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# Long Stress Induced Non-Coding Transcripts 5 (LSINCT5) Promotes Hepatocellular Carcinoma Progression Through Interaction with High-Mobility Group AT-hook 2 and MiR-4516

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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**Background:** Long non-coding RNAs (lncRNAs) have been implicated in various human cancer types. However, the underlying mechanisms involved in hepatocellular carcinoma (HCC) progression remain poorly understood.

**Material/Methods:** In this study, lncRNA array was used to identify HCC related lncRNAs. RNA immunoprecipitation (RIP) followed mass spectrometry was used to explore lncRNA binding proteins. Western blot, quantitative PCR, tumor sphere formation, migration and viability assay were performed to evaluate the oncogenic role of lncRNAs.

**Results:** We identified a novel lncRNA named long stress induced non-coding transcripts 5 (LSINCT5) which facilitates HCC progression. LSINCT5 was significantly upregulated in both HCC specimens and cell lines and correlates with poor survival. *In vitro* experiments showed that LSINCT5 promoted migration and viability of HepG2 and Huh7 cells. The *in vivo* xenograft mouse model also confirmed an oncogenic role for LSINCT5. RIP in combination with mass spectrometry identified HMGA2 as the LSINCT5 binding partner. LSINCT5 could bind to HMGA2 and decrease proteasome-mediated HMGA2 degradation leading to EMT activation. LSINCT5 also served as a competing endogenous RNA (ceRNA) for miR-4516, resulting in increased STAT3/BclxL expression and attenuated apoptosis.

**Conclusions:** Our data have collectively established a lncRNA LSINCT5 mediated process during HCC carcinogenesis and might have provided novel insight into therapeutic targeting.

**MeSH Keywords:** Carcinoma, Hepatocellular • HMGA2 Protein • MicroRNAs • RNA, Long Noncoding

**Full-text PDF:** https://www.medscimonit.com/abstract/index/idArt/911179







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## Background

Hepatocellular carcinoma (HCC) is among the most common neoplasms and contributes largely to the occurrence of cancer death. Hepatitis B virus (HBV) is the major etiologic agent responsible for HCC, especially in China. About 80% of HCC cases are correlated with HBV infection [1]. Although surgical treatments and chemotherapy are commonly used in clinical treatment, the 5-year survival for HCC patients is desperately poor primarily owing to the late diagnosis and high rate of metastasis [2]. Therefore, it is important to identify the underlying mechanisms for HCC pathogenesis to facilitate the development of effective therapeutic strategies to improve the prognosis of HCC incidence.

The long non-coding RNAs (lncRNAs) have emerged as critical factors for gene expression [3]. LncRNAs represent a class of non-coding RNAs with more than 200 nucleotides in length without protein coding capacity [4]. LncRNAs have been reported to be correlated with a huge array of biological activities such as autophagy, myocardial infarction, epigenetic modulation, senescence, and chromatin remodeling [1,5–7]. The expression of lncRNAs is also cell type specific and may correlate with recurrence of cancer and poor prognosis [8]. The lncRNAs involved in tumorigenesis and metastasis may act through multiple ways such as sponging or silencing tumor suppressors or activation of oncogenic factors [9]. Specifically, many studies have clarified that lncRNAs are critically involved in HCC progression. For example, lncRNA HULC can stabilize Sirt1 to trigger autophagy and reduce the chemosensitivity of HCC cells [10]. Li et al. found that lncRNA-ZEB1-AS1 can behave in physical contiguity with ZEB1 and upregulate ZEB1 expression [11]. A large array of other lncRNAs can also play diverse roles during HCC development through lncRNA sequencing [12]. A recently identified lncRNA named long stress induced non-coding transcripts 5 (LSINCT5) is a 2.6-kb transcript which is polyadenylated and transcribed from a negative strand between *IRX4* and *IRX2* sites without protein coding activities [13]. However, the potential involvement of LSINCT5 and its possible mechanism for HCC progression remains elusive.

In our current study, we investigated the contribution of lncRNAs in HCC progression. By lncRNA profiling, we identified a novel lncRNA-LSINCT5 which is highly expressed in HCC cell lines and tumors. Higher expression of LSINCT5 indicated poor prognosis. LSINCT5 overexpression by lentiviral transfection can faithfully promote viability and migration of HCC cells *in vitro*. The oncogenic role of LSINCT5 was also evident in an *in vivo* xenograft mouse model. Mechanistically, we found that LSINCT5 interacted with high-mobility group AT-hook 2 (HMGA2) and increased stability of HMGA2 protein to accelerate epithelial-mesenchymal transition (EMT). Furthermore, LSINCT5 could also bind microRNA-4516 (miR-4516) and act as a competing endogenous RNA (ceRNA) to favor STAT3 activation and BclxL upregulation. Taken together, our data support novel evidence that lncRNA-LSINCT5 could contribute to HCC tumorigenesis possibly by collaborative inhibition in apoptotic pathway and promotion in EMT process.

## Material and Methods

### Cell culture and antibodies

The HCC cell lines 97L, HepG2, Hep3B, 7721, and Huh7, the 293T cells, and the normal human hepatocyte L02 cells were all obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in humidified atmosphere with 5% $CO_2$ in Dulbecco's Modified Eagle Media (Invitrogen) plus 3% fetal bovine serum (FBS, Invitrogen) and 100 μg/mL streptomycin (Invitrogen). The antibodies for HMGA2 (no.HPA039076), E-cadherin (no.5085), GAPDH (no.AMAB91153), GSK3β (no.SAB5300175), STAT3 (no.S5933), pSTAT3 (pTyr$^{705}$, no.AB4300033), and ubiquitin (no.SAB1306582) were obtained from Sigma. BclxL (no.PA5-21676) and SNAIL1 (no.14-9859-82) antibodies were purchased from Invitrogen. The proteasome inhibitor MG132 was obtained from Sigma (no.M8699).

### Human samples

The HCC samples (n=126) were obtained from surgical archives from patients registered at Shanghai Eastern Hepatobiliary Surgery Hospital from October 2012 to July 2013. All patients provided informed and written consent. The specimens were immediately frozen in liquid nitrogen and stored at -80°C refrigerator before usage. The protocols of experimental procedures using human specimens were approved by the Human Research Ethics Committee of Shanghai Eastern Hepatobiliary Surgery Hospital.

### The lncRNA microarray

Total RNA was extracted by TRIzol and then sent to Beijing Genomics Institute (BGI) for sequencing. Array data were then subjected to background subtraction and quality control by GeneSpring software. Probes were regarded as differentially expressed genes with fold change (FC) >2 and *P* values <0.05.

