

MAZ-LINC00645-GP73 Axis Promotes Hepatocellular Carcinoma Proliferation and Metastasis

Yang Chen

Tumor Hospital of Harbin Medical University

Huiyan Li

Tumor Hospital of Harbin Medical University

Chunxun Liu

Tumor Hospital of Harbin Medical University

Yongmei Han

Tumor Hospital of Harbin Medical University

Yubao Zhang

Tumor Hospital of Harbin Medical University

Yanlong Liu

Tumor Hospital of Harbin Medical University

Haitao Xu (✉ xuhaitao@hrbmu.edu.cn)

Tumor Hospital of Harbin Medical University <https://orcid.org/0000-0002-0317-8643>

Primary research

Keywords: LINC00645, MAZ, GP73, Progression, hepatocellular carcinoma (HCC), miR-141-3p

Posted Date: August 28th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-65401/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

BACKGROUND: Long non-coding RNAs (lncRNA) have been shown to play important roles in the development and progression of hepatocellular carcinoma (HCC). In this report, we examined the role of lncRNA LINC00645 in HCC.

MATERIAL AND METHODS: Based on public databases and integrating bioinformatics analyses, the over-expression of LINC00645 in HCC tissues was detected and further validated in a cohort of liver tissues. A series of in vitro and in vivo functional experiments were executed to investigate the role of LINC00645 in the carcinogenesis and development of HCC. Comprehensive transcriptional analysis, chromatin immunoprecipitation (ChIP) assay, dual-luciferase reporter assay and western blot etc. were performed to explore the molecular mechanisms underlying the functions of LINC00645.

RESULTS: LINC00645 was significantly upregulated in HCC cell lines and HCC tissues, which was correlated with poor prognosis in HCC patients. LINC00645 knockdown remarkably suppressed tumor growth in vitro and in vivo. Mechanistically, LINC00645 could competitively bind with miR-141-3p to prevent the degradation of its target gene GP73, which acts as a tumor-promoter in HCC. Furthermore, the ChIP assay showed that the transcription factor MAZ could bind to the LINC00645 promoter and increase its transcription.

CONCLUSIONS: Collectively, this study demonstrated that LINC00645 plays a critical regulatory role in hepatocellular carcinoma cells and LINC00645 may serve as a potential diagnostic biomarker and therapeutic target of HCC. Thus, targeting MAZ/LINC00645/miR-141-3p/GP73 signaling axis may prevent the progression of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and ranks as the third most common cause of cancer-related death[1]. Recently, great advances have been gained for the pathogenesis, diagnosis and treatment of HCC. However, the molecular mechanisms underlying HCC pathogenesis have not been fully understood and the survival of patients remains poor [2, 3]. Hence, much hope is placed in understanding the pathogenesis and exploring a novel strategy for the treatment of HCC.

Large amounts of studies report that dysregulation of oncogenes and tumor suppressor genes contribute to HCC tumorigenesis and progression, but most of them focus on protein-coding genes [4]. Only 2% of the human genome accounts for protein coding genes, while about 70% of the genome is identified as non-coding RNAs (ncRNAs) due to the great progressions of genome and transcriptome sequencing[5]. Long noncoding RNAs (lncRNAs), transcripts longer than 200nt that lack an extended open reading frame and thus do not code for proteins, have emerged as major regulators of a wide range of cellular processes [6]. In human cancer, the aberrant expression of lncRNAs has been associated with tumor development and progression. Moreover, lncRNAs regulate malignant behaviors of cancer cells [7], such

as proliferation, apoptosis resistance, migration, invasion and drug resistance. Aberrant expression of lncRNAs has been frequently observed in cancers. Mechanistically, lncRNAs may influence the function of transcriptional complexes, modulate chromatin structures by serving as scaffolds between proteins, or act as microRNA sponges [8]. Long intergenic non-protein coding RNA 645 (LINC00645) was first annotated as a long intergenic noncoding RNA (lincRNA) on chromosome 14q12. Recent studies have reported that LINC00645 plays an oncogenic role in glioma and it may serve as a prognostic biomarker and a potential therapeutic target for the treatment of glioma in humans[9]. However, the function and mechanism of LINC00645 as ceRNAs in HCC remain unclear.

Golgi protein 73 (GP73), a Golgi glycoprotein, is mainly expressed in bile duct epithelial cells, whereas it is rarely or seldom expressed in hepatocytes. [10, 11]. The serum GP73 levels increase in patients with viral hepatitis B or C, or other chronic liver diseases, which are superior to those of AFP[12, 13]. GP73 is a serum biomarker of liver fibrosis [14]and a potential biomarker for HCC [15]. However, the upstream regulatory factors of GP73 in HCC progression and metastasis have not been fully identified, this study was planned to fill this gap in literature.

Here, this study demonstrated that LINC00645, which was upregulated in HCC tissues compared with adjacent non-tumor tissues and that elevated LINC00645 levels were associated with poor prognosis in HCC patients. Moreover, the knockdown of LINC00645 significantly inhibited the malignant proliferation, invasion and metastasis of HCC cells. Mechanistically, LINC00645 promotes tumor proliferation by sponging miR-141-3p to regulate GP73. Consequently, establishing a new regulatory axis of the “MAZ-LINC00645- miR-141-3p -GP73” could better explore the cancer-promoting mechanism of LINC0645 in HCC.

Materials And Methods

Clinical Sample and Tissue Specimen Acquisition

Hepatocellular carcinoma specimens and adjacent noncancerous tissues were obtained from Harbin Medical University Cancer Hospital (HMUCH), and patients with a histological diagnosis of hepatocellular carcinoma who had received neither chemotherapy nor radiotherapy before surgical resection were recruited for the present study between 2009 and 2019. This study conformed to clinical research guidelines and was approved by the research ethics committee of Harbin Medical University Cancer Hospital. We obtained written informed consent from all patients.

Cell Culture

The human immortalized normal hepatocyte cell line LO2 and HCC cell lines HepG2, Hep3B, Huh7 and SMMC-7721 were obtained from the Chinese Academy of Sciences Cell Bank and Cellbio (China) and were cultured according to the suppliers' instructions. All cell lines were cultured according to instructions.

Plasmids and Transfection

LINC00645 and sh-LINC00645 and controls were constructed by Genechem (Shanghai, China). Concentrated viruses were used to infect 5×10^5 cells in a 6-well plate with 4–6 $\mu\text{g}/\text{ml}$ Polybrene. The infected cells were then subjected to selection with 1 $\mu\text{g}/\text{ml}$ puromycin (Cat#540411, Calbiochem, USA) for two weeks. Stable over-expression cell lines or knockdown cell lines were identified using qRT-PCR or western blotting. The miR-141-3p mimic and inhibitor were purchased from Ribobio (Guangzhou, China). Oligonucleotide or plasmid transfection into HCC cells was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were collected for further experiments at 48 hours after transfection. The shRNA sequences are listed in Table S1.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was prepared with a Transcriptor First Strand cDNA Synthesis Kit (Cat# 04897030001, Roche, USA). Real-time PCR was performed using FastStart Universal SYBR Green Master (ROX) (Cat#04913914001, Roche) on a 7500 Fast Real-Time PCR system (ABI, USA). For the quantification of gene expression, we used the $2^{-\Delta\Delta\text{Ct}}$ method. GAPDH or U1 expression was used for normalization. The primer sequences were synthesized by Genepharm (Shanghai, China). All the primer sequences are available in Table S2.

Subcellular Fractionation

Nuclear/cytoplasmic fractionation was performed by NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Cat#78835, Thermo Fisher) according to the manufacturer's protocols. U1 was used as a nuclear control, while GAPDH was used as a cytoplasmic control.

Cell Proliferation and Colony Formation Assay

Cell proliferation was measured by the CCK-8 method and colony formation method. Briefly, in the CCK-8 experiment, 1×10^3 cells were cultured in 96-well plates at 37°C . 96 well plates were incubated with 10 μl of CCK-8 solution per well for 1 hour. Cell proliferation curves were drawn by measuring the absorbance at 450 nm at each indicated time point. The cell proliferation curves were plotted by measuring the 450 nm absorbance at each indicated time point. Experiments were performed in triplicate. For the colony formation assay, cells were exposed to the indicated treatments, were seeded in 6-well plates and were cultured for 2 weeks. Cell colonies were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and imaged using an optical microscope.

Ethynyl Deoxyuridine (EdU) Incorporation Assay

A Cell-Light™ EdU Apollo567 In Vitro Kit (Catalogue Number C10310–1, RiboBio, China) was used to perform the EdU proliferation assay according to the manufacturer's instructions as previously described.

