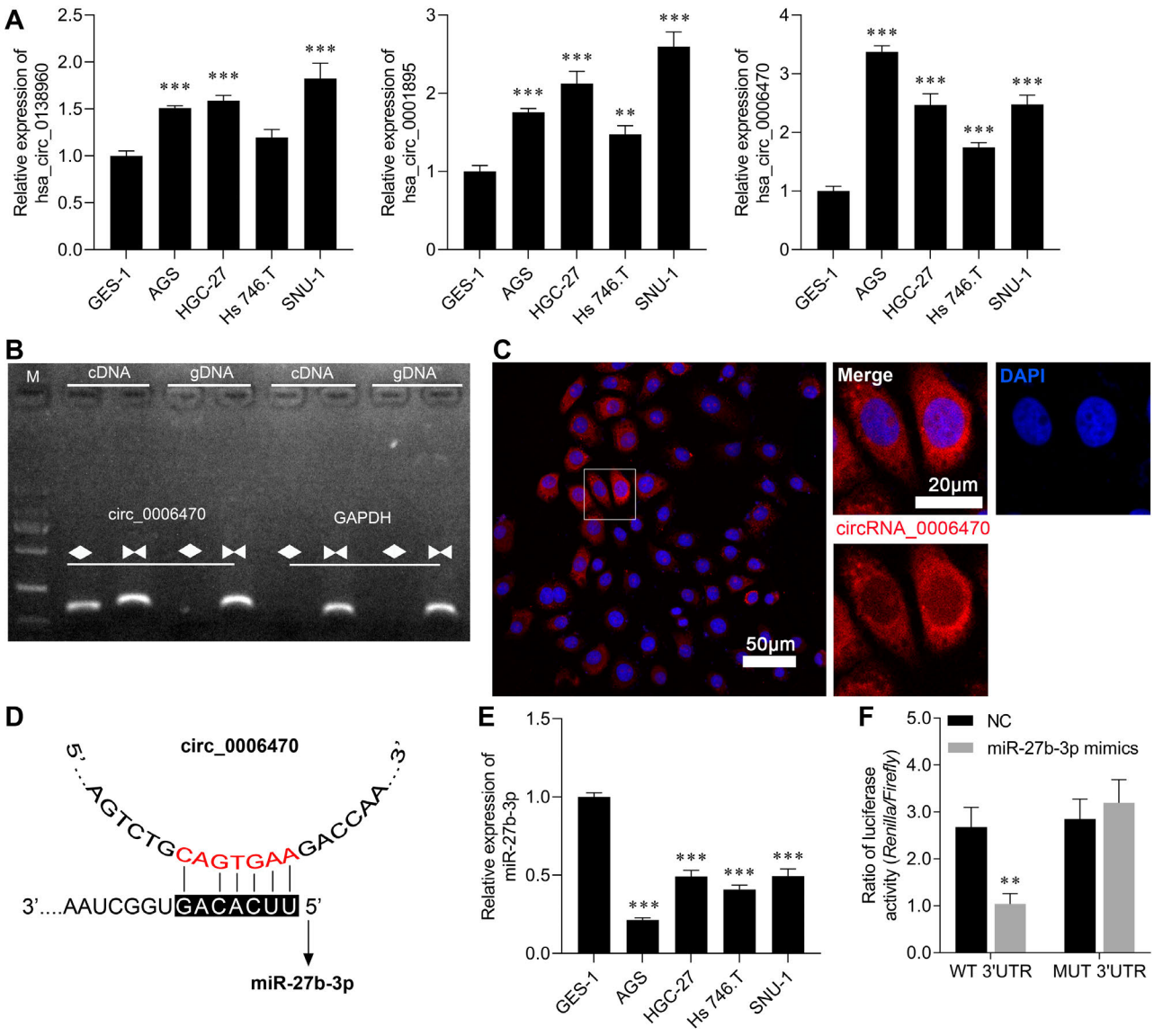

Figure 1. Differential expression of circRNAs and microRNA in GC cells. A) The relative expressional levels of three circular RNAs in GC cell lines AGS, HGC-27, Hs 746.T, SNU-1, and human gastric mucosa epithelial cell line GES-1. Non-coding RNA levels were detected through the quantitative RT-PCR method. B) Validation of the hsa_circ_0006470 formation in GC cells by the PCR method. The circular and linear RNAs were detected using divergent and opposite-directed primer pairs respectively. Both cDNA and genomic DNA samples were used as the templates. Primers targeting the GAPDH gene were applied as the control. C) The subcellular distribution of hsa_circ_0006470 in cancer cells was analyzed via the FISH method. D) Direct association of hsa_circ_0006470 with miR-27b-3p. E) The relative expressional levels of miR-27b-3p in GC cells and GES-1 were detected through the quantitative RT-PCR. F) The binding of hsa_circ_0006470 with miR-27b-3p in AGS cells was validated via the dual-luciferase reporter assay. Abbreviations: cDNA-complementary DNA; gDNA-genomic DNA; WT-wide type; NC-negative control; MUT-mutant; **p<0.01; ***p<0.001