### Migration assay

HepG2 and Huh7 cells were starved for 12 hours and loaded into the upper chamber (~$10^5$ cells/well). The lower chambers were supplemented with DMEM with additional 0.5% FBS (Sigma). Following incubation for 24 hours, the upper chambers were removed and the cells moving into the lower chambers were fixed with 5% polytetrafluoroethylene (PFA) and stained with crystal violet. Results were assessed with a Leica fluorescent microscope (DM-IRB; Leica Microsystems GmbH, Wetzlar, Germany).





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### Viability assay

The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to measure the viability. Indicated cells were re-suspended and seeded into a 12-well plate (~$10^5$ cells/well) for 5 days. Then, 20 µL CCK-8 solution was added to the culture (final concentration, 10 mg/mL). After being shaken for 5 min, the culture was evaluated once a day, for a total of 5 days. Dissolved crystalline formazan was added into 100 µL 10% sodium dodecyl sulfate (SDS) solution for 24 hours and the optical density (OD) at 450 nm was calculated by Spectramax M5 microplate monitor (Molecular Devices, LLC, Sunnyvale, CA, USA) following the manufacturer's instruction.

### Tumor sphere formation

Single-cell suspensions were plated in 100ϕ dish ultra-low attachment plates (~$10^5$ cells/well; Corning, NY, USA) in serum-free MEM supplemented with 20 ng/mL bFGF, 20 ng/mL EGF, 200 units/mL penicillin, 200 ng/mL streptomycin, and B27 (Life Technologies). Tumor spheres were cultured for 5 days, and cells collected from non-adherent cultures were evaluated with a Multisizer 3 Coulter Counter (Beckman).

### *In vivo* tumorigenesis

HepG2 cells transfected with indicated plasmids maintained in DMEM described for 24 hours. Then, cells were resuspended and ~2×$10^6$ cells were subcutaneously injected into nude mice (female, 4~6 weeks old, n=6 in each group). All animals were housed at ~20°C at a 12/12 light/dark cycle with access to water and food ad libitum. After 4 weeks, mice were sacrificed, and tumors were harvested, weighed, and tumor slides were stained with hematoxylin-eosin (Sigma). Cells were fixed in 4% formaldehyde for 20 minutes and then washed by phosphate buffer saline (PBS). Fixed cells were treated with pepsin (1%) and dehydrated by 90% ethanol. Then the dried cells were incubated with 20 nM fluorescent *in situ* hybridization (FISH) probes (designed and synthesized by Sigma) in hybridization buffer for 5 minutes at 80°C (100 mg/mL dextran sulfate, 10% formamide in 2×SSC). Hybridization was carried out at 50°C for 2.5 hours and then the slides were washed and dehydrated. At last, the slides were covered with Prolong Gold Antifade Reagent with DAPI. The animal studies were conducted in accordance with an Institutional Animal Care and Use Committee (IACUC) in Shanghai Eastern Hepatobiliary Surgery Hospital.

### Metastatic mouse model

For each mouse (n=6 in each group), ~1×$10^6$ HepG2 cells with indicated transfection plasmids were suspended in 100 µL PBS (Sigma). Each nude mouse was orthotopically inoculated into the spleen under anesthesia. Animals were sacrificed after 5~6 weeks with their livers resected, fixed in 10% buffered formalin for further analysis.

### Western blot

The HCC cell lines were harvested using lysis buffer (13% glycerol and 2% NP-40) purchased from Sigma (Beijing, China). Samples were centrifuged at 12 000 x *g* at 4°C for 15 minutes. Protein extracts (i.e., 100 µg each) were electrophorized using 8% SDS-PAGE and migrated to nitrocellulose membranes (Bio-Rad Laboratories, CA, USA). The blot was blocked with 4% non-fat milk for 30 minutes at room temperature. The membrane was first coated with primary antibodies with dilution 1: 1000 at 4°C overnight. Then, horseradish peroxidase-conjugated secondary antibodies (1: 1000, no.SAB5201369, Sigma) were used for inoculation at 20°C for 1 hour. The blots were quantified automatically using ImageJ software (NIH, Bethesda, MD, USA).

### RNA fluorescence *in situ* hybridization (RNA-FISH)

DIG (digoxigenin) labeled lncRNA-LSINCT5 probes were used for RNA fluorescent *in situ* hybridization (FISH). HepG2 cells were fixed in 4% formaldehyde and permeabilized with 0.5% Triton X-100 for 10 minutes. Then, cells were washed with PBS 3 times and once in 2×saline sodium citrate (SSC) buffer (no.S8015, Sigma). Hybridization was performed by DNA probes at 20°C for 12 hours. Images were obtained with a FV1000 confocal laser microscope (Olympus).

### Plasmid construction

The full-length cDNA of LSINCT5 was cloned by Invitrogen (Beijing, China) and inserted into the lentiviral vector pWPXL. *HMGA2* was inserted into pCMV-Flag vector for overexpression (represented as "oe" thereafter). Cells were co-transfected with the lentiviral vector packaging system using Lipofectamine 2000. Small hairpin RNAs (shRNAs) for *LSINCT5* and *HMGA2* were designed and synthesized by Invitrogen (Beijing, China) and verified by sequencing (Table 1). A non-targeting, scramble silencing RNA was used as negative control (shCtrl).

### Quantitative PCR (qPCR)

The total RNA (HCC cell lines, samples, and normal adjacent samples) were extracted with TRIzol reagent (Invitrogen) following the manufacturer's instruction. RNA quantification was performed with Nanodrop 2000 (Thermo Scientific). For lncRNA expression, the first strand in cDNA was synthesized using Superscript I (Invitrogen) with random primers. qPCR assays were performed with a miScript SYBR Green PCR Kit (Qiagen). The primers used in current study are listed in Table 2.