Cell Migration and Invasion Assay

Cell migration and invasion assays were performed as described previously. In a wound healing assay, cells were seeded in 6-well plates to form a confluent monolayer. Then, a scratch wound was induced by

a pipette tip. Photographs of cells migrating to the scratched area were taken and the data were shown as a percentage of the control group. In the transwell invasion and migration assay, cells (5×10^4 cells per well) were seeded in the upper chambers of the transwell plate and placed in FBS-free medium with or without matrix gel. After 24 hours of incubation, the cells that invaded/migrated to the lower surface of the membrane were fixed, stained with crystal violet and observed using an inverted microscope.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using a commercially available kit (Beyotime) according to the manufacturer's protocol. Briefly, cells were cross-linked with 1% formaldehyde and were sonicated on ice to create 200–500 bp fragments. Stained chromatin was cultured overnight with an anti-MAZ antibody (Novus Biologicals, NB100-86984, 1:50) or IgG (Cell Signaling Technology, Cat#3900) as an isotype control. The precipitated chromatin DNA was recovered and analyzed by qRT-PCR. The primer sequences are shown in Table S2

RNA Immunoprecipitation (RIP)

RIP assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation (RIP) Kit (EMD Millipore). HepG2 cells lysis solution (Sigma Aldrich Chemical Company, USA) was added to 3 mg of cells and left to incubate at 4 °C for 1 h. Cells were centrifuged at 12,000 g at 4 °C for 10 min in order to collect the supernatant, which was employed for RIP experiments using an anti-AGO2 antibody (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.. The RNA fraction isolated by RIP was subjected to qRT-PCR analysis to identify the direct binding between linc00645 and miR-141-3p. These experiments were repeated three times.

Western Blot Analysis

Cells were lysed with RIPA extraction reagent (Beyotime) supplemented with a protease inhibitor cocktail (Roche). Proteins were separated by 6%-15% SDS-PAGE, transferred to 0.22 mm polyvinylidene fluoride membranes (Millipore), and then incubated with antibodies. The bands on the blots were captured by using an Odyssey Infrared Imaging System (LI-COR Biosciences) and were quantified with Odyssey v1.2 software (LI-COR Biosciences). GAPDH was used as the internal controls. Antibodies against the following proteins were used. MAZ (Abcam, ab85725,1:1000), GP73 (Santa Cruz, sc-365817,1:500), E-cadherin(Cell Signaling Technology, Cat#3195,1:1000), N-cadherin(Cell Signaling Technology ,Cat#4061,1:1000), Cyclin D1 (Cell Signaling Technology ,Cat#2978,1:1000), GAPDH (Cell Signaling Technology Cat#5174,1:1000). Alexa Fluor® 800 goat anti-mouse (LI-COR Biosciences, Cat#926-32210, 1:10000) or anti-rabbit (LI-COR Biosciences, Cat#926-32211, 1:10000) was used as a secondary antibody.

Immunohistochemical (IHC) Analysis

The paraffin-embedded sections were dewaxed in xylene and rehydrated in alcohol. Endogenous peroxidase was blocked by 3% H₂O₂, and microwave heating was performed for antigen retrieval. After blocking nonspecific antigen binding with 5% BSA at 37 °C for 1 h, the sections were incubated with a specific primary antibody against Ki67 (Abcam, ab15580, 1:1000), at 4 °C overnight. After incubating with

the corresponding secondary antibodies (Abcam, ab205718) at 37 °C for 1 h, the sections were stained with diaminobenzidine and counterstained with hematoxylin. Representative images were taken using an Olympus light microscope

Luciferase Reporter Assays

HepG2 cells were seeded at 5×10^4 cells/well in 24-well plates and were cultured overnight. On the next day, the cells were cotransfected with pmirGLO-LINC00645-WT, pmirGLO-LINC00645-MUT, pmirGLO-GP73-3'UTRWT, pmirGLO-GP73-3'UTR-MUT reporter plasmids (Genechem, Shanghai), NC mimics or miR-141-3p mimics. Twenty-four hours posttransfection, cells were lysed using passive lysis buffer (Promega), and the luciferase activity was measured by a GloMax20/20 Luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega) and was normalized to the Renilla luciferase activity.

Tumor Xenograft Model

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Harbin Medical University Cancer Hospital and was performed in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council). Mouse xenograft models were established using 4-week-old BALB/c nude female mice. HepG2 cells (5×10^5 per injection) that were transfected with sh-LINC00645 and sh-control, respectively, were implanted into the mice via subcutaneous injection. Tumor volumes were measured every 3 days after being apparently observed and calculated with the following formula: Volume = (length \times width²)/2. After 4 weeks, all mice were sacrificed under anesthesia.

Bioinformatics Analysis

The Cancer Genome Atlas (TCGA) (<https://tcga-data.nci.nih.gov>) was used to compare the expression of LINC00645 in HCC tissues and normal by using R software. The LncBook (<https://bigd.big.ac.cn/lncbook>), starBase V3.0 (<http://starbase.sysu.edu.cn/>) and TargetScan (<http://www.targetscan.org/>) databases were used to examine putative miRNA interactions between LINC00645 and GP73. The MAZ binding motif in the promoter region of LINC00645 was identified by TRAN SFAC (<http://gene-regulation.com/>) and JASPAR (<http://jaspar.genereg.net/>).

Statistical Analysis

Data are expressed as mean \pm SEM. And all data represent at least three independent experiments. Statistical analysis was performed using unpaired Student t-test or 1-way ANOVA followed by Tukey post hoc analysis. $P < 0.05$ was considered statistically different and indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

LINC00645 Expression Is Up-regulated in HCC Cell lines, HCC Tissues and Associated With Poor Prognosis

To investigate the expression of LINC00645 in HCC, we search TCGA data from starBase V3.0. The results showed that LINC00645 in HCC tissues was significantly higher than that in normal liver tissues (Figure.1A, $p < 0.01$). To further validate this result, we investigated the expression of LINC00645 in 40 paired HCC tissues and adjacent non-tumor tissues from Harbin Medical University Cancer Hospital (HMUHC). These results showed that the expression of LINC00645 was markedly increased in HCC tissues compared with adjacent non-tumor tissues (Figure.1B, $p < 0.001$). Meanwhile, we observed that the expression of LINC00645 also observed in HCC cell lines (HepG2, Hep3B, SMMC-7721 and Huh7) compared to LO2 cells (Figure.1C, $p < 0.001$). Moreover, the patients were divided into high ($n = 23$) or low ($n = 17$) LINC00645 level group according to the mean level of LINC00645 in cancer tissues. The patients with high LINC00645 level displayed poor overall survival (OS) compared with those in low expression group ($p = 0.0389$) (Figure.1D). To further explore the clinical characteristics connected with LINC00645 in HCC, we examined the correlation of LINC00645 expression with patients' clinicopathological characteristics in HCC. As illustrated in Table 1, patients with high LINC00645 expression exhibited a dramatic association with tumor size ($P = 0.014$), TNM stage ($P = 0.012$), venous invasion ($P = 0.08$), and lymph node metastasis ($P = 0.017$), while there were no significant association between LINC00645 expression with Gender, age, liver cirrhosis, and alpha fetoprotein (AFP) level ($P > 0.1$). The results of nuclear/cytoplasmic RNA fractionation from the subcellular distribution assay confirmed that LINC00645 was mainly located in the cytoplasm (Figure.1E and F). Collectively, these findings revealed that high expression of LINC00645 may be involved in tumor cell proliferation, invasion and LINC00645 may be an oncogene in HCC.

Table 1
Correlation between LINC00645 expression and the clinicopathological features of HCC patients

Characteristics	No.	LINC00645 expression	P-value
	(n = 40)	Low (n = 17)	High (n = 23)
Gender			
Male	33	12	20
Female	7	5	3
Age(y)			
< 55	18	9	9
≥ 55	22	8	14
Tumor size			
< 5 cm	23	10	9
≥ 5 cm	17	7	14
TNM stage			
I + II	20	12	8
III + IV	20	5	15
Liver criihosis			
Without	5	2	4
With	45	15	19
AFP levels(ng/ml)			
< 20	11	4	7
≥ 20	29	13	16
Venous invosion			
Absent	19	13	8
Present	11	4	5
Lymph node metastasis			
no	23	14	10
yes	17	6	13

LINC00645 Knockdown Inhibited HCC Cell Proliferation and Invasion in vitro

To determine the biological function of LINC00645 in HCC cells, short interference shRNAs against human LINC00645 (Sh-1 and Sh-2) were applied to knockdown LINC00645 expression, whereas full-length recombinant plasmid (Lv-LINC00645) with LINC00645 was used to increase LINC00645 expression. The knockdown and over-expression efficiency were confirmed by RT-PCR (Fig. 2A, $p < 0.001$ and Fig. 2D, $p < 0.001$). CCK-8 and colony formation assays revealed that depletion of LINC00645 inhibited the growth and proliferation of HepG2 and Hep3B cells (Fig. 2B-C, $p < 0.001$), while LINC00645 over-expression promoted cell growth and proliferation in HepG2 cell lines (Fig. 2E and F, $p < 0.001$). EdU incorporation assays also indicated that knocking down LINC00645 prominently suppressed the growth of HepG2 and Hep3B cells (Fig. 2G, $p < 0.01$). Furthermore, we then investigated the role of LINC00645 in the motility of HCC cells. The results showed that LINC00645 knockdown significantly impaired the migration and invasion of HCC cells (Fig. 2H, $p < 0.001$). Given that epithelial-mesenchymal transition (EMT) is one of the major mechanisms for cancer metastasis, we further evaluated the effect of LINC00645 on EMT-related markers[9]. Western blot analysis showed that LINC00645 knockdown could increase the expression of epithelial markers (E-cadherin) and decrease the expression of mesenchymal markers (N-cadherin) (Fig. 2I), indicating that LINC00645 could regulate the EMT process to modulate HCC progression. Taken together, these data show that LINC00645 promotes HCC cell growth and invasion of HCC in vitro.

