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Long noncoding RNA HAGLROS promotes cell proliferation, inhibits apoptosis and enhances autophagy via regulating miR-5095/ATG12 axis in hepatocellular carcinoma cells

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ABSTRACT

In this research, we planned to dig the possible influences and mechanism of long noncoding (lnc) RNA HAGLROS in the development and progression of hepatocellular carcinoma (HCC). The levels of lncRNA HAGLROS in HCC tumor samples and their relationship with clinicopathological characteristics and prognosis of patients with HCC were studied. Subsequently, overexpression and silenced approaches were used in HCC cells for detecting the effects of lncRNA HAGLROS on cell viability, apoptosis, and autophagy. Furthermore, we investigated whether HAGLROS could function as a competing endogenous RNA (ceRNA) to regulate miR-5095 expression in HCC cells, and explored the correlation between miR-5095 and ATG12. Besides, the correlation of HAGLROS, the consequent PI3K/AKT/mTOR signaling pathway was further explored. The level of HAGLROS was higher in HCC tissues and correlated with clinical performances including tumor stages or tumor differentiation. In contrast to the lower level, a higher level of HAGLROS correlated with a shorter survival time of patients with HCC. The suppression of HAGLROS decreased cell viability, promoted apoptosis, and inhibited autophagy. Moreover, HAGLROS negatively regulated miR-5095 expression, which further regulated HCC cell viability, apoptosis, and autophagy. In addition, ATG12 was targeted by miR-5095 and was then involved in miR-5095-regulated HCC cell biological processes including viability, apoptosis, and autophagy. Furthermore, overexpression of HAGLROS activated PI3K/AKT/mTOR signals. Our results revealed that HAGLROS is highly expressed in HCC, and its high level may correlate with the progression and development of HCC involving the processes of cell viability, apoptosis, and autophagy through the miR-5095/ATG12 axis and PI3K/AKT/mTOR signals.

1. Introduction

Hepatocellular carcinoma (HCC) remains to be one of the most prevalent cancers worldwide, with high rates of recurrence and poor prognosis [1,2]. Patients with HCC are at high risk for liver cirrhosis and other symptoms such as ascites, pain, and fatigue [3]. Surgical resection has been considered as the most effective therapy for HCC patients [4]; however, it probably generates new metastases and promotes the growth of existing micro-metastases [5]. Therefore, great efforts on exploring the molecular mechanism underlying HCC may throw light on the identification of molecular diagnostic markers or therapeutic targets for HCC.

Vast evidence has highlighted the regulatory effects of noncoding RNAs such as long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) in various human cancers [6–8]. LncRNAs are transcribed RNAs with a length of > 200 nucleotides and have little or no protein-coding capability [9,10]. In various cell biological performances of HCC, lncRNAs have been identified as key players [11–13].

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Furthermore, the competing endogenous RNAs (ceRNAs) hypothesis has attracted increasing attention. The hypothesis proposes that diverse RNA transcripts, such as lncRNAs and circRNAs, could be at crosstalk with each other by competing for shared miRNA binding sites and consequently maintain balance under normal circumstances or participate in disease conditions [14–16]. Various lncRNAs function as ceRNAs and play a crucial role in the pathogenetic mechanisms of HCC. For instance, lncRNA FAL1 is oncogenic and may promote HCC cell proliferation and invasion by functioning as a ceRNA of miR-1236 [17]. lncRNA KRAL may reverse the resistance of HCC cells to 5-fluorouracil by acting as a ceRNA against miR-141 [18]. lncRNA LINC01287 may regulate the pathological progress of HCC by acting as a ceRNA of miR-298 [19]. Despite this knowledge, the key lncRNAs closely associated with HCC remain to be further identified that could be promising biomarkers and potential targets for HCC.

HAGLROS is a 699-bp lncRNA that can function as a ceRNA to sponge miR-100-5p and consequently promote the malignant progression of gastric cancer [20]. The downregulation of HAGLROS may induce apoptosis and inhibit autophagy in colorectal cancer cells through sponging of the miR-100/ATG5 axis [21]. Furthermore, lncRNA LOC105369748 functions as a suppressor in the biology of HCC through the miR-5095/MBD2 axis [22], and SNHG7 is involved in the process of glioblastoma through miR-5095 [23]. However, to our knowledge, the roles and regulatory molecular mechanism of HAGLROS in HCC remain undiscovered.

In this study, we analyzed the level of HAGLROS in HCC tissues as well as its correlation with clinicopathological characteristics and prognosis of patients with HCC. Overexpression and silenced approaches were used for detecting the effects of lncRNA HAGLROS on cell viability, apoptosis, and autophagy. Furthermore, we investigated whether HAGLROS could function as a ceRNA to regulate miR-5095 expression in HCC cells, and explored the regulatory correlation between miR-5095 and ATG12. The regulatory correlation between HAGLROS and PI3K/AKT/mTOR signals was also detected. Our findings will facilitate understanding of HCC pathogenesis and development of effective therapies.

2. Materials and methods

2.1. Patient samples

Sixty-eight pairs of HCC tissues and matched nontumor tissues were taken from patients who were diagnosed with primary HCC after surgical resection. None of the patients enrolled in this study receive radiotherapy or chemotherapy before surgery. Clinical information of patients is given in Table 1. The researchers obtained permission from the ethics committee of our hospital (Approval No. DUHS-17-041). Written consents for the use of patients' tissues in the research were obtained from each patient.

2.2. Cell lines and cell culture

A normal liver cell line HL-7702 and hepatoma cell lines SK-Hep1, MHCC97L, MHCC97H, Huh7, and HepG2.2.15 were purchased from American Type Culture Collection. All cell lines were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS), 100 mg/mL streptomycin, and 100 U/mL penicillin, and then maintained in 5% CO_2 at 37 °C.