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**Table 1.** Sequences for shRNA.

| Names | Sequences |
|---|---|
| *shLSINCT5#1* | CGAAAGCACGTAATCGCCGGTGTAACGAATTACACCGGCGATTAC |
| *shLSINCT5#2* | CGAAGCCAAGTCCGAGTTCCATTTCTTCGAAAAGAAATGGAACTCGG |
| *shHMGA2* | GCTATACTCTTCGGAGAACTTCTTCGAGC |

**Table 2.** Primers for RNAs.

| Names | Forward | Reverse |
|---|---|---|
| *LSINCT5* | | |
| 1-525 | GACTAATACGACTCACTATAGGGA | GAGAGAACTTTCGTGT |
| 1-1347 | GACTAGGGAGAGAAGATGGAGCAGTGA | GATTATACATAGGAACAT |
| 1348-1864 | TGCTTGCGAGATTGTAT | GATAGCCTTGCATGA |
| 1865-2647 | GGATCCATGTCGCTTTCTAACAAG | CGGAATTCTCAAGTGGCAG |
| FL | TATGCTGAAAATATTCCAAGGTTTAT | ATAGGGAGTTTCACTGTGT |
| Antisense | TAATACGACTCACTGGGAGAGGGGCAAGT | GGGCAAGTGCTTTCAAC |
| HMGA2 | CACTGTGGCTTCTGGCAT | ATGAGAGCTTTGGTTCG |
| GAPDH | CGCTTCGGCAGCACATATACTA | TCCTTAATGTACGACGAT |
| miR-23b-5p | GGGCAGGGCTTCTGAGCT | |
| miR-23a-5p | ATGTAAGGAAGTGTGTG | |
| miR-1197 | AGGCGGGGCGCCGCGG | |
| miR-222-5p | TGGTAAAATGGAACCAAAT | |
| miR-6747-3p | GTGGTGTTGGGACAGCTCCGT | |
| miR-4516 | TGGAGTGTGACAATGGTGTT | |
| miR-6515-5p | GTTATCCGTGTTGCCTTCG | |
| miR-7-2-3p | TGGCAGTGTCTTAGCGCT | |
| miR-7-1-3p | GGGCTTCTGAGCTCCT | |
| miR-4478 | TGAGGAGGAACAAGAAGATG | |

### RNA immunoprecipitation (RIP)

The MS2bp-MS2bs-based RNA immunoprecipitation (RIP) assay was performed as previously described [14]. In brief, the pcDNA3.1-Flag-MS2bp and pcDNA3.1-LSINCT5-MS2bs were co-transfected into HepG2 cells. After 48 hours, cells were harvested and then lysed by mild lysis buffer together with RNase inhibitor. Immunoprecipitation was performed with anti-FLAG M2 magnetic beads (Invitrogen). Complexes of RNA and co-isolated RNA-binding partners were extracted by TRIzol (Invitrogen) for further evaluation.

### RNA pulldown assay and mass spectrometry

Biotin-labeled RNAs were transcribed with Biotin RNA Labeling Mix and T7 RNA polymerase (Roche) followed by purification with the RNeasy Mini Kit (Qiagen). Total RNAs were then annealed to form a proper secondary structure, mixed with cytoplasm extract in RIP buffer and incubated for 2 hours at 20°C. Streptavidin agarose beads were incubated at 20°C for 1 hour. Beads were extracted by TRIzol reagent for quantitative PCR (*q*PCR). Electrophoresis was used to separate specific bands which were further investigated by mass spectrometry. Samples from mass spectrometry were sent and analyzed in Shanghai Mass Spectrometry Center, Chinese Academy of Sciences.

### Luciferase reporter assay

LSINCT5 expressing plasmid was co-transfected with miR-4516 mimic or miR-NC into tumor cells by Lipofectamine 2000. Luciferase activities were measured using a dual luciferase reporter assay system (Riobo).





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### MicroRNA inhibitors and mimics

MicroRNA-4516 (miR-4516) inhibitors, mimics were designed and purchased from Qiagen. SiRNA duplexes with nonspecific sequences were used as a negative control (NC) and were also purchased from Qiagen. The miRNeasy mini kit (no.217004) and miScript II RT kit (no.218160) were purchased from Qiagen according to the manufacturer's instructions.

### Statistical analysis

Statistical results were analyzed by SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) using the Mann-Whitney test. Statistical differences among multiple groups were analyzed by one-way ANOVA. The log-rank test was used to determine whether the survival was significantly different between the "high" and "low" expression groups. All experiments were performed in at least triplicate. Data were represented as mean ± standard deviation (SD). $P<0.05$ was considered statistically significant.

## Results

### Identifying LSINCT5 as a putative oncogenic lncRNA

To identify the lncRNAs involved in HCC tumorigenesis, an integrative analysis was performed. The genome-wide lncRNA profiling was implemented between tumor specimens and normal adjacent tissues (Figure 1A). We found 49 specifically upregulated lncRNAs in tumor samples which might be cancer-derived lncRNAs (Figure 1A). Similar strategies were used in L02/7721 and L02/HepG2 pairs (Figure 1B, 1C). Specifically, 33 and 25 upregulated lncRNAs were identified, respectively (Figure 1B, 1C, bottom). The Venn plot showed that 3 lncRNAs were consistently upregulated in all 3 pairs (Figure 1D, 1E). The lncRNA-MUF and HOTTIP, however, have been investigated previously [14,15] and were excluded from current study. Therefore, we focused on lncRNA-LSINCT5 to evaluate its expression pattern and biologic function in HCC.

### LSINCT5 is upregulated in HCC and predicts poor survival

To further explore the clinical significance of LSINCT5, we measured LSINCT5 expression in 126 HCC tumor specimens and matched normal adjacent tissues (NATs). We found that LSINCT5 was significantly upregulated in HCC samples (Figure 2A). Furthermore, higher LSINCT5 expression correlated with advanced TNM stages (Figure 2B, Table 3). The expression of LSINCT5 was also markedly increased in patients with metastasis (Figure 2C, Table 3). LSINCT5 expression was also positively associated with tumor size but not associated with age and gender (Table 3). Furthermore, higher LSINCT5 substantially correlated with poor overall survival in HCC patients (Figure 2D). Additionally, LSINCT5 also showed higher expression in HCC cell lines (Figure 2E). These data demonstrated that LSINCT5 was frequently overexpressed in HCC and might indicate poor prognosis.

### LSINCT5 promotes HCC migration and viability *in vitro*

We next checked whether LSINCT5 played an oncogenic role in HCC cell lines. Lentiviral transfection was used to alter the expression of LSINCT5 in HCC cell lines (Figure 3A, 3B). LSINCT5 overexpressing and knockdown efficiency was verified in Huh7 and HepG2 cells, respectively (Figure 3A, 3B). Notably, shLSINCT5#1 showed higher efficiency and was selected (Figure 3B). LSINCT5 overexpression substantially promoted the migration of HepG2 and Huh7 cells (Figure 3C, 3D). Lowering LSINCT5 instead significantly attenuated migration (Figure 3C, 3D). Furthermore, LSINCT5 overexpression facilitated HepG2 and Huh7 viability (Figure 3E, 3F). Reducing LSINCT5 consistently decreased the viability (Figure 3E, 3F). These results suggested that LSINCT5 promoted HCC migration and viability *in vitro*.