GC cells, when compared with that in GES-1 cells (Figure 1E). Through dual-luciferase report assay, we found that the luciferase activities were greatly reduced by transfection with miR-27b-3p mimics in AGS cells expressing the wild-type (WT) hsa_circ_0006470 sequences, but not in those cells expressing the mutant (mut) hsa_circ_0006470 sequences, showing the direct binding of hsa_circ_0006470 with miR-27b-3p in GC cells (Figure 1F). These results revealed the formation and expressional increase of hsa_circ_0006470 in GC cells, as well as the significant decrease of miR-27b-3p in GC cells.

hsa_circ_0006470 promoted GC cell proliferation and migration as a ceRNA. To investigate the relationship between these opposite expressional alterations of hsa_circ_0006470 and miR-27b-3p in GC cells, we then tested the potential of hsa_circ_0006470 as miRNA sponges

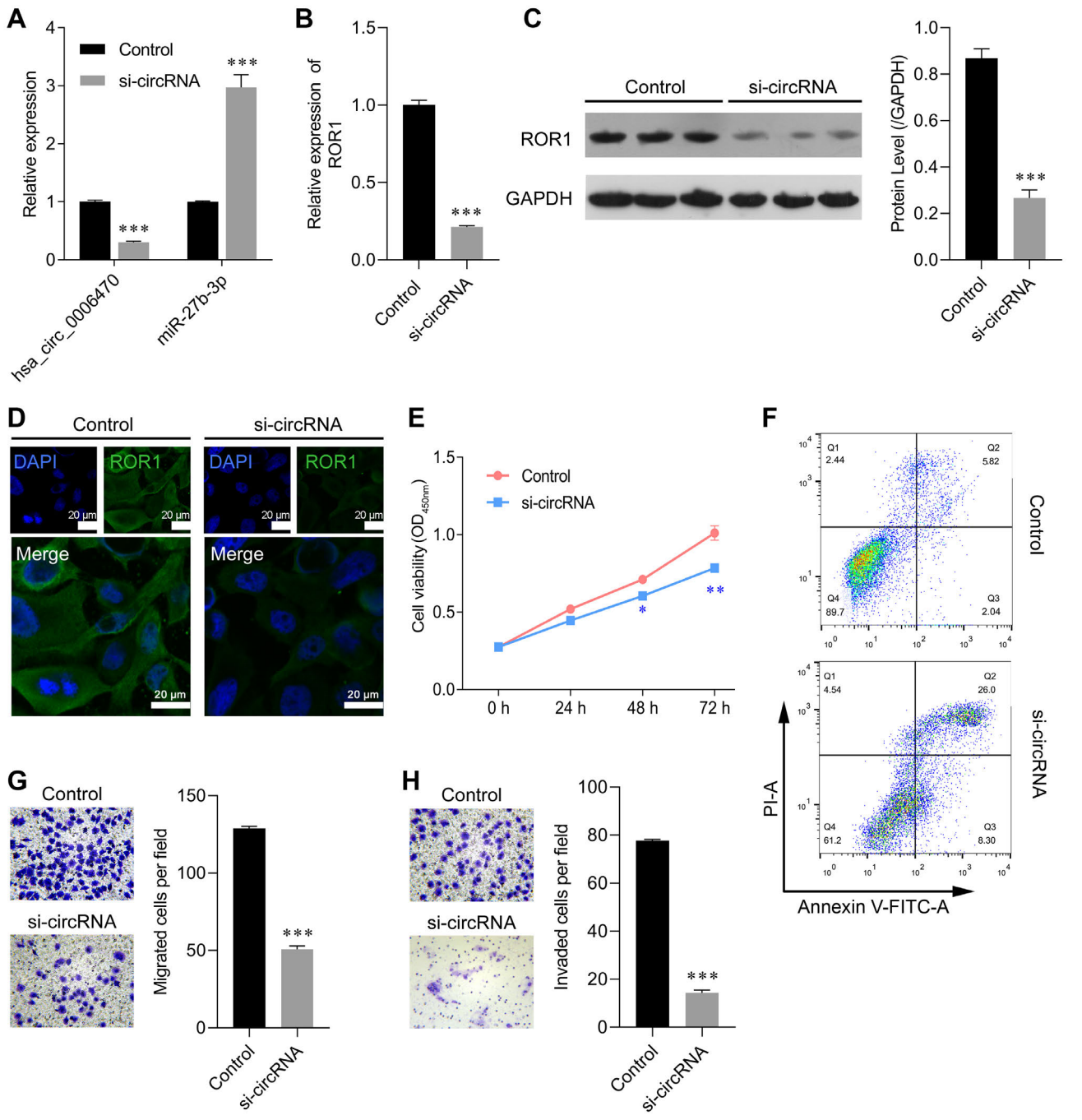

Figure 2. Knockdown of hsa_circ_0006470 inhibited proliferation, migration, and invasion of AGS cells by regulating miR-27b-3p/ROR1. A, B) The relative expressional levels of hsa_circ_0006470, miR-27b-3p, and ROR1 in AGS cells transfected with hsa_circ_0006470 siRNA. C, D) Protein level of ROR1 detected by western blotting and immunofluorescence assay. E) Cell viability of AGS cells transfected with hsa_circ_0006470 siRNA was detected by the CCK-8 assay. F) Apoptosis was detected by the flow cytometry. (G, H) Cell migration and invasion were measured by the Transwell assay. *p<0.05; **p<0.01; ***p<0.001