2.3. Transient transfection

Short hairpin RNA for HAGLROS (sh-HAGLROS) (100 nM, Sangon Biotech, Shanghai, China) and expression plasmids for HAGLROS (pEX2-HAGLROS, Sangon Biotech) were transfected in Huh7 or HepG2.2.15 cells to suppress or overexpress HAGLROS, respectively. sh-NC and empty vector pEX2 were used as the negative controls for si-HAGLROS and pEX2-HAGLROS, respectively. To regulate miR-5095 expression in cells, miR-5095 mimic (50 nM), mimic control (50 nM), miR-5095 inhibitor (150 nM), and inhibitor NC (150 nM) (Sangon Biotech) were also introduced in Huh7 or HepG2.2.15 cells. To overexpress ATG12, the full-length ATG12 coding sequence was inserted into pcDNA3.1 and then transfected in Huh7 or HepG2.2.15 cells, and the empty vector pcDNA3.1 was chosen as the negative control. Cell transfection was conducted using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, the cells were harvested for subsequent experiments.

Table 1
Correlation between HAGLROS expression and clinicopathological characteristics of patients with hepatocellular carcinoma.

Characteristics		Cases	HAGLROS expression		P value
			Low	High	
Gender	Male	38	21	17	0.468
	Female	30	17	13	
Age	< 60	28	16	12	0.653
	≥60	40	22	18	
T stage	T1–2	20	13	7	0.026
	T3–4	48	25	23	
N stage	N0	36	28	8	0.032
	N1–2	22	10	12	
M stage	M0	48	36	12	0.002
	M1	20	2	18	
Differentiation	Low	12	3	9	0.033
	Medium	48	30	18	0.033
	High	8	5	3	
Size	< 4.5 cm	32	18	14	0.536
	≥4.5 cm	36	20	16	

2.4. Real-time quantitative PCR (qPCR)

Total RNA from tissues or cells was extracted using Trizol reagent (Invitrogen), followed by reverse-transcription for complementary DNA (cDNA) synthesis with the M-MLV Reverse Transcriptase kit (Invitrogen). For detecting gene expression levels, real-time qPCR was performed with the standard SYBR Green PCR kit (Toyobo, Osaka, Japan) protocol and then run on Rotor-Gene RG-3000A (Corbett Life Science, Sidney, Australia). The obtained DNA samples were analyzed for relative quantitation of gene expression levels using the $2^{-\Delta\Delta Ct}$ method. U6 and β-actin were chosen as the internal controls for miRNAs and RNAs, respectively.

2.5. Cell viability assay

For detecting cell viability after treatment, approximately 2×10^3 Huh7 or HepG2.2.15 cells were seeded into a 96-well plate. After 24 h of incubation with different treatments, 20 μL of 5 mg/mL MTT (Sigma-Aldrich, St. Louis, USA) was added to each well, and the cells were incubated at 37 °C for 4 h. After centrifugation was performed for removing supernatant, 150 μL of dimethyl sulfoxide was mixed with the cells for 10 min. The absorbance of each well was measured at 470 nm using an MRX II absorbance reader (DYNEX Technologies, Chantilly, VA, USA).

2.6. Cell apoptosis assay

In order to assess cell apoptosis, flow cytometry was carried out. After 24 h of transfection, Huh7 or HepG2.2.15 cells at a cell density of 1×10^6 cells/mL were harvested, and then washed twice with pre-chilled PBS. Then, the cells were double-stained with FITC-Annexin V and propidium iodide (PI) according to the Annexin V-FITC Apoptosis Detection Kit (Sangon Biotech). The apoptotic cells were then analyzed by the BD LSRII Flow Cytometer System with FACSDiva Software