### LSINCT5 played an oncogenic role *in vivo*

To further investigate the function of LSINCT5 *in vivo*, xenograft mouse models were used. The tumor volume was substantially increased with LSINCT5 overexpression (Figure 4A, 4B). ShRNA mediated LSINCT5 knockdown markedly weakened xenograft tumor growth (Figure 4A, 4B). The tumor weight was also decreased with LSINCT5 knockdown and elevated with LSINCT5 overexpression (Figure 4C, $P<0.01$). Furthermore, higher LSINCT5 expression was detected in tumor slides from LSINCT5 overexpressing group by an *in situ* hybridization assay (Figure 4D). Using a metastatic model, we found that LSINCT5 depletion significantly repressed metastatic capacity *in vivo* (Figure 4E). Consistently, LSINCT5 expression in tumor tissues with pWPXL-LSINCT5 was higher than the control groups (Figure 4F). These results further demonstrated an oncogenic role of LSINCT5 *in vivo*.

### LSINCT5 functioned as a ceRNA of miR-4516 to promote HCC progression

Accumulating evidence has suggested that lncRNAs can behave like ceRNAs to regulate miRNA function [16,17]. To examine whether LSINCT5 could also bind endogenous miRNAs, we predicted the putative target miRNAs using miRDB (*http://www.mirdb.org/miRDB/custom.html*). Ten hits were identified with target score >70. Furthermore, RIP and RNA pulldown assays showed that miR-4516 were consistently enriched in LSINCT5 group compared with the control (Figure 5A, 5B). Thus, we mainly focused on LSINCT5 binding to miR-4516. Luciferase activity together with AGO2 RIP assay revealed that LSINCT5 indeed interact with miR-4516 (Figure 5C). There were totally





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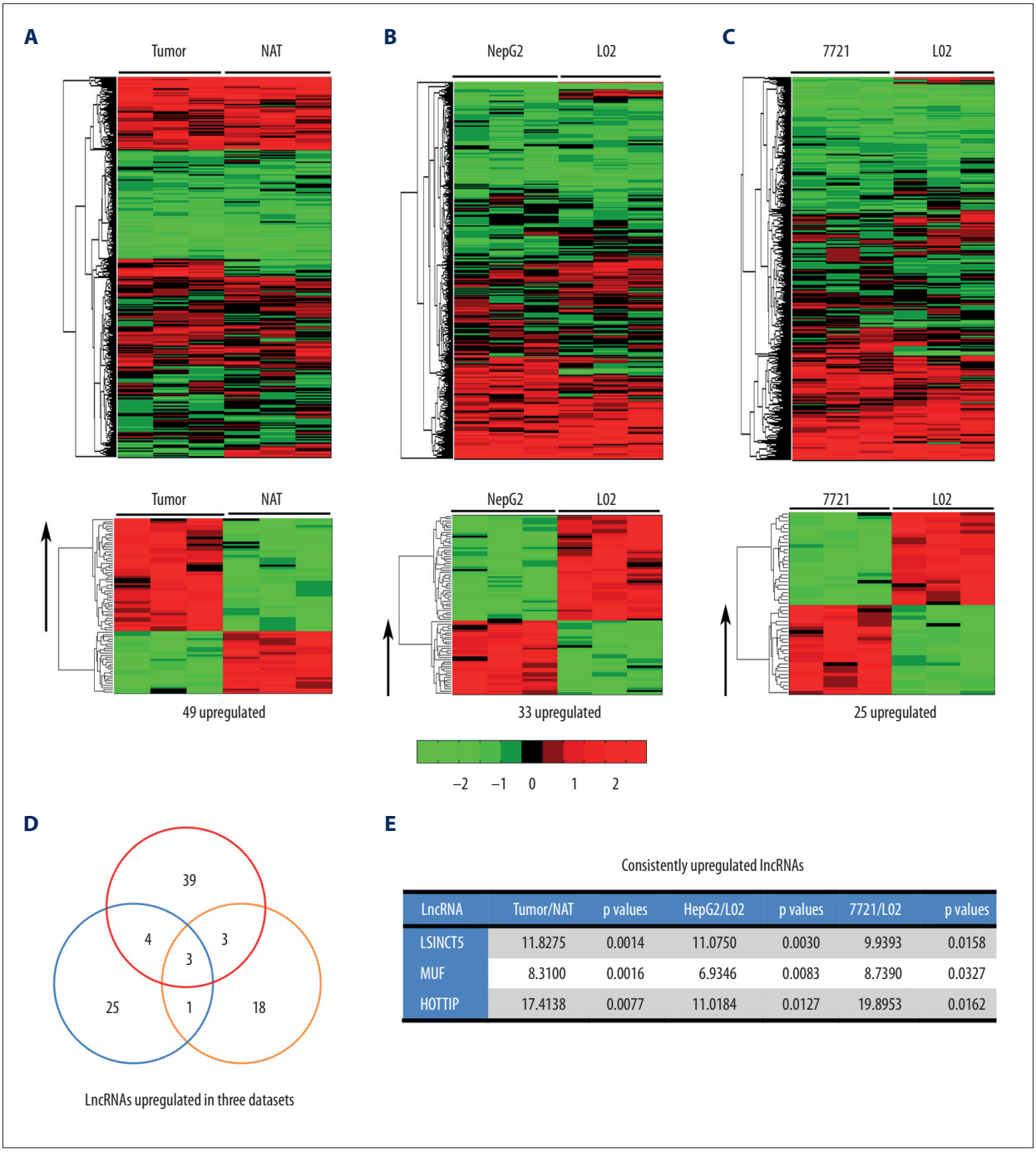


| LncRNA | Tumor/NAT | p values | HepG2/L02 | p values | 7721/L02 | p values |
|---|---|---|---|---|---|---|
| LSINCT5 | 11.8275 | 0.0014 | 11.0750 | 0.0030 | 9.9393 | 0.0158 |
| MUF | 8.3100 | 0.0016 | 6.9346 | 0.0083 | 8.7390 | 0.0327 |
| HOTTIP | 17.4138 | 0.0077 | 11.0184 | 0.0127 | 19.8953 | 0.0162 |

**Figure 1.** The lncRNA LSINCT5 was consistently upregulated in HCC. Heatmaps for lncRNA expression in (**A**) paired tumor and normal adjacent tissues (NATs) (**B**) HepG2 and L02 pairs, and (**C**) 7721 and L02 pairs. Samples are shown in columns and lncRNAs are in rows. Arrows denote upregulated lncRNAs. The upregulated lncRNAs (shown in red) are displayed in the bottom panels. (**D**) Venn diagram indicating consistently upregulated (n=3) lncRNAs in lncRNA array data. (**E**) Identification of consistently upregulated lncRNAs. HCC – hepatocellular carcinoma; lncRNAs – long non-coding RNAs; LSINCT5 – long stress induced non-coding transcripts 5.