in regulating miR-27b-3p expression. We knocked down hsa_circ_0006470 in AGS cells, subsequently, the expression of miR-27b-3p was found significantly high by quantitative RT-PCR (Figure 2A). One reported target gene of miR-27b-3p, ROR1, was investigated. Results showed that it was significantly inhibited by hsa_circ_0006470 silencing both at the transcriptional level and protein level (Figures 2B–2D). Furthermore, we observed that the proliferation rates were significantly repressed by the transfection of hsa_circ_0006470 siRNAs after 48 h (Figure 2E)

while inducing increased apoptosis in AGS cells (Figure 2F). Moreover, we found using the Transwell system that silencing of hsa_circ_0006470 expression with specific siRNAs substantially suppressed the migration in invasion capacities of AGS cells (Figures 2G, 2H). These results suggested that hsa_circ_0006470 may promote GC cell proliferation and migration through regulating the miR-27b-3p/ROR1 axis.

hsa_circ_0006470 repressed the autophagy in GC cells by regulating miR-27b-3p/PI3KCA. To explore the potential molecular mechanisms mediating the tumorigenic roles of hsa_circ_0006470 in GC, we then detected the autophagy machinery in GC cells with altered hsa_circ_0006470 expression. We observed that hsa_circ_0006470 silencing caused significant increases of LC3BII (Microtubule-associated protein11ight chain 3 II) and Beclin-1 proteins and remarkably reduce P62 proteins in the AGS cells (Figure 3C). In consistence, we found hsa_circ_0006470 siRNAs promoted the autophagosome formation in AGS cells compared with the control group (Figure 3E). To elaborate the regulatory mechanism of hsa_circ_0006470 to autophagy, we investigated potential target genes of miR-27b-3p through the TargetScan database (http://www.targetscan.org/vert_72/) and found maybe miR-27b-3p regulates phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA) (Figure 3A). Dual-luciferase report assay also revealed that miR-27b-3p may regulate the expression