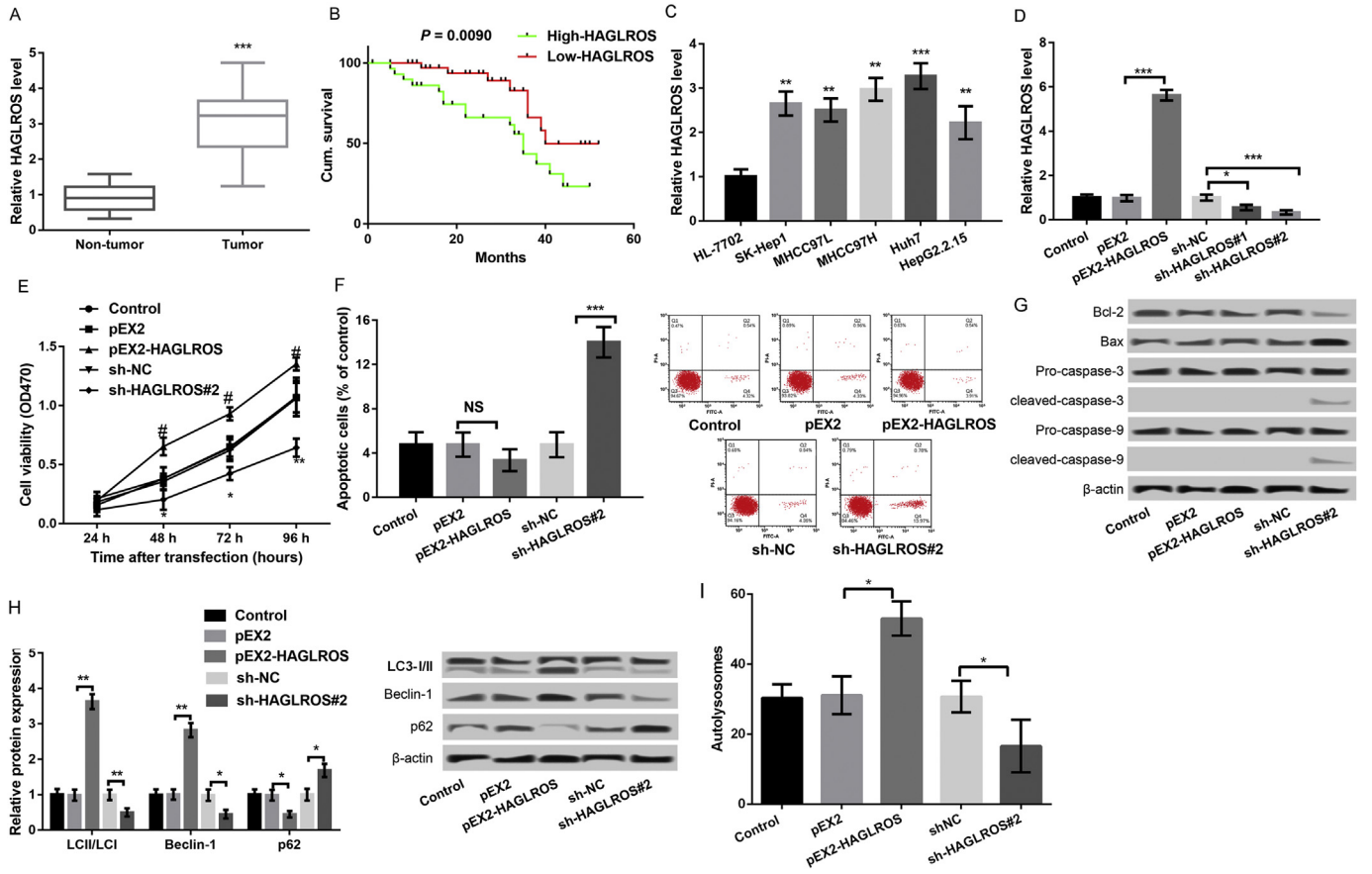

Fig. 1. High expression of HAGLROS in hepatocellular carcinoma (HCC) tissues and its correlation with shorter survival time in HCC patients, and suppression decreasing cell viability, promoting apoptosis, and inhibiting autophagy. A: High expression of HAGLROS in HCC tissues in comparison to that in the matched non-tumor tissues. B: Survival analysis revealing the correlation between higher expressions of HAGLROS and shorter survival time of patients with HCC. C: High expression of HAGLROS in hepatoma cell lines, including SK-Hep1, MHCC97L, MHCC97H, Huh7 and HepG2.2.15 in comparison with that in a normal liver cell line HL–7702. D: Overexpression and suppression of HAGLROS in Huh7 cells after transfection by pEX2-HAGLROS and sh-HAGLROS, respectively. E: MTT assay showing Huh7 cell viability in different transfected groups. F: Flow cytometry showing the percentage of apoptosis cells in different transfected groups. G: Western blot showing the expression levels of apoptosis-related proteins. H: Western blot showing the expression levels of autophagy markers in different transfected groups. I: LC3 II autolysosomes in cells with the silenced and up-regulated HAGLROS.
Data are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with corresponding controls.

within 1 h.

2.7. LC3 II punctuation assay

Huh7 cells were transfected with two vectors (GFP-LC3 and si-HAGLROS) for 24 h. The cells were then added into a 24-well plate for another 24-h incubation. Then, the cells were fixed and permeabilized with DAPI for 15 min. Autolysosomes in the cells were countered.

2.8. Dual-luciferase reporter assay

The pmiR-REPORT-HAGLROS-wt/mut and pMIR-REPORT-ATG12–3′UTR-wt/mut (Sangon Biotech) were constructed. Subsequently, Huh7 or HepG2.2.15 cells were transfected with these luciferase reporters along with miR-5095 mimic or mimic control. After 48 h of transfection, luciferase activities of these reporters were detected using a Dual-Luciferase Reporter Assay System (E1910, Promega, WI, USA). The targeted sequences (represented with bold and italic) in HAGLROS and miR-5095 used in this study are as follows: for HAGLROS, 5′-…GC ***GGUUC*** (position 90–94)…***CGCCUG*** (position 205–210) CUGGGGAG…-3′; for miR-5095, 3′-…GCGCCA ***CCAAG*** U ***GCGGAC*** AUU-5′.

2.9. RNA-binding protein immunoprecipitation

An RNA-Binding Protein Immunoprecipitation kit (17e700, Millipore, Billerica, MA, USA) was used to detect the relationship between HAGLROS and miR-5095. The kit included steps such as lysate collection, preparation of magnetic beads, immunoprecipitation, RNA purification, and qPCR analysis. For enrichment of HAGLROS and miR-5095, antiAgo2 (Millipore) was used with normal mouse Anti-IgG (Millipore) as a negative control.

2.10. Western blotting

Following different transfections, cells (Huh7 or HepG2.2.15) were obtained by centrifugation and were lysed with a cell lysis buffer (Sangon Biotech) for collecting total proteins. The proteins (50 μg/lane) were separated onto 12% SDS-polyacrylamide gels, and, subsequently, were transferred onto polyvinylidene fluoride membranes (Millipore). After incubation with the primary antibodies overnight at 4 °C (LC3 I, LC3 II, Beclin-1, P62, Bax, Bcl-2, pro-caspase-3, cleaved-caspase-3, pro-caspase-9, cleaved-caspase-9, ATG12, PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, and β-actin, 1:1000, Abcam, Cambridge, UK), the membranes were further incubated with the corresponding secondary antibodies. The protein signals were analyzed using the enhanced chemiluminescence method.