LINC00645 Acted as a Sponge For miR-141-3p in HCC Cells

Recently, many lncRNAs have been reported to function as competing endogenous RNAs (ceRNAs) in modulating the expression and biological functions of miRNAs[16, 17]. Since LINC00645 was distributed predominantly in the HCC cell cytoplasm, we hypothesized that LINC00645 might act as a miRNA sponge to prevent miRNAs from binding with their target mRNAs. Through prediction of the bioinformatics database [microrna.org](http://microrna.org/microrna/getMirnaForm.do) (<http://microrna.org/microrna/getMirnaForm.do>) and [LncBook](https://bigd.big.ac.cn/lncbook) (<https://bigd.big.ac.cn/lncbook>), we found that there were potential binding sites between LINC00645 and miR-141-3p. To validate the above theory, we subcloned the wild-type (LINC00645-WT) and mutated (LINC00645-MUT) miR-141-3p binding sites into dual-luciferase reporters (Fig. 3A). The luciferase assay showed that transfection of miR-141-3p mimics significantly reduced the relative luciferase activity of LINC00645-WT-treated HCC cells, but did not affect that of LINC00645-MUT-treated HCC cells (Fig. 3B). The AGO2 immunoprecipitation assay showed that the AGO2 antibody was able to pull down both endogenous LINC00645 and miR-141-3p (Fig. 3C and D). To determine the relationship between LINC00645 and miR-141-3p, we used RT-qPCR assay to evaluate miR-141-3p expression in HCC patients. Interestingly, the level of miR-141-3p significantly decreased in HCC tissues compared with non-tumor liver tissues (Fig. 4E, $p < 0.01$). Analysis of our HCC database revealed that LINC00645 was inversely correlated with the expression of miR-141-3p in the HCC tissues (Fig. 3F). In contrast to LINC00645, miR-141-3p expression in HCC cell lines was much lower than that in LO2 cells (Fig. 3G). In addition, miR-141-3p was upregulated when the HCC cells were transfected with LINC00645 shRNAs (Fig. 3H). However, LINC00645 was down-regulated when the HCC cells were transfected with miR-141-3p mimics (Fig. 3I).

Taken together, the above data suggested that LINC00645 acts as a molecular sponge for miR-141-3p in HCC cells.

GP73 is the Direct Target of MiR-141-3p in HCC Cells

By using starBase V3.0, miRWalk, miRPathDB, and TargetScan databases, we found that Golgi protein 73 (GP73) was a potential target of miR-141-3p (Fig. 4A). Recent studies reported that GP73, a golgi glycoprotein, was reported to be oncogenes in HCC[18, 19]. According to this inference, we performed the luciferase reporter assays and confirmed that the repression of luciferase activity was diminished in HepG2 and Hep3B cells cotransfected with the miR-141-3p mimics and GP73-WT 3'-UTR (Fig. 4B). Meanwhile, we examined a set of HCC tumors and paired adjacent normal tissues from HCC patients. qRT-PCR results showed that GP73 expression was significantly increased in tumors compared to adjacent non-tumor tissues (Fig. 4C). Then, we detected the mRNA levels of GP73 after miR-141-3p over-expression in HepG2 and Hep3B cells. We found that miR-141-3p over-expression reduced the level of GP73 mRNA (Fig. 4D-E). Furthermore, GP73 protein expression was significantly down-regulated by miR-141-3p mimics in both HepG2 and Hep3B cells (Fig. 4F). In short, these data implied that GP73 was a direct target of miR-141-3p in HCC cells.

LINC00645-miR-141-3p-GP73 Axis Promotes HCC cells Proliferation and Metastases

In light of the above findings, we hypothesized that LINC00645/miR-141-3p/GP73 axis might play a role in the progression of HCC. Restored experiments were performed introducing the miR-141-3p inhibitor into cells of LINC00645 knockdown. Remarkably, a reduction in the GP73 mRNA (Fig. 5H, $p < 0.01$) and protein amount (Fig. 5I, $p < 0.01$) in HepG2 and Hep3B cells, as a result of LINC00645 knockdown, were countered when the miR-141-3p inhibitor was co-transfected. The recovery of GP73 by the mediation of miR-141-3p inhibitor inhibited the effects of the LINC00645 silencing on HepG2 and Hep3B cells proliferation (Fig. 5A and B, $p < 0.01$), colony formation (Fig. 5C and D, $p < 0.001$), migration and invasion (Fig. 5E and F, $p < 0.001$). Western blot analysis showed that the expression of E-cadherin was decreased and the N-cadherin was increased (Fig. 5G). Taken together, the LINC00645 could play an oncogenic role by miR-141-3p/GP73 axis in HCC cells.

The Downregulation of LINC00645 Inhibited HCC Tumor Growth In Vivo

To elucidate the biological roles of LINC00645 in HCC tumorigenesis in vivo, we inoculated nude mice with HepG2 cells that stably expressed lentiviral shLINC00645 to suppress LINC00645 expression (Fig. 6A). Tumor xenografts with down-regulated LINC00645 showed markedly reduced volumes and weights compared to control xenografts (Fig. 6B and E). Tumor tissues were harvested for qRT-PCR analysis of LINC00645. We confirmed that lower expression of LINC00645 was detected in tumor tissues arising from the LINC00645 knockdown group compared to the control group (Fig. 6F). Moreover,

immunohistochemistry (IHC) assays confirmed that LINC00645 knockdown caused increased Ki67 expression (Fig. 6G), indicating reduced cell proliferation. Besides, GP73 and miR-141-3p expression in the tumor tissues were detected by the qRT-PCR analysis. GP73 expression was observed to be reduced (Fig. 6I), whereas miR-141-3p expression was enlarged (Fig. 6H). Similarly, GP73 protein was sharply down-regulated in the sh-LINC00645 group compared with the sh-NC group (Fig. 6J) by Western blot analysis. Together, these results suggested that knockdown LINC00645 significantly suppressed HCC tumorigenesis *in vivo*.

MAZ Transcriptionally Regulates LINC00645 Expression in HCC Cells

To examine the possible transcription factor-binding sites in promoter loci of LINC00645 in hepatocellular cancer, we searched the TRAN SFAC (<http://gene-regulation.com/>) and JASPAR (<http://jaspar.genereg.net/>) databases to identify transcription factors that may regulate LINC00645. The transcription factor Myc-associated zinc finger protein (MAZ) was predicted by both the TRAN SFAC and JASPAR databases with high scores (Supporting Information, Table S3). The predicted binding sites of MAZ in the LINC00645 promoter sequence are illustrated in Fig. 7A. To determine the differences between the expression levels of MAZ in HCC and normal tissues, the mRNA levels of MAZ in HCC and normal tissues were analyzed based on TCGA and GTEx data in the GEPIA platform and HMUCH (Harbin Medical University Cancer Hospital) database respectively. The results from different databases showed to be similar results from each other. The mRNA expression levels of MAZ were up-regulated in patients with HCC in GEPIA and HMUCH database, respectively (Fig. 7B and C). To explore whether MAZ regulated LINC00645 expression, we knocked down MAZ by transfecting with siRNA in HepG2 cells, which led to a significant decrease in LINC00645 expression (Fig. 7D and E). Furthermore, the over-expression of MAZ significantly elevated LINC00645 expression in HepG2 cells (Fig. 7F and G). Moreover, ChIP assays showed that the LINC00645 promoter was specifically pulled down by a MAZ-specific antibody but not the control antibody (Fig. 7H). Taken together, these findings suggested that MAZ is a bona fide transcriptional activator of LINC00645.

Discussion

Emerging evidence indicates key regulatory roles of lncRNAs in HCC [20, 21]. However, evidence of lncRNAs with clinical prognostic value is still limited. Ideally, in addition to exhibiting HCC-specific expression patterns, lncRNAs should be demonstrated to regulate clear mechanistic pathways that drive the growth of HCC to support their potential use as a therapeutic target during clinical treatment [22]. By using clinical specimens, *in vitro* hepatoma cell lines, and xenograft/orthotopic mice models, we demonstrated that a novel lncRNA, LINC00645, plays a key role in HCC growth. Our data showed that over-expression of LINC00645 could be used to predict the prognostic outcome of HCC patients' overall survival ratios. Alteration of endogenous cellular LINC00645 expression influenced the sensitivity of both hepatoma cells and HCC tumors. In addition, LINC00645 promoted HCC growth partly via binding to miR-

141-3p to accelerate GP73 expression in HCC progression. Therefore, our study provides clinical and mechanistic data to support the role of this lncRNA in HCC.