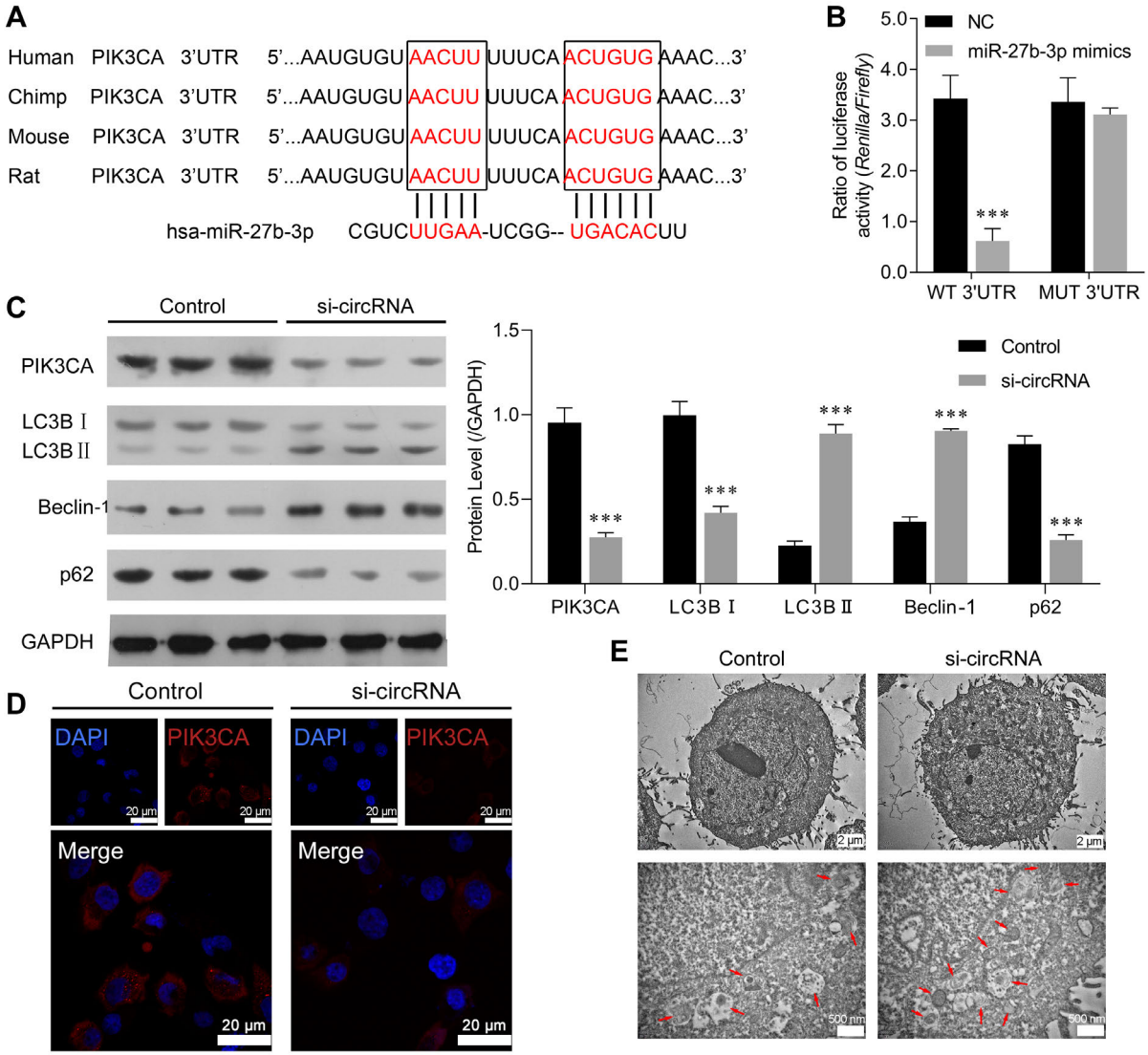

Figure 3. Knockdown of hsa_circ_0006470 promoted autophagy by regulating miR-27b-3p/PI3KCA. A) Binding site and direct association of miR-27b-3p with PI3KCA. B) The binding of miR-27b-3p with PI3KCA 3'UTR in AGS cells was validated via the dual-luciferase reporter assay. C) Protein levels of PI3KCA, LC3B, Beclin-1, and p62 showed by western blotting. D) Expression of PI3KCA showed by immunofluorescence assay. E) Cell morphology and autophagosome showed by transmission electron microscopy. Abbreviation: 3'UTR-3'-Untranslated Regions; ***p<0.001

of PI3KCA (Figure 3B). Furthermore, results of western blotting and immunofluorescence assay showed that hsa_circ_0006470 silencing significantly reduced the expression of PI3KCA (Figures 3C, 3D). These results showed that hsa_circ_0006470 could effectively repress the autophagy activation by regulating the miR-27b-3p/PI3KCA axis in GC cells, which might mediate the regulation of GC proliferation and migration.

Overexpressing PI3KCA attenuated the effect of hsa_circ_0006470 silencing on GC cells. To further prove the regulatory role of hsa_circ_0006470 to PI3KCA, we overexpressed PI3KCA in the AGS cells after hsa_circ_0006470 silencing. Expression of PI3KCA was validated through quantitative RT-PCR and western blotting assay (Figures 4A, 4B). Subsequently, we found that LC3BII and Beclin-1 were significantly reduced by the PI3KCA overexpressing, while P62 was significantly increased by that in AGS cells (Figure 4B). Moreover, the cell activity was significantly increased at 72 h after transfection, and apoptosis was decreased at 48 h after transfection in the si-circRNA+PI3KCA group when compared with the si-circRNA group (Figures 4C, 4D). Cell migration and invasion were also significantly increased by the PI3KCA overexpressing (Figures 5A, 5B), meanwhile, increased autophagy levels induced by hsa_circ_0006470 silencing were also suppressed by overexpression of PI3KCA (Figure 5C). These results showed once again