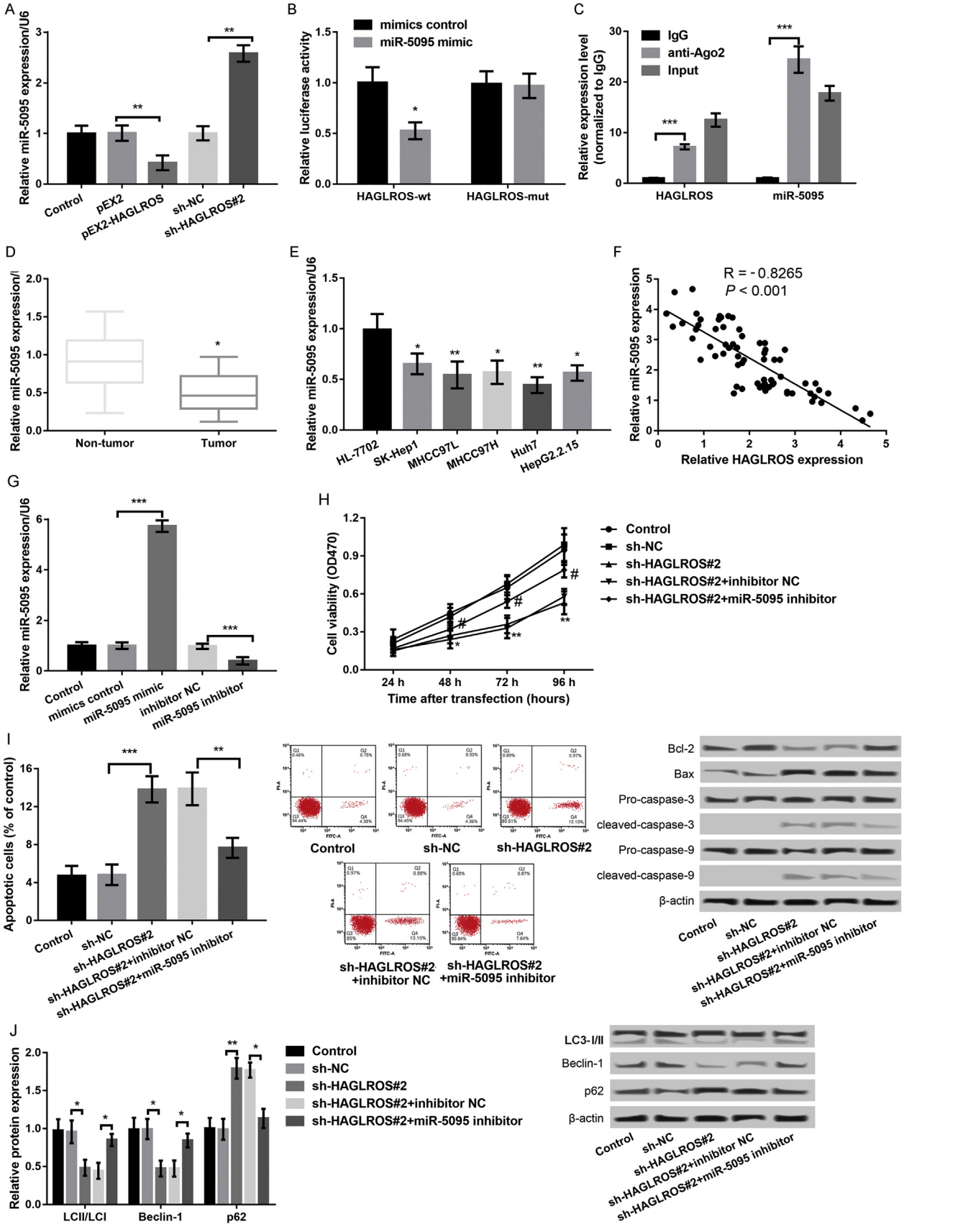

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Fig. 2. Negative regulation between HAGLROS and miR-5095 expressions, and HAGLROS regulating Huh7 cell viability, apoptosis, and autophagy through miR-5095. A: miR-5095 expression being significantly down-regulated in the pEX2-HAGLROS group and markedly up-regulated in the sh-HAGLROS#2 group relative to that in the corresponding control group. B–C: Dual-luciferase reporter and RIP assays further showing HAGLROS and miR-5095 in the same RNA-induced silencing complex. D–E: miR-5095 expression significantly decreases in HCC tissues, as well as in hepatoma cell lines. F: Pearson's correlation analysis confirming a negative correlation between HAGLROS expression and miR-5095 expression. G: miR-5095 overexpressed and inhibited in Huh7 cells by transfection with miR-5095 mimic and miR-5095 inhibitor, respectively. H: MTT assay showing Huh7 cell viability in different transfected groups. I: Flow cytometry showing the percentage of apoptosis cells in different transfected groups and western blot showing the expression levels of apoptosis-related proteins. J: Western blot showing the expression levels of autophagy markers in the different transfected groups. Data are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with corresponding controls.

2.11. Statistical analysis

All experiments were conducted independently for three times. The data are presented as the mean ± standard deviation (SD). All statistical analyses among the groups were analyzed using two-tailed Student's *t*-tests or Turkey's post-hoc tests in one-way ANOVA by using SPSS Statistics 20.0 software (IBM, Armonk, NY, USA). The correlation between the level of HAGLROS and clinicopathological characteristics of HCC patients was analyzed by chi-square test. The correlation between HAGLROS and miR-5095 was evaluated using Pearson's correlation coefficient. Survival analysis was analyzed by the Kaplan-Meier method, followed by the difference analysis between the survival curves by the log-rank test. $P < 0.05$ was chosen as a statistically significant result.