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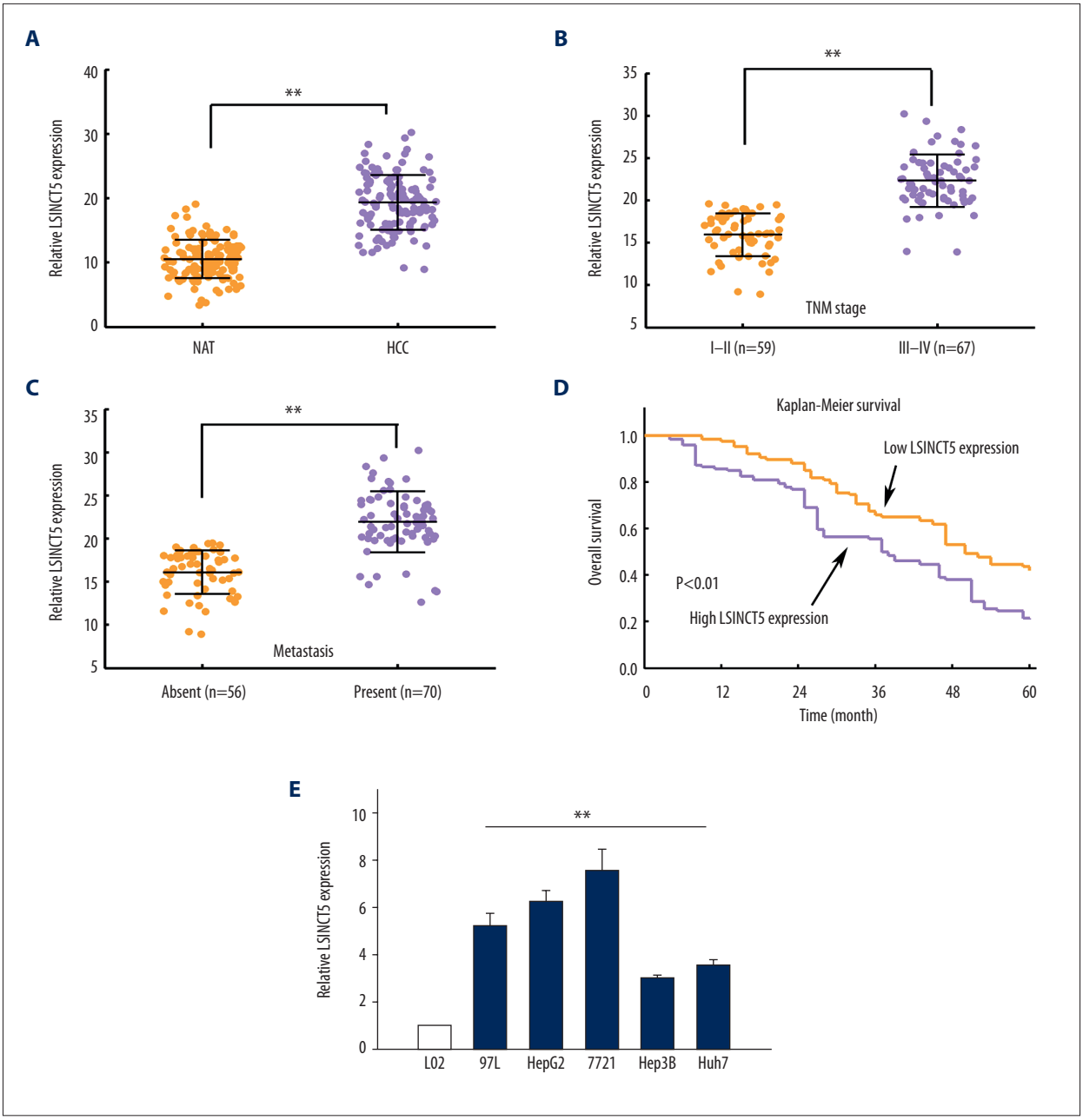


**Figure 2.** (**A–E**) LSINCT5 overexpression in HCC tissues. The level of LSINCT5 in HCC and pair normal adjacent tissues (n=126). Normalization to GAPDH is shown. Clinical significance for LSINCT5. Higher LSINCT5 expression was associated with advanced TNM stages (III/IV) and metastasis. Kaplan-Meier survival curves for patients with HCC. *P*<0.01 by log-rank test. The median value was used as the cutoff. HCC – hepatocellular carcinoma; LSINCT5 – long stress induced non-coding transcripts 5.

3 predicted binding sites between LSINCT5 and miR-4516 (Figure 5D). RNA-FISH assays also confirmed miR-4516 binding to LSINCT5 (Figure 5E). Next, we noticed that miR-4516 could inhibit HepG2 and Huh7 cell viability (Figure 5F). However, LSINCT5 and miR-4516 co-transfection was performed in HepG2 and Huh7 cells, and LSINCT5 might counteract inhibitory role of miR-4516 on viability (Figure 5F) and tumor sphere formation (Figure 5G). Previous results showed that miR-4516 targets *STAT3* transcript and downregulates STAT3, pSTAT3, and BclxL expression [18]. Consistently, we found that LSINCT5 upregulation increased the expression of BclxL and (p-)STAT3 using western blot (Figure 5H). These results suggested that LSINCT5 might function as a ceRNA of miR-4516 in HCC cells.





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**Table 3.** Correlation between clinicopathological parameters and LSINCT5 expression (n=126).

| Clinicopathological parameters | Patient no. | LSINCT5 | | *P* values |
|---|---|---|---|---|
| | | Low (N=63) | High (N=63) | |
| Gender | | | | 0.142 |
| Male | 65 | 29 (44.6%) | 36 (55.4%) | |
| Female | 61 | 34 (55.7%) | 27 (44.3%) | |
| Age | | | | 0.296 |
| <55 | 60 | 32 (53.3%) | 28 (46.7%) | |
| ≥55 | 66 | 31 (47.0%) | 35 (53.0%) | |
| TNM stage | | | | 0.006** |
| I–II | 59 | 37 (62.7%) | 22 (37.3%) | |
| III–IV | 67 | 26 (38.8%) | 41 (61.2%) | |
| Tumor size | | | | 0.002** |
| <3 cm | 53 | 35 (66.0%) | 18 (34.0%) | |
| ≥3 cm | 73 | 28 (38.4%) | 45 (61.6%) | |
| Metastasis | | | | 0.003** |
| Absent | 56 | 36 (64.3%) | 20 (35.7%) | |
| Present | 70 | 27 (38.6%) | 43 (61.4%) | |

TNM denotes tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M) (* $P<0.05$, ** $P<0.01$).