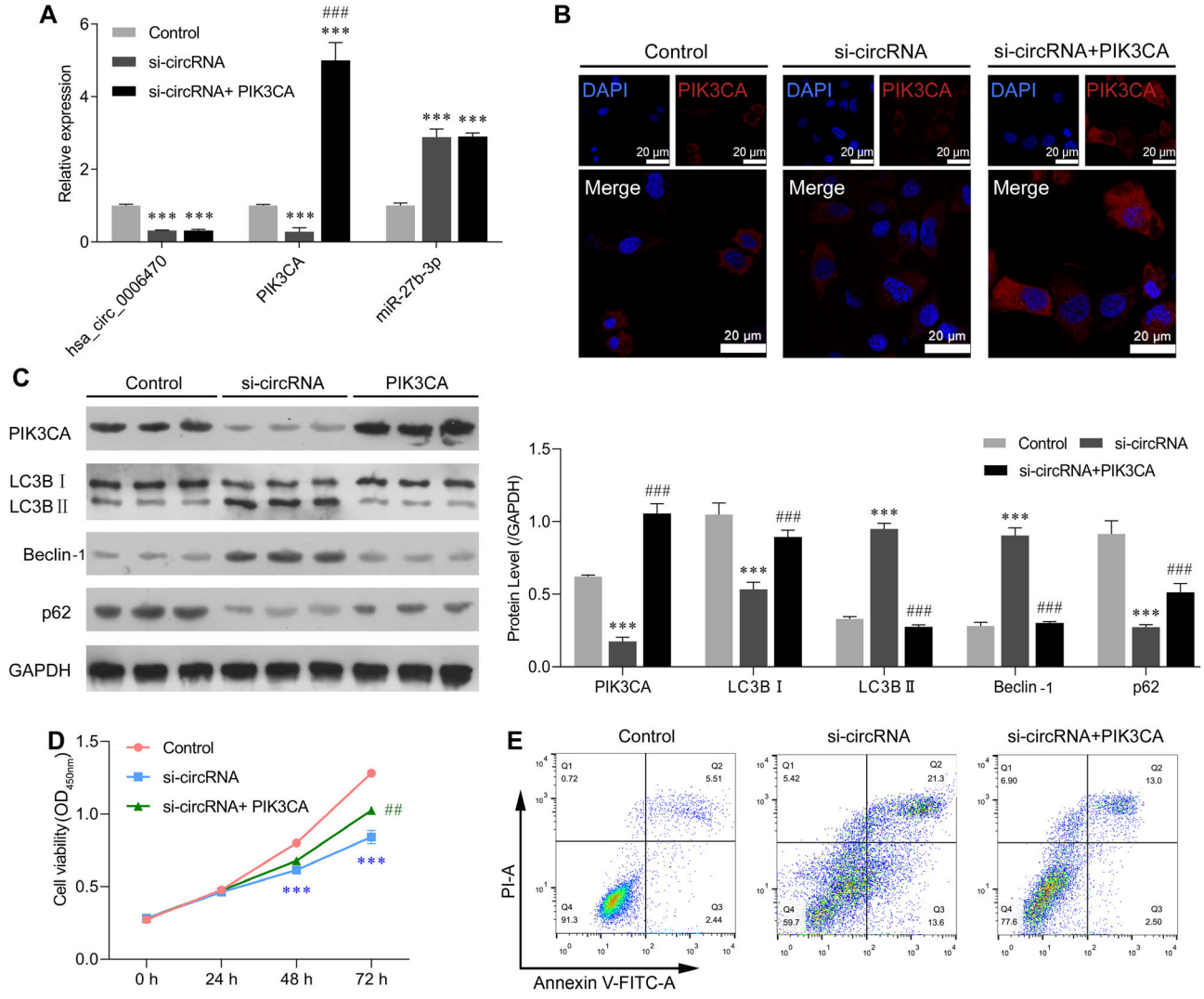

Figure 4. Overexpression of PI3KCA promoted the proliferation of AGS cells. PI3KCA was overexpressed in the AGS cells after hsa_circ_0006470 silencing. A) The relative expressional levels of hsa_circ_0006470, miR-27b-3p, and PI3KCA in AGS cells 48 h after transfection. B) Expression of PI3KCA showed by the immunofluorescence assay. C) Protein levels of PI3KCA, LC3B, Beclin-1, and p62 showed by western blotting. D) Cell viability of AGS cells after transfection was detected by the CCK-8 assay. E) Apoptosis was detected by the flow cytometry. ***p<0.001 vs. control group; ##p<0.01, ###p<0.001 vs. si-circRNA group