3. Results

3.1. HAGLROS level is high in HCC tissues and its level is correlated with clinical characteristics of patients with HCC

We investigated the correlation between HAGLROS and the clinical characteristics of patients with HCC and found that HAGLROS level was significantly higher in HCC tissues compared with that in the matched nontumor tissues ($P < 0.001$, Fig. 1A). According to the mean expression levels of HAGLROS in the total enrolled 68 HCC patients, 30 patients were classified into a high-HAGLROS expression group and the remaining 38 patients were classified into a low-HAGLROS expression group. Further survival analysis revealed that higher expression of HAGLROS was correlated with a shorter survival time of patients with HCC ($P = 0.0090$, Fig. 1B). Moreover, there were significant correlations between HAGLROS expression and tumor stage or tumor differentiation (Table 1).

3.2. Influences of HAGLROS suppression on cell viability, apoptosis, and autophagy

Fig. 1C shows that in comparison with a normal liver cell line, HL-7702, HAGLROS was also highly expressed in hepatoma cell lines, including SK-Hep1, MHCC97L, MHCC97H, HepG2.2.15 (all $P < 0.01$), and Huh7 ($P < 0.001$). Among these hepatoma cell lines, HAGLROS level was the highest in Huh7 cells; consequently, Huh7 cells were selected for the following experiments. HAGLROS was successfully overexpressed and suppressed in Huh7 cells by transfection with pEX2-HAGLROS and sh-HAGLROS, and the high transfection efficiency was confirmed by qPCR ($P < 0.001$, Fig. 1D). sh-HAGLROS#2 was selected for suppression experiments for its stronger inhibitory effects compared with sh-HAGLROS#1. Further experiments showed that suppression of HAGLROS by transfection of sh-HAGLROS#2 in Huh7 cells significantly decreased cell viability ($P < 0.05$, Fig. 1E) and markedly increased cell apoptosis ($P < 0.001$, Fig. 1F). Moreover, the suppression of HAGLROS remarkably inhibited Bcl-2 expression and enhanced the levels of apoptotic-related proteins including Bax, cleaved-caspase-3, and cleaved-caspase-9 in Huh7 cells (Fig. 1G). Furthermore, overexpression of HAGLROS at a high level significantly resulted in an increase in LC3II/LC3I, Beclin-1, and the total number of LC3 II autolysosomes, but it decreased P62 expression, and suppression of HAGLROS had opposite effects (all $P < 0.05$, Fig. 1H and I). All these results indicate that the suppression of HAGLROS could inhibit autophagy in Huh7 cells.

3.3. Negative regulation exists between HAGLROS and miR-5095

Previous evidence revealed that miR-5095 is targeted by lncRNA SNHG7 in glioblastoma [23], and is also involved in HCC via regulation by lncRNA LOC105369748 [22]. We thus investigated the correlation between HAGLROS and miR-5095 in HCC. miR-5095 was markedly downregulated in the pEX2-HAGLROS group compared with the pEX2 group, but it was markedly upregulated in the sh-HAGLROS#2 group compared with the sh-NC group ($P < 0.01$, Fig. 2A), implying a negative interaction between HAGLROS and miR-5095. Dual-luciferase reporter and RNA-binding protein immunoprecipitation (RIP) assays further presented that HAGLROS and miR-5095 were in the same RNA-induced silencing complex ($P < 0.05$, Fig. 2B and C), thus verifying their relationship. To further study the role of miR-5095 in HCC, we analyzed miR-5095 in HCC tissues and hepatoma cell lines. Data revealed that miR-5095 expression was markedly decreased in HCC tissues compared with matched nontumor tissues ($P < 0.05$, Fig. 2D), as well as in hepatoma cell lines, including SK-Hep1, MHCC97L, MHCC97H, Huh7, and HepG2.2.15, in comparison with a normal liver cell line, HL-7702 ($P < 0.05$, Fig. 2E). Furthermore, Pearson's correlation analysis confirmed that HAGLROS negatively interacted with miR-5095 ($R = -0.8265$, $P < 0.001$, Fig. 2F).

3.4. HAGLROS regulates cell viability, apoptosis, and autophagy through miR-5095 in Huh7 cells

In order to further explore whether HAGLROS regulated HCC development through miR-5095, miR-5095 was upregulated or suppressed in Huh7 cells by transfection with an miR-5095 mimic or an miR-5095 inhibitor, respectively, and their transfection efficiency was high enough for the following experiments ($P < 0.001$, Fig. 2G). The cells were then cotransfected with sh-HAGLROS#2 and an miR-5095 inhibitor. The results showed that the effects of HAGLROS suppression on Huh7 cell viability (Fig. 2H), apoptosis and apoptosis-related proteins (Fig. 2I), and autophagy-related markers (Fig. 2J) were markedly reversed by miR-5095 inhibition (all $P < 0.05$), indicating that the effects of HAGLROS on the viability, apoptosis, and autophagy of Huh7 cells were achieved by the negative regulation of miR-5095.

3.5. ATG12 is a target of miR-5095

A previous study showed that ATG12 expression is remarkably increased in hepatitis B virus-associated HCC [24]. ATG12 was reported as a potential target of miR-5095 based on the information of TargetScan (http://www.targetscan.org/cgi-bin/targetscan/vert_71/, Fig. 3A). A luciferase reporter assay showed that cotransfection with miR-5095 mimic markedly suppressed the luciferase activity of ATG12 3′UTR-wt ($P < 0.05$, Fig. 3B) but not ATG12 3′UTR-mut, meaning that miR-5095 could target ATG12. Furthermore, ATG12 mRNA and protein levels were markedly decreased in the miR-5095 mimic group but were markedly increased in the miR-5095 inhibitor group in relation to the levels in a corresponding control group ($P < 0.01$, Fig. 3C and D). All