### LSINCT5 interacted with HMGA2 to promote EMT process

LncRNAs can also act through RNA-protein interactions to regulate tumorigenesis [14]. To further clarify the function of LSINCT5, we used RNA pulldown assays accompanied by silver staining and mass spectrometry to identify LSINCT5-protein interactions (Figure 6A). We identified 6 putative proteins and by verification HMGA2 was found to physically bind to LSINCT5 (Figure 6B). HMGA2 was found in the biotin-labeled sense LSINCT5 group by RNA pulldown assay (Figure 6B). The interaction between LSINCT5 and HMGA2 was also confirmed using RIP assays (Figure 6C). Furthermore, a series of truncated LSINCT5 were constructed and the results showed that the C-terminal domain might contribute to LSINCT5 and HMGA2 binding (Figure 6D). Additionally, LSINCT5 alteration did not affect the *HMGA2* transcript levels (Figure 6E) but instead positively modulated the protein abundance of HMGA2 (Figure 6F). Furthermore, HMGA2 degradation was inhibited after administration of the proteasome inhibitor MG132 (Figure 6G) implying that LSINCT5 repressed proteasome-mediated degradation of HMGA2. Ubiquitination of HMGA2 was markedly inhibited when LSINCT5 was overexpressed while shLSINCT5 increased ubiquitin ligation of HMGA2 (Figure 6H). HMGA2 has been shown to be a positive regulator of EMT process by binding to the *Snail1* promoter, inducing *Snail1* expression and repressing E-cadherin expression [19]. As expected, HMGA2 overexpression can partially reverse the inhibitory effect of shLSINCT5 for HCC cell migration (Figure 6I). SNAIL1 expression was significantly attenuated and E-cadherin was upregulated when LSINCT5 was knocked down (Figure 6J). HMGA2 overexpression again partially rescued the EMT phenotype with LSINCT5 knockdown (Figure 6J). These results suggested that LSINCT5 could interact with and stabilize HMGA2 to facilitate EMT process.

## Discussion

Intense studies over the past few years have suggested a critical role of lncRNAs during tumorigenesis and aberrant expression of lncRNAs is significantly associated with cancer development [20,21]. In our current study, we explored the function of lncRNA LSINCT5 in promoting HCC progression. Moreover, LSINCT5 markedly enhanced HCC tumorigenesis and some EMT traits. LSINCT5 physically interacted with HMGA2 to reduce its proteasome-mediated degradation and thereby stabilizing HMGA2. Furthermore, LSINCT5 can also bind to miR-4516 to participate in the regulation of apoptotic pathway. Collectively, these data in our current study present a novel mechanistic angle to this research field by which lncRNA LSINCT5 can facilitate HCC development. Noticeably, we only investigated the effect of LSINCT5 in HCC and whether LSINCT5 also plays an oncogenic role in other types of cancer remains to be determined.





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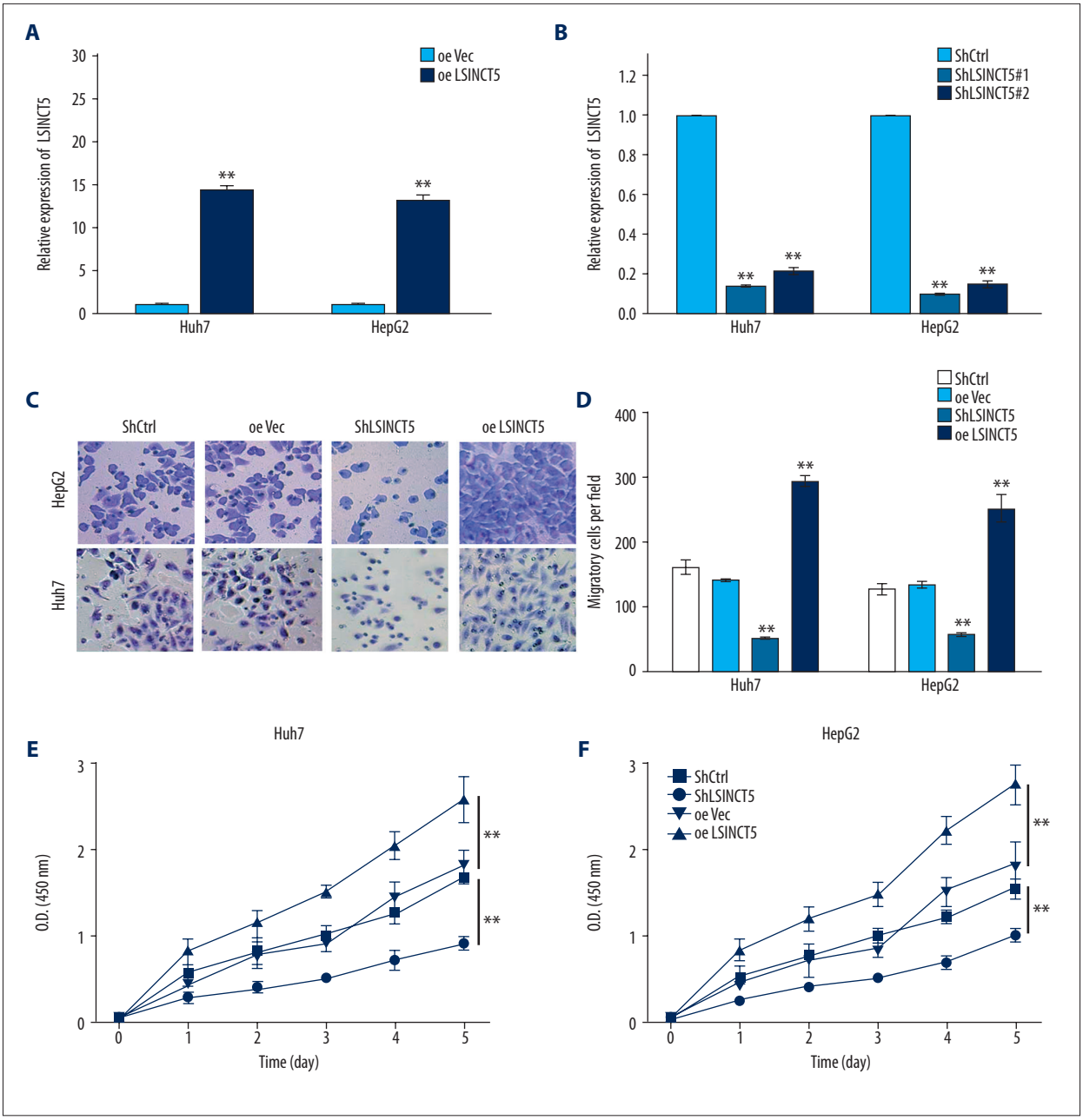