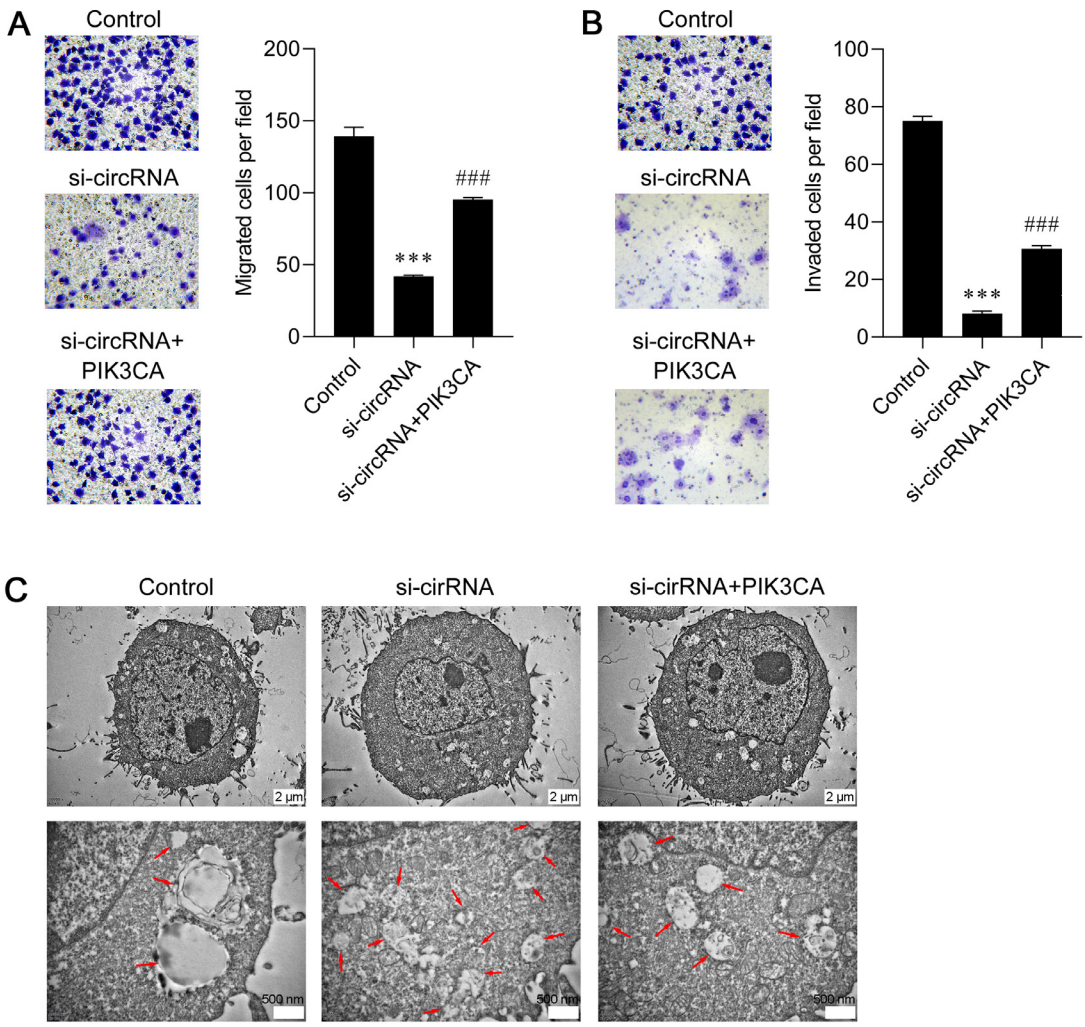

Figure 5. Overexpression of PI3KCA promoted migration, invasion, and inhibited autophagy in AGS cells. PI3KCA was overexpressed in the AGS cells after hsa_circ_0006470 silencing. A, B) Cell migration and invasion were measured by the Transwell assay. C) Cell morphology and autophagosome showed by transmission electron microscopy. ***p<0.001 vs. control group; ###p<0.001 vs. si-circRNA group

that hsa_circ_0006470 represses autophagy by regulating miR-27b-3p/PI3KCA in GC cells.

Inhibition of miR-27b-3p repressed the autophagy in GC cells. To further investigate the role of miR-27b-3p in GC, we knocked down the miR-27b-3p expression in AGS cells. Results showed that the expression of hsa_circ_0006470 and PI3KCA both significantly increased after inhibiting miR-27b-3p (Figure 6A), and the protein of PI3KCA was also significantly accumulated (Figure 6B, 6C). Further, the protein level of LC3BII and Beclin-1 was significantly reduced by inhibiting miR-27b-3p, while that of p62 was significantly increased. Moreover, the cell activity was significantly increased at 48 h after transfection in the miR-27b-3p inhibitor group (Figure 6D), meanwhile, cell migration and invasion were also significantly promoted by inhibiting miR-27b-3p (Figure 6E, 6F). In addition, inhibiting miR-27b-3p decreased the number of autophagosomes in AGS cells (Figure 6G). These results proved again that miR-27b-3p plays as a tumor suppressor in GC.

Discussion

GC is one major severe human malignant disorder and its pathogenesis and responses to current therapeutic regimens are closely associated with the cellular autophagy machinery. Non-coding RNAs such as circRNAs and microRNAs have been characterized as important regulators of the autophagy progression associated with GC development [17, 18] but their specific cellular roles in GC initiation and progression and underlying mechanisms deserve further investigations. In the present study, we showed that the expression of one circular RNA hsa_circ_0006470 was greatly increased in GC