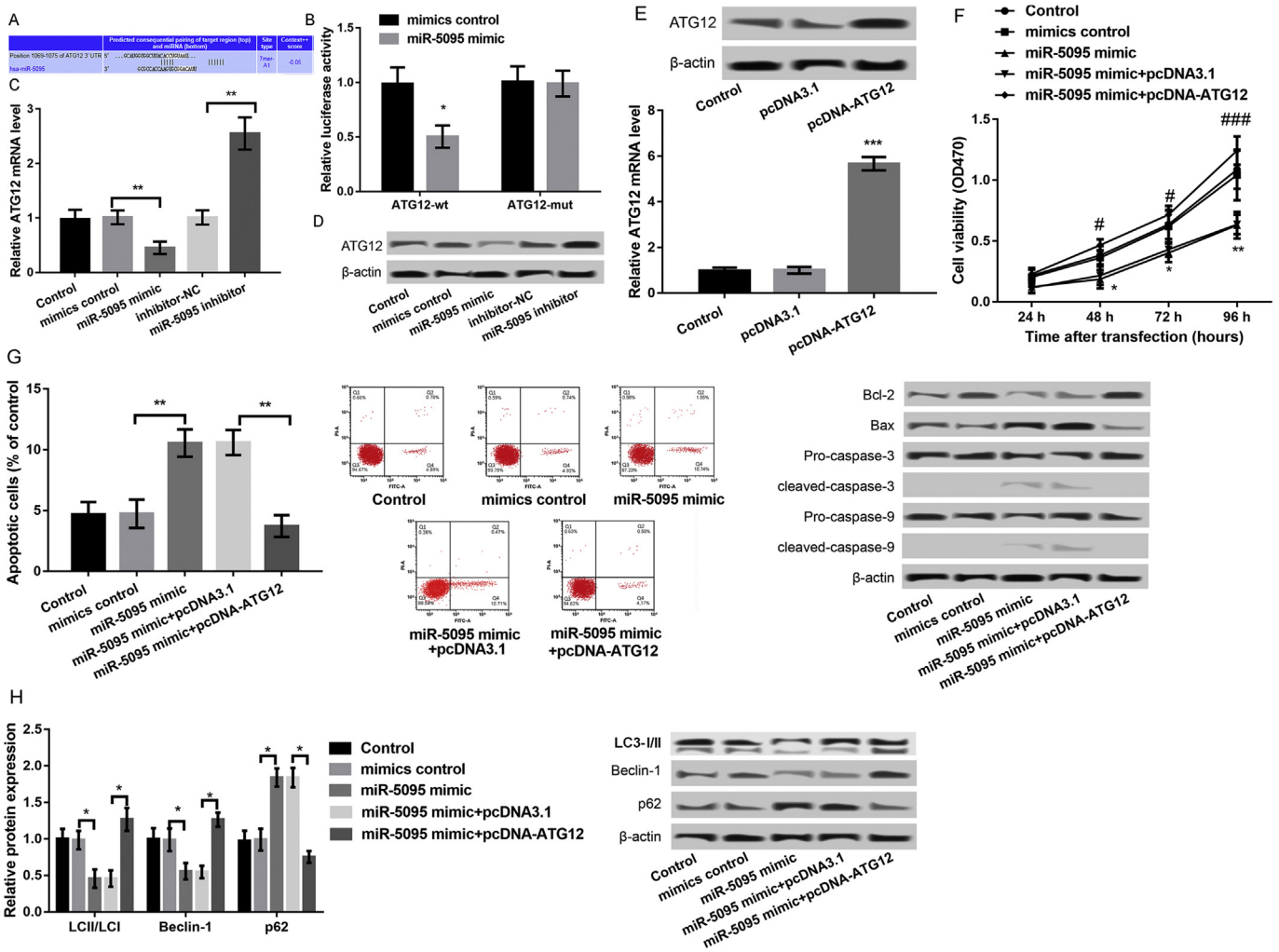

Fig. 3. ATG12 as the target of miR-5095, and miR-5095 regulating Huh7 cell viability, apoptosis, and autophagy via targeting ATG12. A: ATG12 identified as a potential target of miR-5095 based on the information of TargetScan. B: Luciferase reporter assay showing that co-transfection with miR-5095 mimic significantly inhibits the luciferase activity of ATG12 3′UTR-wt but not that of ATG12 3′UTR-mut. C–D: The expression of ATG12 at both mRNA and protein levels significantly decreases in the miR-5095 mimic group but markedly increases in the miR-5095 inhibitor group in relation to that in the corresponding control group. E: ATG12 overexpressed in Huh7 cells by transfection with pcDNA-ATG12. F: MTT assay showing Huh7 cell viability in the different transfected groups. G: Flow cytometry showing the percentage of apoptosis cells in the different transfected groups and western blot showing the expression levels of apoptosis-related proteins. H: Western blot showing the expression levels of autophagy markers in the different transfected groups. Data are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with corresponding controls.

these results show that ATG12 is negatively regulated by miR-5095.

3.6. miR-5095 regulates cell viability, apoptosis, and autophagy via targeting ATG12 in Huh7 cells

In order to confirm further whether miR-5095 regulated HCC cell viability, apoptosis, and autophagy by targeting ATG12, we overexpressed ATG12 in Huh7 cells by transfection with pcDNA-ATG12. The data revealed that ATG12 was significantly upregulated in Huh7 cells after transfection with pcDNA-ATG5 compared with pcDNA3.1 ($P < 0.001$, Fig. 3E). The results showed that the effects of miR-5095 overexpression on HCC cell viability (Fig. 3F), apoptosis, and expression of apoptosis-related proteins (Fig. 3G), and autophagy-related markers (Fig. 3H) were significantly reversed after synergistic co-transfection of an miR-5095 mimic and pc-ATG12 (all $P < 0.05$), confirming that ATG12 was a functional target of miR-5095, which could mediate the role of miR-5095 in HCC cells.