Previously defined mechanisms of lncRNAs in cancer growth include regulation of viability, proliferation, immortality, mobility and angiogenesis [23–25]. Additionally, HCC-related lncRNAs that have previously been characterized as target mRNAs [26], promoter regions [27, 28], or proteins [26] to exert their regulatory effects on HCC cells. Our study provides a novel lncRNA LINC00645 to regulate cancer growth and to serve as a potential prognostic marker.

MiRNAs are usually acted as the regulatory targets of lncRNAs that involved in the development of human cancers. MiRNAs regulate target genes in two different ways: 1) At the transcriptional level, miRNAs can activate or suppress target genes by binding to their promoters [29, 30]; 2) At the post-transcriptional level, miRNAs can repress target gene translation or cause target mRNA degradation by binding to their 3'UTR regions [31, 32]. In addition, miR-141-3p has been verified to suppress the growth and metastasis of tumors in multiple diseases in hepatocellular carcinoma [33] and its expression was substantially reduced in HCC cells and tissue samples [34]. In this study, LINC00645 primarily localized to the cytoplasm of HCC cells, suggesting that LINC00645 might exert the functions of ceRNA. Next, LncBook (<https://bigd.big.ac.cn/lncbook>) was employed as a means of predicting LINC00645 targeting miRNAs, with the identified miR-141-3p being of particular interest based on its biological function in HCC. MiR-141-3p was down-regulated in HCC tissues, serving as a tumor suppressor that targets several genes to inhibit proliferation and invasion in these cancer cells.[35–37] Luciferase reporter and RIP assays further confirmed miR-141-3p was a LINC00645 target in HCC. miR-141-3p expression was substantially decreased in HCC tissues, and its expression was negatively correlated with LINC00645. Moreover, changes to cell proliferation, cycle arrest, migration and invasion upon LINC00645 knockdown could be partially reversed by miR-141-3p inhibitors in HCC cells. These results suggested that LINC00645 promoted HCC progression via modulating miR-141-3p.

In this study, we chose GP73, a specific serum marker for HCC diagnosis and prognosis, as a target gene to identify upstream miRNAs that potentially regulates HCC progression. GP73 plays important roles in regulating HCC cellular and molecular processes. It has been reported that GP73 is a promising serum marker for diagnosis of HCC with high sensitivity (76%), specificity (86%), and diagnostic odds ratio (18.59) [38]. GP73-SphK1sR-Ad5 serves as a novel OA and can inhibit HCC progression with high specificity and efficacy [39]. Some studies have indicated that highly expressed GP73 promotes the migration and invasion of HCC[19], but the molecular processes are far more complete. In the previous study, it has been found that miR-141-3p over-expression up-regulated E-cadherin protein expression (epithelial marker) [40], and down-regulated N-cadherin and vimentin expression (mesenchymal markers), whereas over-expression of GP73 reversed expression levels of these markers. We found that miR-141-3p has the greatest potential to regulate GP73 expression and affect HCC progression. Our luciferase assay showed that miR-141-3p mimics significantly inhibited GP73 wild type 3'UTR activity, whereas the mimics had no effects on mutant 3' UTRs, suggesting that miR-141-3p regulates GP73 expression at the post-transcriptional level.

In conclusion, LINC00645 was up-regulated in HCC tissues and correlated with the poor prognosis of HCC patients. Silencing the expression of LINC00645 inhibited the proliferation, migration and invasion, and promoted the apoptosis of HCC cells via targeting miR-141-3p. Down-regulated expression of LINC00645 also repressed the xenograft tumor growth in rats. Moreover, we provided evidence that MAZ has potential binding sites with LINC00645 and down-regulated expression of MAZ repressed the LINC00645 expression in HCC cells, which suggested that MAZ transcriptionally regulates LINC00645 expression in hepatocellular carcinoma cells. Thus, MAZ and LINC00645 may serve as potential prognostic biomarkers and therapeutic targets for HCC.

Declarations

Conflict of Interest

The authors declare that they have no conflict of interests.

Funding

The research and manuscript preparation are funded by Haitao Xu.

This work was supported by the Foundation of Education Department of Heilongjiang Province (No. 12541411).

Ethics approval

This study conformed to clinical research guidelines and was approved by the research ethics committee of Harbin Medical University Cancer Hospital. We obtained written informed consent from all patients. The animal study protocol was approved by the Institutional Animal Care and Use Committee of Harbin Medical University Cancer Hospital and was performed in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council).

Availability of data and material

The data and material are available on reasonable request.

Consent for publication

Written informed consent for publication was obtained from all participants.

Authors' Contribution

Guarantor of integrity of the entire study: Haitao Xu; Study concepts: Haitao Xu; Study design: Haitao Xu; Literature research: Yang Chen; Clinical studies: Huiyan Li; Animal experiments: Yang Chen, Chunxun Liu; Molecular assay: Yongmei Han, Yubao Zhang; Data acquisition: Yang Chen; Statistical analysis: Yanlong

Liu, Haitao Xu; Manuscript preparation: Yang Chen; Manuscript editing: Yang Chen; Manuscript review: Haitao Xu, Yang Chen.

Acknowledgements

N/A.

References

1. Bertuccio P, Turati F, Carioli G, Rodriguez T, La Vecchia C, Malvezzi M, Negri E. Global trends and predictions in hepatocellular carcinoma mortality. *J Hepatol*. 2017;67(2):302–9.
2. Chen PJ, Furuse J, Han KH, Hsu C, Lim HY, Moon H, Qin S, Ye SL, Yeoh EM, Yeo W. Issues and controversies of hepatocellular carcinoma-targeted therapy clinical trials in Asia: experts' opinion. *Liver Int*. 2010;30(10):1427–38.
3. Bruix J, da Fonseca LG, Reig M. Insights into the success and failure of systemic therapy for hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol*. 2019;16(10):617–30.
4. Hemminki K, Hemminki A, Forsti A, Sundquist K, Li X. Genetics of gallbladder cancer. *Lancet Oncol*. 2017;18(6):e296.
5. Yan X, Hu Z, Feng Y, Hu X, Yuan J, Zhao SD, Zhang Y, Yang L, Shan W, He Q, et al. Comprehensive Genomic Characterization of Long Non-coding RNAs across Human Cancers. *Cancer Cell*. 2015;28(4):529–40.
6. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet*. 2016;17(1):47–62.
7. Beermann J, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches. *Physiol Rev*. 2016;96(4):1297–325.
8. Schmitt AM, Chang HY. Long Noncoding RNAs in Cancer Pathways. *Cancer Cell*. 2016;29(4):452–63.
9. Li C, Zheng H, Hou W, Bao H, Xiong J, Che W, Gu Y, Sun H, Liang P. Long non-coding RNA linc00645 promotes TGF-beta-induced epithelial-mesenchymal transition by regulating miR-205-3p-ZEB1 axis in glioma. *Cell Death Dis*. 2019;10(10):717.
10. MO R, C FSHL, BC P SSB, N P-H PS. C H, B M et al: Golgi phosphoprotein 2 (GOLPH2) expression in liver tumors and its value as a serum marker in hepatocellular carcinomas. *Hepatology*. 2009;49(5):1602–9.
11. RD K, X C, GA B, EM B. CJ F: Expression of GP73, a resident Golgi membrane protein, in viral and nonviral liver disease. *Hepatology*. 2002;35(6):1431–40.
12. X HWBLRZ. H, Y H, Y Q, J H, X L, X L: Serum GP73, a marker for evaluating progression in patients with chronic HBV infections. *PloS one*. 2013;8(2):e53862.
13. Z X, K W LLXP, H MWLL. Y, Q L: Serum Golgi protein 73 (GP73) is a diagnostic and prognostic marker of chronic HBV liver disease. *Medicine*. 2015;94(12):e659.