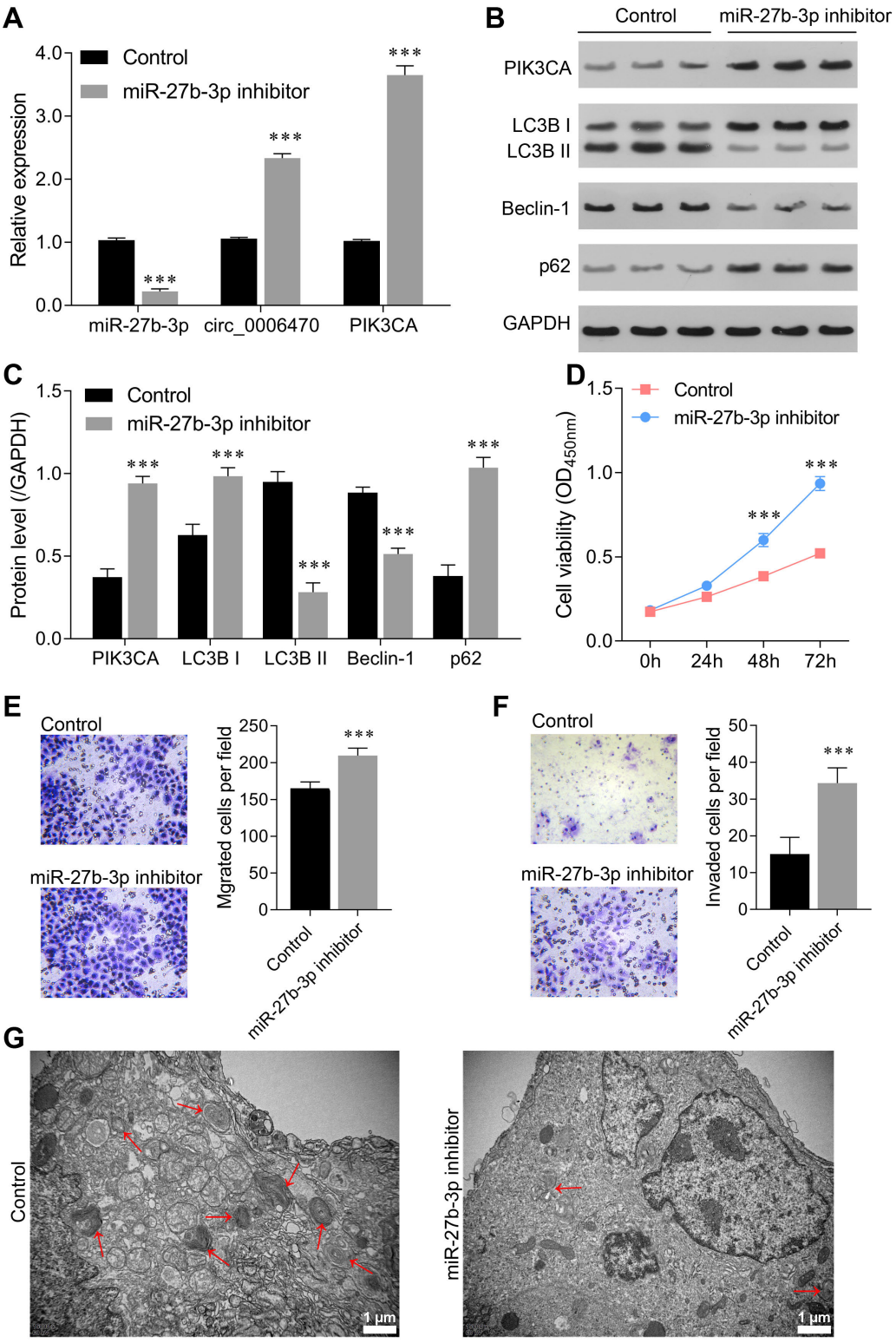

Figure 6. Knockdown miR-27b-5p promoted migration, invasion, and inhibited autophagy in AGS cells. The miR-27b-5p inhibitor was transfected into AGS cells. A) The relative expressional levels of hsa_circ_0006470, miR-27b-3p, and PI3KCA in AGS cells 48 h after transfection. B, C) Protein levels of PI3KCA, LC3B, Beclin-1, and p62 showed by western blotting. D) Cell viability of AGS cells after transfection was detected by the CCK-8 assay. E, F) Cell migration and invasion were measured by the Transwell assay. G) Cell morphology and autophagosome showed by transmission electron microscopy. ***p<0.001

cells, and was mainly distributed in the cytosols and repressed the expression of the microRNA miR-27b-3p via direct binding. Moreover, silencing of hsa_circ_0006470 resulted in substantial inhibition of GC cell proliferation and migration. In addition, hsa_circ_0006470 silencing enhanced the autophagy activation in GC cells. These results revealed the oncogenic roles of hsa_circ_0006470 in GC development, which was mediated by the modulation of the autophagy machinery.