3.7. Verification experiments in HepG2.2.15 cells

In order to verify the effects of the HAGLROS-miR-5095-ATG12 axis in HCC, verification experiments were performed in HepG2.2.15 cells. Fig. 4A–C shows that HAGLROS, miR-5095, and ATG12 were successfully suppressed in HepG2.2.15 cells by transfection with sh-HAGLROS, an miR-5095 inhibitor, and si-ATG12, respectively (all $P < 0.01$). Further experiments showed that suppression of HAGLROS markedly decreased HepG2.2.15 cell viability ($P < 0.05$, Fig. 4D), promoted apoptosis ($P < 0.001$, Fig. 4E), decreased the expression levels of LC3II/LC3I and Beclin-1, but increased P62 expression ($P < 0.05$, Fig. 4F), which were markedly counteracted by the upregulation of HAGLROS and miR-5095 synchronously. Furthermore, silencing of ATG12 further reversed the combined inhibitory effects of HAGLROS and miR-5095 on HepG2.2.15 cell viability, apoptosis, and autophagy (all $P < 0.05$, Fig. 4D–F).

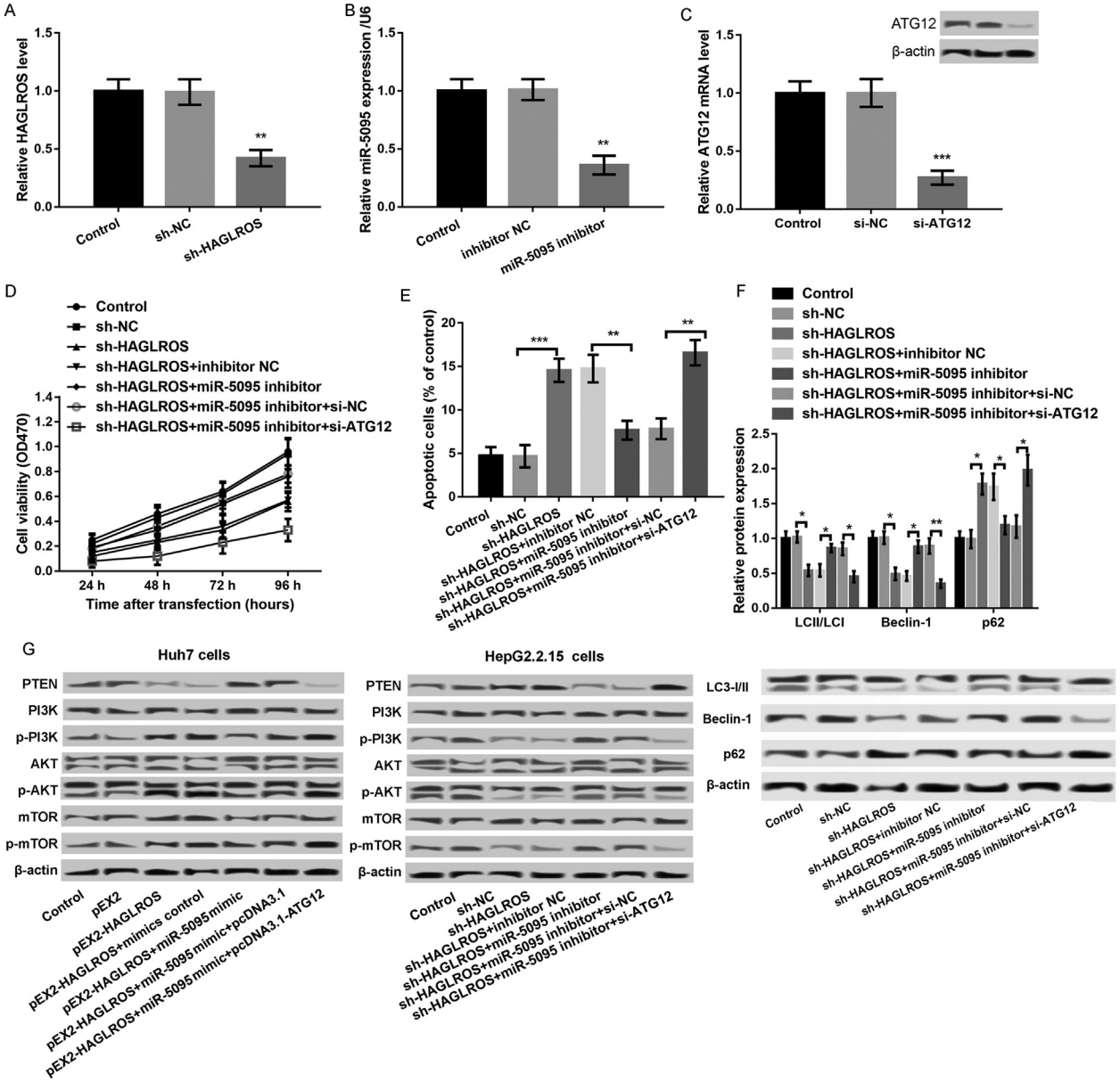

Fig. 4. Verification assay in HepG2.2.15 cells and the effects of HAGLROS-miR-5095-ATG12 axis in HCC cells achieved by PI3K/AKT/mTOR signals. A–C: Suppression of HAGLROS, miR-5095, and ATG12 in HepG2.2.15 cells by transfection with sh-HAGLROS, miR-5095 inhibitor, and si-ATG12, respectively. D: MTT assay showing HepG2.2.15 cell viability in different transfected groups. E: Flow cytometry showing HepG2.2.15 cell apoptosis in different transfected groups. F: Western blot showing the expression levels of autophagy markers in different transfected HepG2.2.15 cells. G: Western blot showing the expression levels of PI3K/AKT/mTOR signal-related proteins in transfected Huh7 and HepG2.2.15 cells. Data are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with corresponding controls.