**Figure 3.** The *in vitro* effect of LSINCT5. (**A**) Verifying the effect of lentiviral transfection to overexpress LSINCT5. ** *P*<0.01. (**B**) The efficiency of stable LSINCT5 knockdown. The shRNA sequence #1 showed higher efficiency and was therefore selected (i.e., shLSINCT5 thereafter). (**C**) Effect of LSINCT5 knockdown or overexpression on migratory capacity of HCC cell lines. Quantification of migration data was shown in (**D**). ** *P*<0.01. (**E, F**) A 5-day viability assay for (**E**) Huh7 cells and (**F**) HepG2 cells either transfected with shCtrl, shLSINCT5, overexpression (oe) Vec or oe LSINCT5. ** *P*<0.01. HCC – hepatocellular carcinoma; LSINCT5 – long stress induced non-coding transcripts 5.

We used lncRNA microarray analyses to identify potential mechanisms of LSINCT5 in HCC progression. We uncovered a novel binding partner HMGA2 in physical interaction with LSINCT5. HMGA2 is a transcription factor already known to be linked to mesenchymal cell differentiation [22]. In mammary epithelial cells, TGF-β can induce the expression of HMGA2 via Smad pathways. Then, HMGA2 bind to the *Snail1* promoter to induce massive *Snail1* expression, E-cadherin inhibition, and EMT phenotype [23]. Furthermore, HMGA2 can cooperatively associates with Smad to induce ZEB1/2 and Twist expression [23]. These induced repressors collaboratively inhibit E-cadherin and therefore facilitate EMT occurrence. These findings argue that





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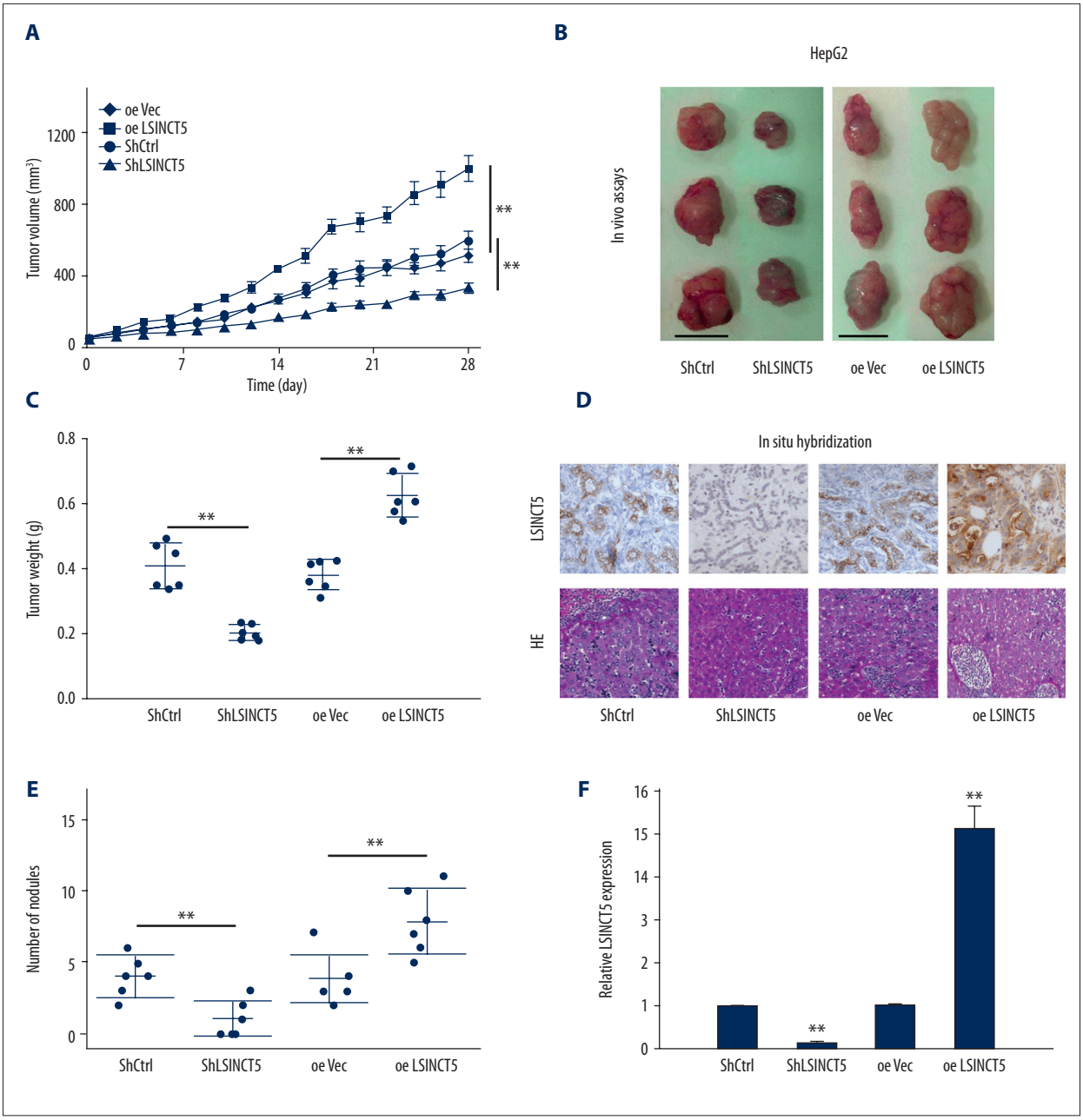


**Figure 4.** LSINCT5 promotes HCC progression *in vivo*. (**A**) Tumor volumes were quantified for 4 weeks in HepG2 xenograft mouse model. (**B**) Representative images of resected tumors for HepG2 xenografts. Scale bar, 1 cm. (**C**) By the end of implantation, xenograft tumors were resected and weighted. ** $P<0.01$. (**D**) *In situ* hybridization was used to detect LSINCT5 level. Tumor slides stained with HE are shown at the bottom. (**E**) Number of metastatic nodules by altering LSINCT5 expression. (**F**) LSINCT5 expression level of nodules in mice. Data were normalized to the shCtrl group. HCC – hepatocellular carcinoma; HE – hematoxylin-eosin; LSINCT5 – long stress induced non-coding transcripts 5.