14. LM W, CJ DHSLFWPF. F: Hepatocyte GP73 expression in Wilson disease. *Journal of hepatology*. 2009;51(3):557–64.
15. Y M, H Y, H X, X L, X S, S D, H Z, W C, Y X, T C et al: **Golgi protein 73 (GOLPH2) is a valuable serum marker for hepatocellular carcinoma**. *Gut* 2010, 59(12):1687–1693.
16. Ren N, Jiang T, Wang C, Xie S, Xing Y, Piao D, Zhang T, Zhu Y: **LncRNA ADAMTS9-AS2 inhibits gastric cancer (GC) development and sensitizes chemoresistant GC cells to cisplatin by regulating miR-223-3p/NLRP3 axis**. *Aging (Albany NY)* 2020, **12**.
17. Gugnoni M, Manicardi V, Torricelli F, Sauta E, Bellazzi R, Manzotti G, Vitale E, de Biase D, Piana S, Ciarrocchi A: **Linc00941 is a novel TGFbeta target that primes papillary thyroid cancer metastatic behavior by regulating the expression of Cadherin 6**. *Thyroid* 2020.
18. Chen X, Wang Y, Tao J, Shi Y, Gai X, Huang F, Ma Q, Zhou Z, Chen H, Zhang H, et al. mTORC1 Up-Regulates GP73 to Promote Proliferation and Migration of Hepatocellular Carcinoma Cells and Growth of Xenograft Tumors in Mice. *Gastroenterology*. 2015;149(3):741–52 e714.
19. Mao Y, Yang H, Xu H, Lu X, Sang X, Du S, Zhao H, Chen W, Xu Y, Chi T, et al. Golgi protein 73 (GOLPH2) is a valuable serum marker for hepatocellular carcinoma. *Gut*. 2010;59(12):1687–93.
20. Q FWHYZDYS. F, Z Y: HOX Antisense lincRNA HOXA-AS2 Promotes Tumorigenesis of Hepatocellular Carcinoma. *Cellular physiology biochemistry: international journal of experimental cellular physiology biochemistry pharmacology*. 2016;40:287–96.
21. C LDJNWH. Y, H L: Upregulation of Long Non-Coding RNA PlncRNA-1 Promotes Metastasis and Induces Epithelial-Mesenchymal Transition in Hepatocellular Carcinoma. *Cellular physiology biochemistry: international journal of experimental cellular physiology biochemistry pharmacology*. 2016;38(2):836–46.
22. G L, R V, M S, F M, A S, M B: **Long non-coding RNAs as novel therapeutic targets in cancer**. *Pharmacological research* 2016, 110:131–138.
23. AM S. HY C: Long Noncoding RNAs in Cancer Pathways. *Cancer cell*. 2016;29(4):452–63.
24. C L, J C, K Z, B F, R W, L C: **Progress and Prospects of Long Noncoding RNAs (lncRNAs) in Hepatocellular Carcinoma**. *Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology* 2015, 36(2):423–434.
25. Neuzillet C, Tijeras-Raballand A, Cohen R, Cros J, Faivre S, Raymond E, de Gramont A. Targeting the TGFbeta pathway for cancer therapy. *Pharmacol Ther*. 2015;147:22–31.
26. JK ZH, C ZYPWH. H: The role of long noncoding RNAs in hepatocellular carcinoma. *Mol Cancer*. 2020;19(1):77.
27. K JWJZBS. Y, J X, W G, L Z: Long noncoding RNA lncTCF7, induced by IL-6/STAT3 transactivation, promotes hepatocellular carcinoma aggressiveness through epithelial-mesenchymal transition. *Journal of experimental clinical cancer research: CR*. 2015;34:116.
28. LX L, XT WD, RP Z, MQ C, YT X, ZJ G, GF PRL, LF W. W: The mechanism of adenosine-mediated activation of lncRNA MEG3 and its antitumor effects in human hepatoma cells. *Int J Oncol*. 2016;48(1):421–9.

29. MA BK, GI A, W SSODWRR. F: Transcriptional control of gene expression by microRNAs. *Cell*. 2010;140(1):111–22.
30. BA J, ST Y, DB H, KE RR. H, DR C: Activating gene expression in mammalian cells with promoter-targeted duplex RNAs. *Nature chemical biology*. 2007;3(3):166–73.
31. G H, PD Z: **A microRNA in a multiple-turnover RNAi enzyme complex**. *Science (New York, NY)* 2002, 297(5589):2056–2060.
32. H F, W B: **Effect of etafenone on total and regional myocardial blood flow**. *Arzneimittel-Forschung* 1975, 25(9):1400–1403.
33. CZ L, ZH Y, RQ JM, HW H, ZG L. P, G C: **A qRT-PCR and Gene Functional Enrichment Study Focused on Downregulation of miR-141-3p in Hepatocellular Carcinoma and Its Clinicopathological Significance**. *Technology in cancer research & treatment* 2017:1533034617705056.
34. L JY. LncRNA SNHG15 promotes hepatocellular carcinoma progression by sponging miR-141-3p. *Journal of cellular biochemistry*. 2019;120(12):19775–83. T, Y F, H X, L W, Y D, K L.
35. YF JX, G NJH, LX P, YH W. Y, YQ L: miR-141 suppresses the growth and metastasis of HCC cells by targeting E2F3. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology Medicine*. 2014;35(12):12103–7.
36. MK K, YA M, CK S. R B, S B, SG Y: Tumor-suppressing miR-141 gene complex-loaded tissue-adhesive glue for the locoregional treatment of hepatocellular carcinoma. *Theranostics*. 2018;8(14):3891–901.
37. SM W, DY HWA, XQ Z, Q H, XL P. Z: MiR-141 targets ZEB2 to suppress HCC progression. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology Medicine*. 2014;35(10):9993–7.
38. X YZ Y, J Y, B Z: **Golgi protein 73 versus alpha-fetoprotein as a biomarker for hepatocellular carcinoma: a diagnostic meta-analysis**. *BMC cancer* 2012, 12:17.
39. YH B, XJ Y. Y X, T Z, X S, YJ G: A novel oncolytic adenovirus inhibits hepatocellular carcinoma growth. *Journal of Zhejiang University Science B*. 2019;20(12):1003–13.
40. X H, X LYXJZL, S X L. G L, J L: Role of microRNA-141-3p in the progression and metastasis of hepatocellular carcinoma cell. *Int J Biol Macromol*. 2019;128:331–9.

Figures

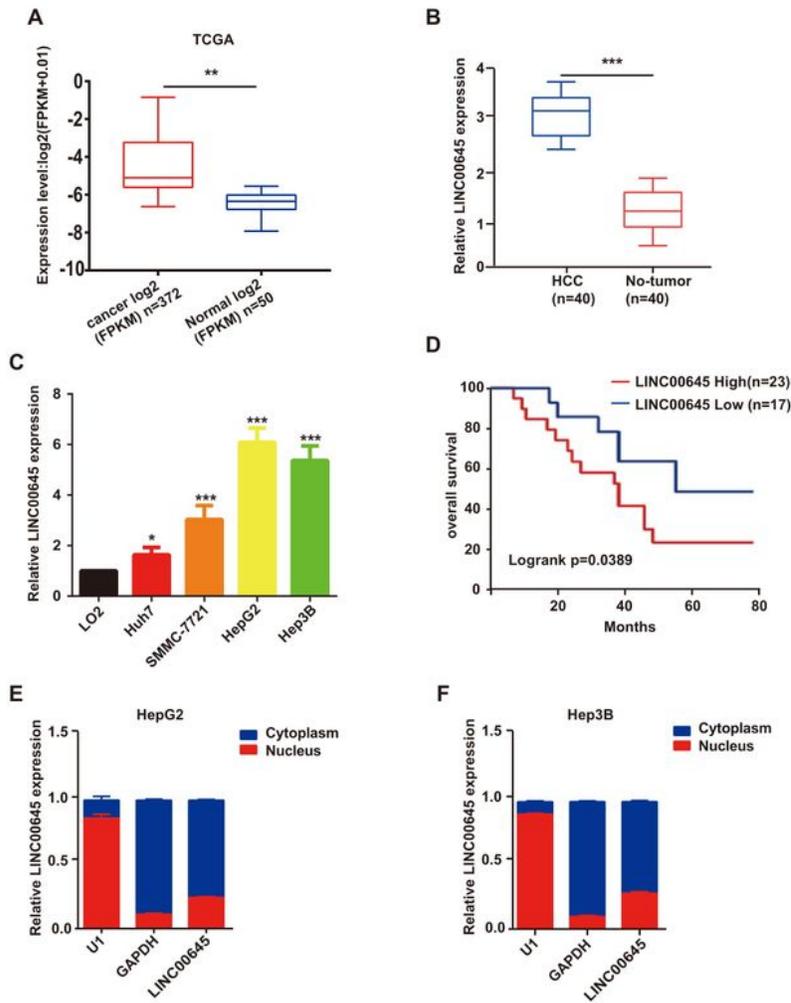


Figure 1

LINC00645 expression is up-regulated in HCC and associated with poor prognosis. (A) The levels of LINC00645 in HCC tissues were obviously higher than that in normal liver tissues in the TCGA database from starBase V3.0 platform. $P < 0.001$ by Student's t-test. (B) The expression of LINC00645 in 40 pairs of HCC and matched noncancerous tissues was measured by qRT-PCR. (C) RT-PCR analysis was used to detect the expression of LINC00645 in HCC cell lines. (D) Kaplan-Meier survival analysis revealed that

HCC patients with high LINC00645 expression showed a significantly poorer overall survival compared to those with low LINC00645 expression. (E and F) The expression levels of LINC00645 in the subcellular fractions of HepG2 and Hep3B cells were detected by qRT-PCR. Error bars represented the mean \pm SD of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