3.8. Effects of the HAGLROS-miR-5095-ATG12 axis in HCC cells are achieved by PI3K/AKT/mTOR signals

The activation of PI3K/AKT/mTOR signals is widely involved in many aspects of biological and pathological processes [25]. We analyzed the influence of HAGLROS on PI3K/AKT/mTOR signals in HCC by the overexpressed and silenced approaches in Huh7 and HepG2.2.15 cells. The results revealed that the high expression of HAGLROS alone distinctly decreased the expression levels of PTEN but enhanced the expression of p-PI3K, p-AKT, and p-mTOR in Huh7 cells, which were remarkably suppressed after cotransfection of an miR-5059 mimic and pEX2-HAGLROS, which was then reversed by the pc-ATG12 transfection (Fig. 4G). Whereas the performances of PTEN, p-PI3K, p-AKT, and p-mTOR were all opposite in HepG2.2.15 cells with the silenced HAGLROS, and their protein levels were reversed by the cotransfection of an miR-5059 inhibitor and sh-HAGLROS, which was then reversed by si-ATG12 transfection (Fig. 4G). All these findings indicated that the effects of the HAGLROS-miR-5095-ATG12 axis in HCC cells were achieved through possible association with PI3K/AKT/mTOR signals.

4. Discussion

HCC is one of the deadliest cancers worldwide with a significant direct impact on public health [26]. Great progress has been made in understanding the molecular mechanisms underlying HCC, especially after the ceRNA hypothesis [14]. lncRNA was firstly discovered by Okazaki et al. [27] in 2002, which has become a research focus after miRNAs or genes. However, the crucial lncRNAs that participate in disease development are yet to be discovered and merit further investigation.

HAGLROS is recently identified in the development of several cancers [20,21,23]. Our study also investigated the role of HAGLROS in HCC. The results showed that HAGLROS was highly expressed in HCC tissues and correlated with shorter survival time, tumor stage, or tumor differentiation in patients with HCC. Moreover, suppression of HAGLROS decreased HCC cell viability, promoted apoptosis, but inhibited autophagy, whereas overexpression of HAGLROS had opposite effects. Given the role of HAGLROS in other cancers, our study data proved that HAGLROS may also play an oncogenic role in HCC.

Based on the ceRNA hypothesis, increasing studies have confirmed that lncRNAs play key roles in many diseases including cancers by functioning as ceRNAs to sponge miRNA and consequently regulate gene expression [28,29]. A previous study reported that LOC105369748 functions as a ceRNA to regulate the miR-5095/MBD2 axis in HCC [22]. miR-5095 mediates the oncogenic role of lncRNA LINC01296 in human cholangiocarcinoma [30], as well as regulates the role of lncRNA SNHG7 in promoting the progression of glioblastoma [23]. In this study, miR-5095 expression was markedly decreased in HCC tissues and hepatoma cells. Although the role of miR-5095 in HCC has been completely elucidated, we speculated that miR-5095 may function as a tumor suppressor in HCC. In addition, we found that miR-5095 inhibition reversed the effects of the suppression of HAGLROS on HCC cell viability, apoptosis, and autophagy, suggesting that miR-5095 inhibition may be pivotal in mediating the oncogenic role of HAGLROS in HCC.

ATG12 was discovered as a functional target of miR-5095. ATG12 is an important protein in the elongation of autophagosomes [31]. ATG12-mediated autophagy regulates radiosensitivity and chemoresistance in cancers [32,33]. Moreover, ATG12 expression was remarkably increased in hepatitis B virus-associated HCC [23]. In this study, the influence of highly expressed miR-5095 on HCC biological performances of viability, apoptosis, and autophagy were markedly reversed by the cotransfection of an miR-5095 mimic with pc-ATG12, suggesting that miR-5095 may regulate HCC development by targeting ATG12.

In addition, the high expression of HAGLROS decreased the level of PTEN but increased the expressions of p-PI3K, p-AKT, and p-mTOR in HCC cells, suggesting that HAGLROS could activate PI3K/AKT/mTOR signals. PI3K downstream signals are crucial in regulating cell proliferation and survival of human cancers [34]. H-116, a PI3K inhibitor, promotes apoptosis, and inhibits angiogenesis of HCC through PI3K/AKT/mTOR signals [34]. Xin et al. revealed that downregulation of NRSN2 could promote HCC cell proliferation and survival by regulating PI3K/AKT/mTOR signals [35]. Yang et al. demonstrated that inhibition of PI3K/AKT/mTOR signals by apigenin could promote apoptosis and autophagy in HCC cells [36]. Additionally, Chen et al. proved that HAGLROS indirectly interacted with an mTOR signal mediated by miR-100 in gastric cancer processes of autophagy and apoptosis [20], which is in accordance with our findings of the expression pattern of HAGLROS versus p-mTOR, as well as the influences of HAGLROS versus p-mTOR on cell autophagy (Fig. 4). After taking all the above findings into consideration, we speculate that HAGLROS is pivotal in contributing to HCC development by regulating the activation of PI3K/AKT/mTOR signals, and may, therefore, serve as a promising therapeutic target for the treatment of HCC.

To sum up, our results revealed that HAGLROS is highly expressed in HCC, and may promote cell proliferation and inhibit apoptosis but enhance autophagy in HCC cells by regulating miR-5095/ATG12 and PI3K/AKT/mTOR signals. The HAGLROS-miR-5095-ATG12 axis may throw a new insight on designing a promising therapeutic strategy for the treatment of HCC. However, we did not perform in vivo animal experiments to confirm the role of the HAGLROS-miR-5095-ATG12 axis. More studies are required to verify our findings.

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Conflict of interest

None.

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