Circular RNAs function as one essential hub of the epigenetic regulation of the functional gene expression regulation and signaling transduction during cancer pathogenesis [22, 23]. In the past decades, circRNAs were shown to be prevalently expressed in cancer cells including GC, which were closely implicated with almost every step during cancer development such as initiation, progression, metastasis, and also responses to current treatments [17, 18]. In GC cells, a large number of circular RNAs were reported to be differentially expressed compared with the non-cancerous tissues, whose upregulated or downregulated expressional alterations were closely correlated with GC pathogenesis [24, 25]. Moreover, the differential expressions of circRNAs were identified as critical regulators of GC cell proliferation, apoptosis, migration, and invasion. For instance, the circRNA circRNA0047905 was recently identified as a new tumor promoter of GC development through regulating the Akt (protein kinase B) and CREB (cAMP response element-binding protein) signaling pathways [26]. In this study, we showed the great capacities of hsa_circ_0006470 in promoting GC cell proliferation and migration, which indicated that hsa_circ_0006470 as a new promoter of GC development and further supported the prevalent roles of circular RNAs in GC development. Also, the expressions of another two circRNAs hsa_circ_0138960 and hsa_circ_0001895 were also significantly elevated in GC cells, whose cellular functions during cancer pathogenesis deserve further investigations.

Among the multiple known functional mechanisms, the sponging of microRNA expression as competing endogenous RNAs (ceRNAs) has been recently characterized as one major mechanism mediating circular functions [27]. For instance, the circRNA hsa_circ_0032627, also known as circDLST encoded by the Digit letter substitution test (DLST) gene, could sponge the miR-502-5p to promote the GC development and metastasis, mediated by the regulation of the NRAS (neuroblastoma Ras viral oncogene) gene expression and the mitogen-activated protein kinase (MEK) and extracellular signal-regulated kinase (ERK) pathways [28]. In the present study, we showed by dual-luciferase reporter gene assay that hsa_circ_0006470 could also directly bind with microRNA miR-27b-3p in GC cells, indicating the microRNA sponge roles of hsa_circ_0006470 during cancer development. Previous reports have already shown that miR-27b-3p played essential roles in cancer cell proliferation, migration, and even multiple resistances to chemotherapies [29, 30]. More importantly, miR-27b-3p was previously shown to repress the proliferation of GC cells via directly targeting the Receptor Tyrosine Kinase Like Orphan Receptor 1 (ROR1) gene [31]. Here, we proved that the knockdown of hsa_circ_0006470 led to a great increase of miR-27b-3p expression in GC cells, which further supported the functions of hsa_circ_0006470 sponging miR-27b-3p in the pathogenesis of GC.

As introduced above, the autophagy machinery was widely implicated in GC development and progression through its effects on many cancer-related processes such as EMT, extracellular matrix maintenance, metastasis, and angiogenesis [11]. Specifically, GC development was previously shown to be promoted by the inhibition of autophagy machinery and resultant alterations of ROS production and metabolisms [12]. The conversion of LC3-I (light chain 3) to LC3-II and the elevation of Beclin-1 protein, as well as the inhibition of P62 protein, have been commonly used as major protein markers of autophagy activation [6, 9]. In the present study, we showed that knockdown of hsa_circ_0006470 resulted in an effective increase of LC3-II and Beclin-1 proteins and significantly decreased the P62 protein level. These results disclosed the great capacity of hsa_circ_0006470 in repressing the machinery activation in GC cells, which was also confirmed by the changes in autophagosome formation, as shown by our following transmission electron microscopy. Furthermore, we found that hsa_circ_0006470 may function through regulating the miR-27b-3p/PI3KCA axis. Previous studies have been reported expression of PI3KCA plays a crucial role in influencing autophagy in cancer cells [32, 33]. More importantly, the enhanced autophagy in GC cells induced by hsa_circ_0006470 silencing was greatly abrogated by the overexpression of PI3KCA, further validating the roles of hsa_circ_0006470 in regulating PI3KCA during GC development. Unlike our results, Sun et al. have found that miR-27b-3p inhibits autophagy by regulating ATG10 expression in colorectal cancer chemoresistance [34]. Due to the multi-target gene characteristics of microRNAs, we can speculate that miR-27b-3p may play different roles in different environments and different cells. Despite studies have reported that low expression of miR-27b-3p was related to chemoresistance in various cancer cell lines [34–36], miR-27b-3p was still found as a tumor suppressor in GC [31], breast cancer [29], lung cancer [37], glioma [38], and esophageal squamous cell carcinoma [39]. Of note, the mechanisms of autophagy inhibition by hsa_circ_0006470 and its interaction with other potential microRNAs during GC development deserve further elucidations.

In summary, we revealed in this study that the circular RNA hsa_circ_0006470 was highly expressed in GC cells, which promoted cell proliferation and migration, suppressed miR-27b-3p expression via direct binding, and inhibited the autophagy machinery activation in GC cells. These results provided new insights into the functions of circular RNAs and their interaction with microRNAs during GC pathogenesis, which could also be explored as new targets for GC

diagnosis and treatment mediated by the non-coding RNAs and epigenetic regulation.

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