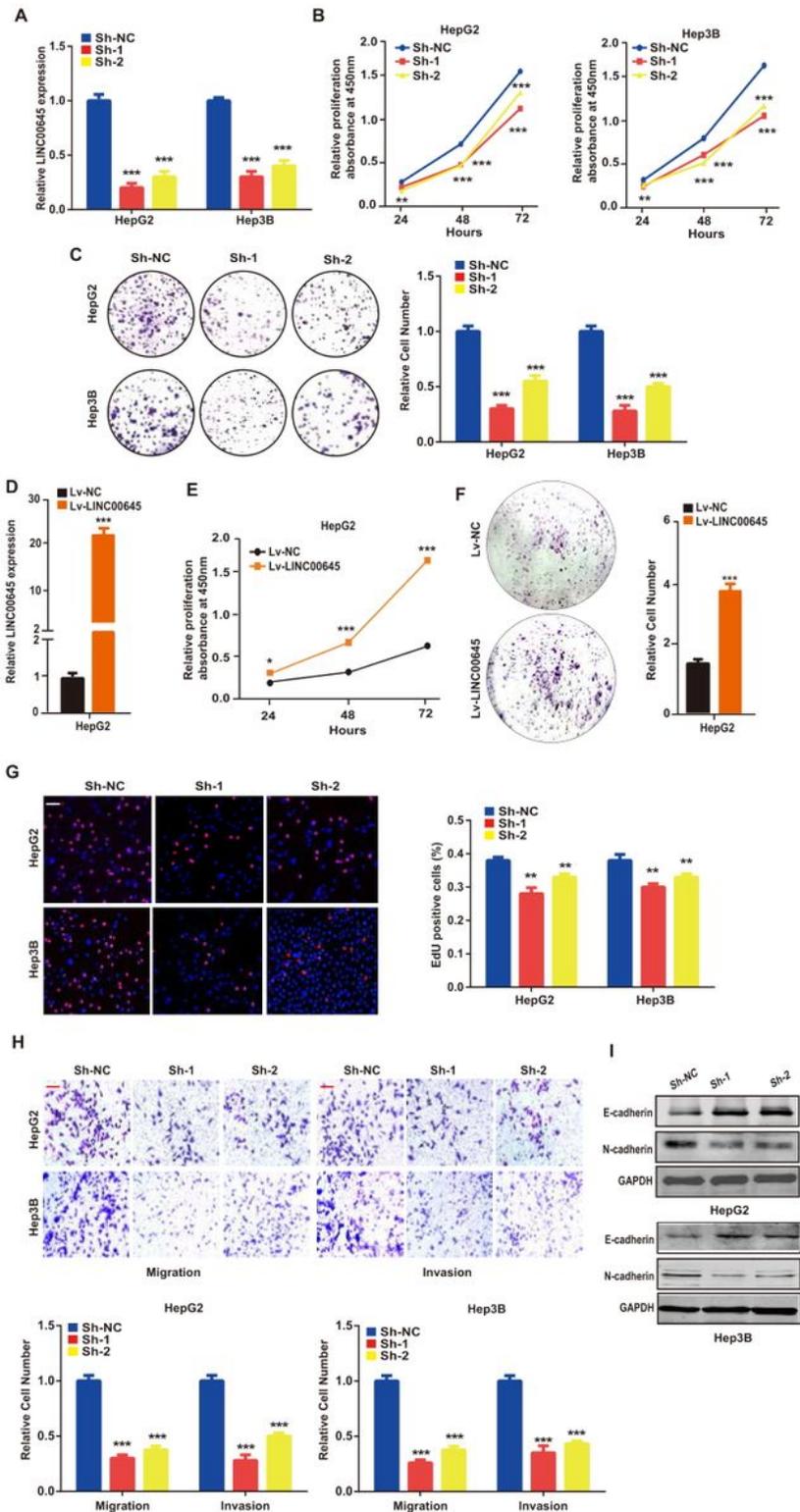


Figure 2

LINC00645 promotes HCC cell proliferation, apoptosis and invasion in vitro. (A) The expression levels of LINC00645 in HepG2 and Hep3B cells after transfection with sh-NC or sh-LINC00645 were detected by RT-PCR. (B-C) The effects of LINC00645 knockdown on the proliferation of HepG2 and Hep3B cells were examined by CCK8 assay (B) and colony formation assays (C). (D) The expression levels of LINC00645 in HepG2 cells after transfection with Lv-NC or Lv-LINC00645 were detected by RT-PCR. (E-F) The effects of LINC00645 overexpression on the proliferation of HepG2 cells were examined by CCK8 assay (E) and colony formation assays (F). (G) EdU assays were used to detect the proliferation rate of HepG2 and Hep3B cells after LINC00645 knockdown. (H) Transwell migration and invasion assays were used to evaluate the motility of HepG2 and Hep3B cells transfected with sh-NC or sh-LINC00645. (I) The expression level of E-cadherin is increased, while the expression levels of N-cadherin are decreased in sh-LINC00645 cells. Error bars represented the mean \pm SD of at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001.

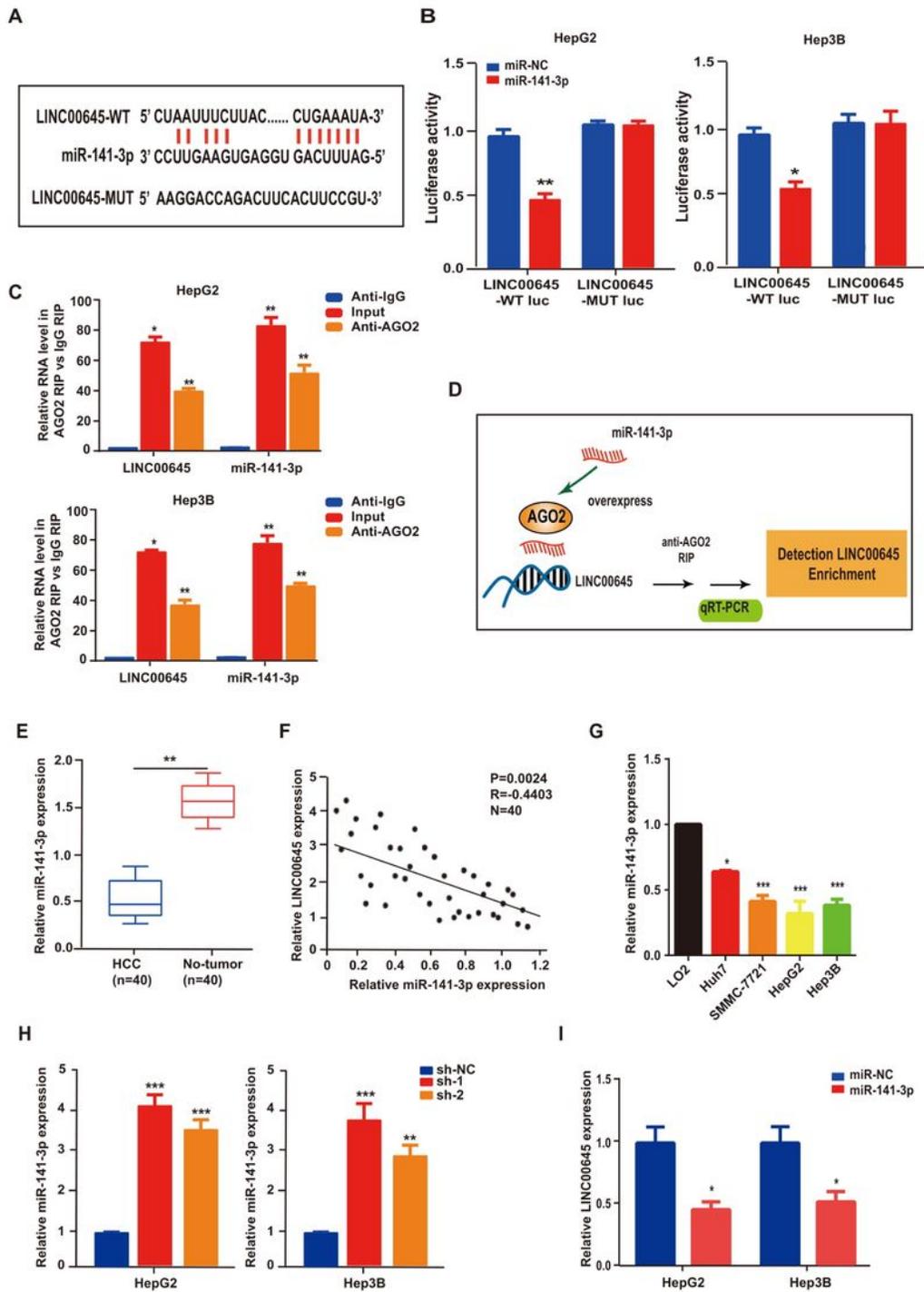


Figure 3

LINC00645 acted as a sponge for miR-141-3p in HCC cells. (A) The potential binding sites of miR-141-3p and LINC00645. (B) Relative luciferase activities of wild type (WT) and mutated (MUT) LINC00645 reporter plasmid in HepG2 and Hep3B cells co-transfected with miR-141-3p mimics. (C and D) Association between LINC00645 and miR-141-3p with Ago2. (E) The RT-qPCR assays were utilized to measure miR-141-3p expression in 40 paired HCC tissues and adjacent non-tumor tissues. (F) Pearson's

correlation analysis determined the relationship between LINC00645 and miR-141-3p expression in 40 HCC tissues. (G) Relative expression of miR-141-3p in HCC cell lines compared with that in LO2 cell. (H) Relative miR-141-3p expression after LINC00645 knockdown in HepG2 and Hep3B cells. (I) Detection of LINC00645 expression by RT-qPCR after over-expression of miR-141-3p in HepG2 and Hep3B cells. Error bars represented the mean \pm SD of at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001.

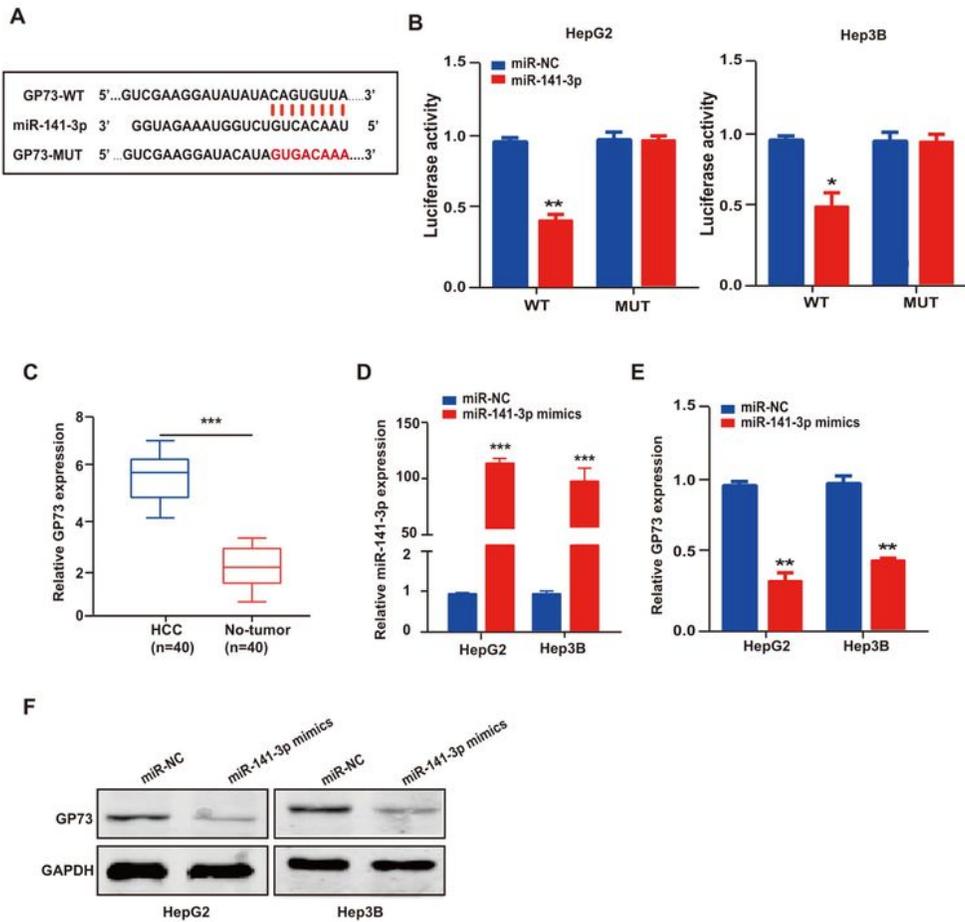


Figure 4

GP73 is a direct target of miR-141-3p in HCC cells. (A) The potential binding sites of miR-141-3p and GP73. (B) The relative luciferase activity of reporters harboring the GP73-Wt or GP73-Mut in HepG2 and Hep3B cells transfected with miR-141-3p mimics. (C) The expression of GP73 in 40 pairs of HCC and matched noncancerous tissues was measured by qRT-PCR. (D) The relative mRNA expression of miR-141-3p was detected in HepG2 and Hep3B cells transfected with miR-141-3p mimics. (E) The relative mRNA expression of GP73 was detected in HepG2 and Hep3B cells transfected with miR-141-3p mimics or miR-NC. (F) The relative protein expression of GP73 was detected in HepG2 and Hep3B cells transfected with miR-141-3p mimics or miR-NC. Error bars represented the mean \pm SD of at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001.

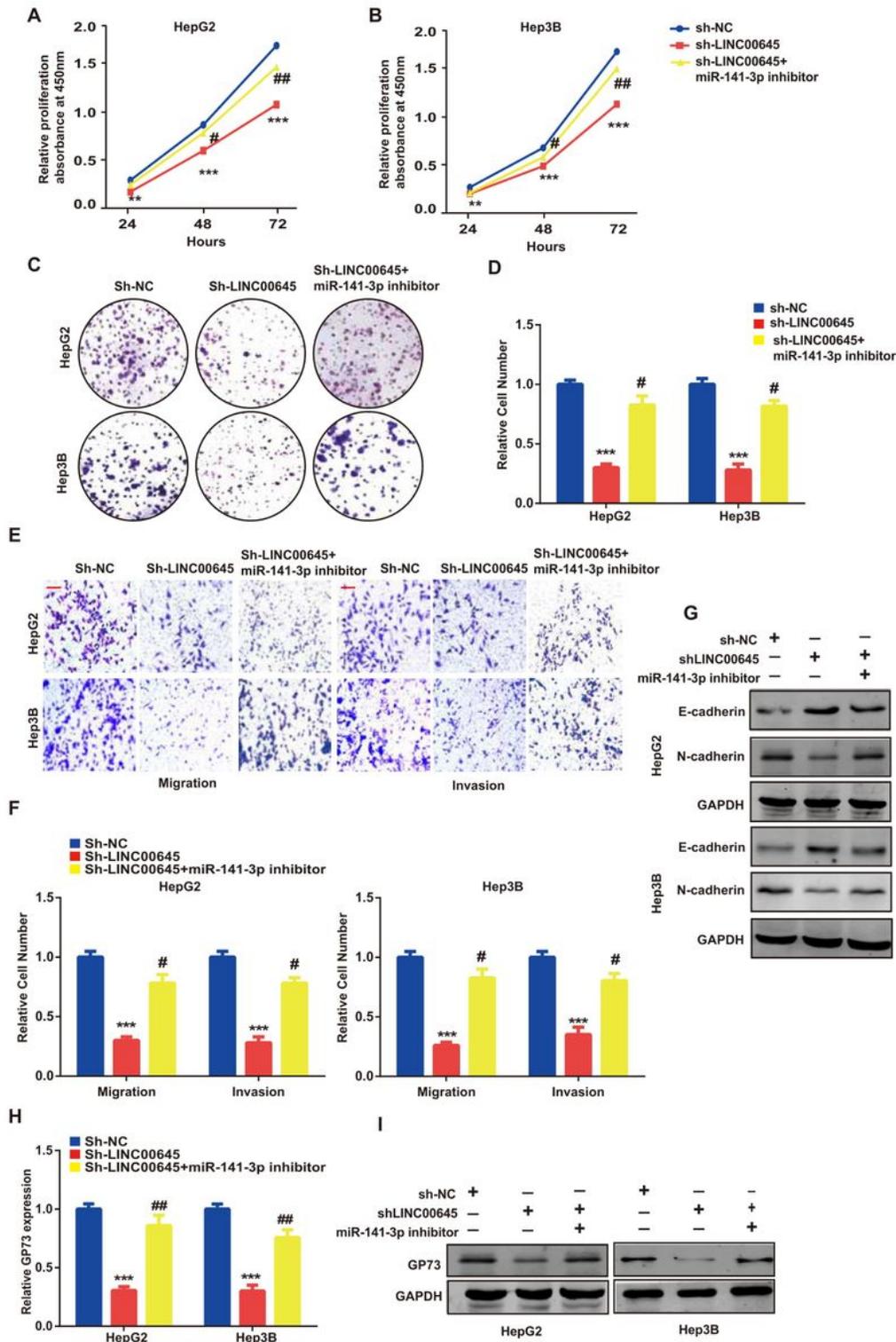


Figure 5

LINC00645-miR-141-3p/632-GP73 axis promotes HCC cell proliferation and metastases. (A-D) The cell proliferation was determined by CCK8 (A and B) and colony formation (C and D) assays in sh-LINC00645 and miR-141-3p inhibitor transfected in HepG2 and Hep3B cells. (E and F) Transwell migration and matrigel invasion assays were conducted to detect the migratory and invasive abilities in sh-LINC00645 and miR-141-3p inhibitor transfected in HepG2 and Hep3B cells. (G) Western blot analysis detected the

expression of E-cadherin and the N-cadherin protein. (H and I) mRNA and protein levels of the GP73 were detected by qRT-PCR assays (H) and western blot (I) in sh-LINC00645 and miR-141-3p inhibitor transfected in HepG2 and Hep3B cells. Error bars represented the mean \pm SD of at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001.

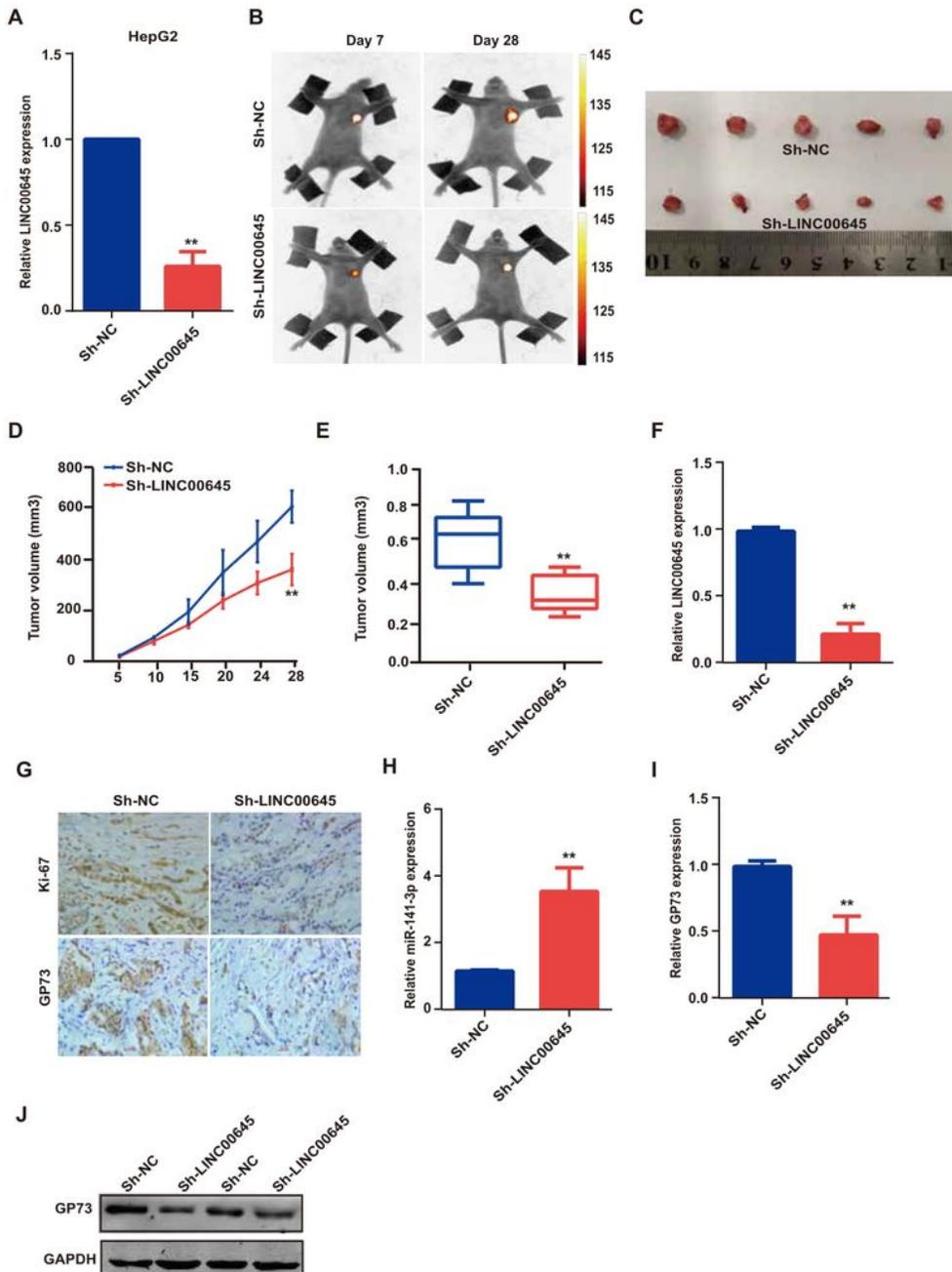


Figure 6

LINC00645 promotes tumor growth in HCC. (A) Knockdown of LINC00645 by shRNA in HepG2 cells was confirmed by qRT-PCR. (B) Luminescence images of subcutaneous tumors in xenograft mouse models bearing tumors generated from HepG2 cells that were stably transfected with sh-LINC00645 or sh-NC on days 7 and 28 after tumor cell injection. (C-E) Tumor volume and weight in mice treated with sh-LINC00645 or sh-NC. Tumor volume was calculated every 5 days, n=5. (F) xenograft mouse tissues were subjected to qRT-PCR for LINC00645 expression. (G) Images of Ki67 staining in the tumor. Immunohistochemical staining revealed that LINC00645 knockdown led to reduced expression of Ki67. (Scale bar: 50 μ m). (H) qRT-PCR for miR-141-3p expression in Xenograft mouse tissues. (I and J) The mRNA and protein levels of the GP73 were detected by qRT-PCR assays (I) and western blot (J) in xenograft mouse tissues. Error bars represented the mean \pm SD of at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001.

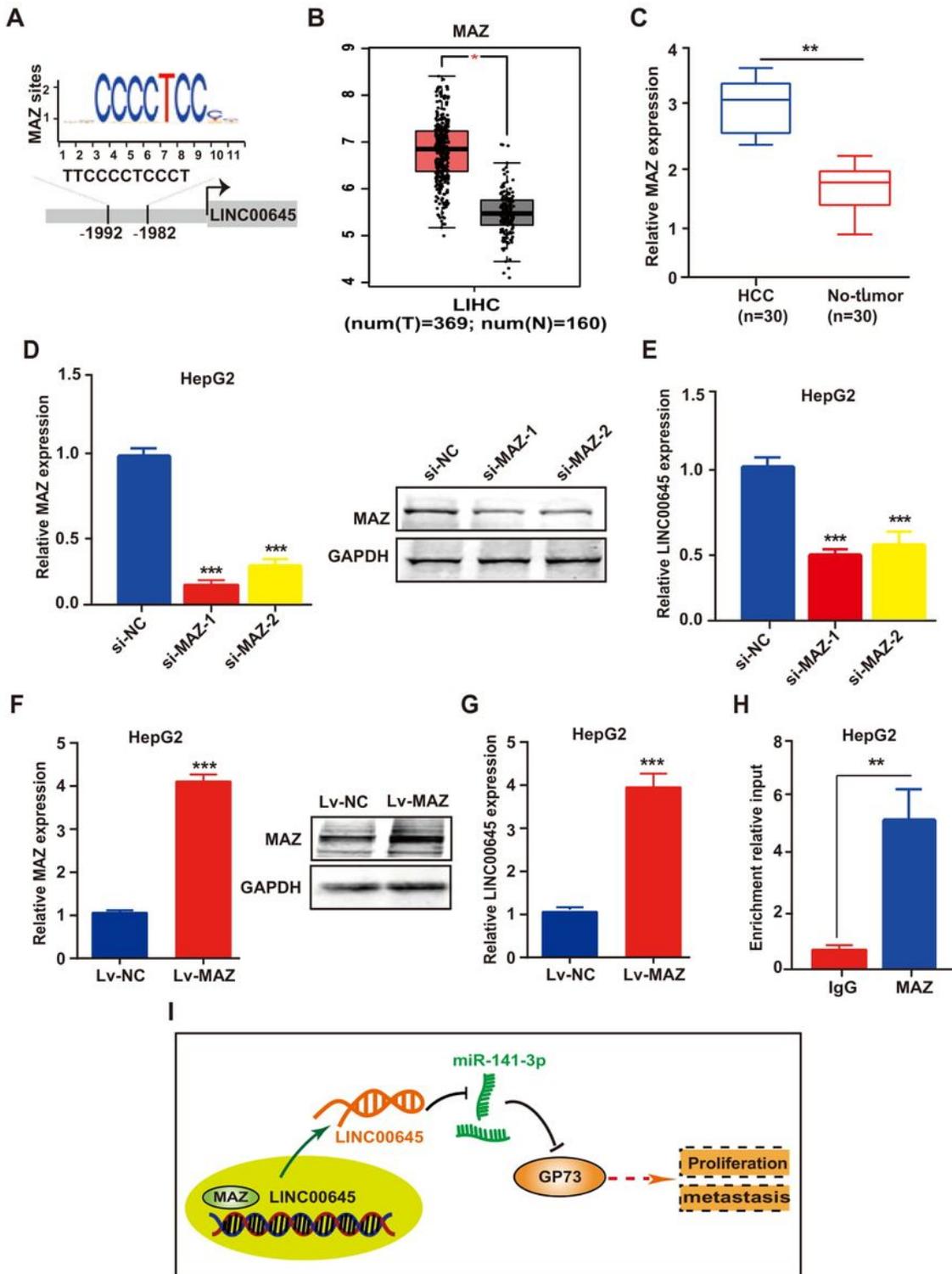


Figure 7

MAZ transcriptional regulates LINC00645 expression in HCC cells. (A) The predicted binding sites of MAZ in the LINC00645 promoter sequence. (B and C) The mRNA levels of MAZ in HCC and normal tissues were detected. (D) Reduced expression of MAZ in HCC cells either by si-MAZ-1 or si-MAZ-2. (E) The interference effect of si-MAZ on the expression of LINC00645 in HCC cells measured by RT-PCR. (F) The over-expression of MAZ in HCC cells by Lv-MAZ. G, the over-expression of MAZ significantly elevated

LINC00645 expression in HepG2 cells. (H) ChIP assays showed that MAZ is a bona fide transcriptional activator of LINC00645 in HCC cells. (I) The mechanism of the regulatory network and function of LINC00645. Targeting the MAZ-LINC00645-miR141-3p-GP73 signaling axis promotes hepatocellular carcinoma proliferation and metastasis. Error bars represented the mean \pm SD of at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001.

Supplementary Files

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

- [SupplementaryinformationTableS1S3.docx](#)