HMGA2 acts as an important activator during EMT development. Noticeably, HMGA2 might contribute to genomic instability and is usually upregulated in various malignant cancer types [24–26] and therefore HMGA2 might serve as a master regulator for neoplasm.

Our results showed that LSINCT5 interacts with HMGA2 and inhibits proteasome mediated HMGA2 degradation. Lowering LSINCT5 fails to protect HMGA2 from degradation. The exact mechanisms by which LSINCT5 impedes HMGA2 degradation by proteasome remains unclear and should be investigated in further study. Notwithstanding, LSINCT5 might fulfill its role





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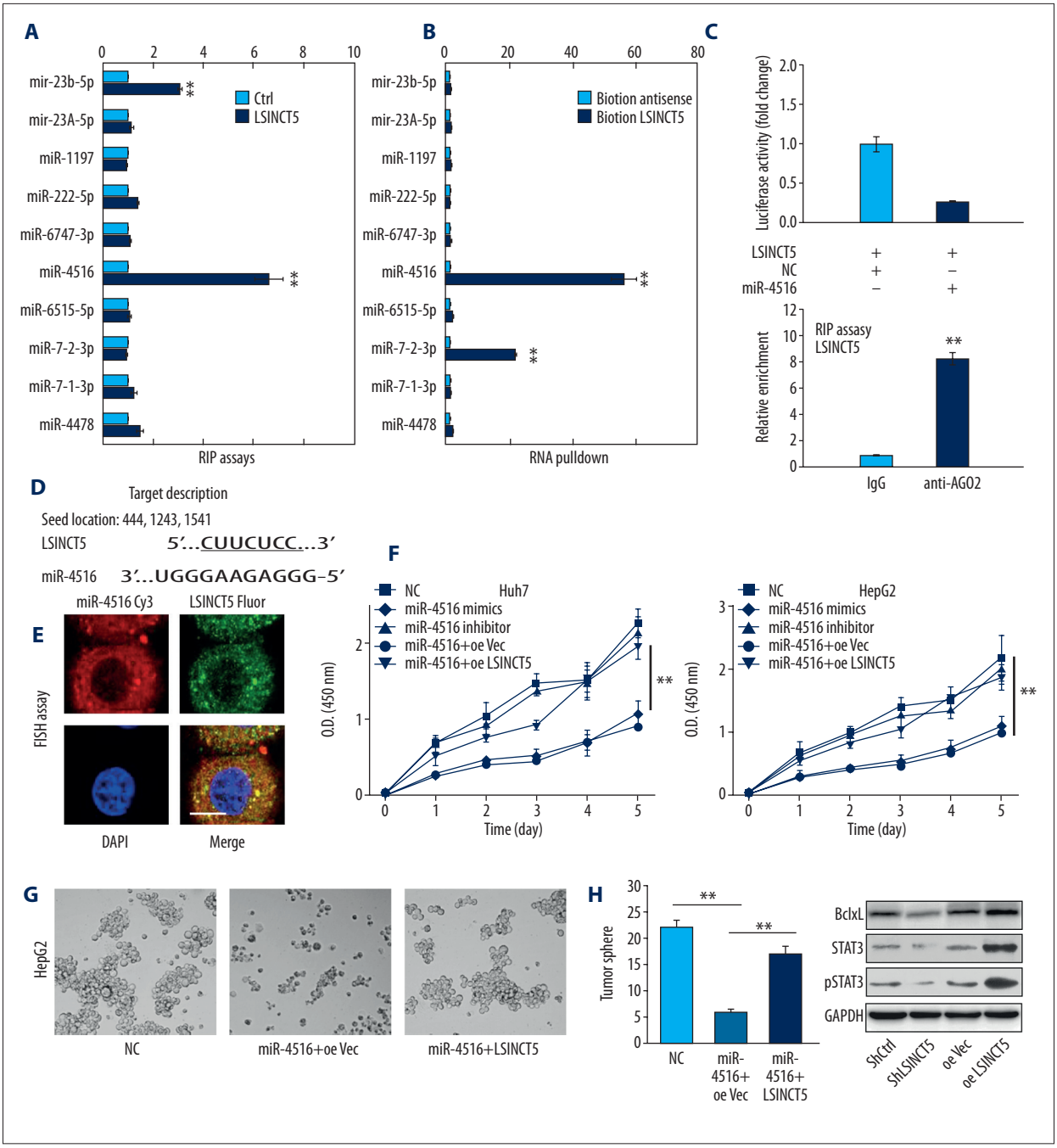


**Figure 5.** LSINCT5 acts as a ceRNA of miR-4516 to promote HCC progression. (**A**) RIP assays followed by qPCR to identify putative miRNAs associated with LSINCT5. (**B**) The miRNAs were verified by RNA pulldown a with biotin-labeled sense or antisense LSINCT5. (**C**) Luciferase reporter assays identified miR-4516 could interact with LSINCT5 (top). RNA pulldown assay with anti-AGO2 was performed in HepG2 cells with miR-4516 overexpression followed by qPCR to enrich LSINCT5 (bottom). (**D**) The predicted base-pairing between LSINCT5 and miR-4516 at miRDB. Three hits were detected. (**E**) RNA-FISH assay revealed the co-localization of LSINCT5 (Alexa Fluor 488, green dots) and miR-4516 (Cy3, red dots) in HepG2 cells. Scale bar, 5 μm. (**F**) CCK-8 viability assay for Huh7 cells and HepG2 cells transfected with plasmids as indicated. (**G**) Tumor sphere formation assay for HepG2 cells transfected with negative control (NC), miR-4516 mimics + oe Vec or miR-4516 mimics + LSINCT5. Quantification results were shown on the right panel. (**H**) Western blot assays for BclxL, STAT3, and pSTAT3 (Tyr705) expression in HepG2 cells by knocking down or overexpressing LSINCT5. CCK-8– cell counting Kit-8; ceRNA – competing endogenous RNA; FISH – fluorescent *in situ* hybridization; HCC – hepatocellular carcinoma; LSINCT5 – long stress induced non-coding transcripts 5; miRNAs – micro RNAs; oe – overexpression; RIP – RNA immunoprecipitation.





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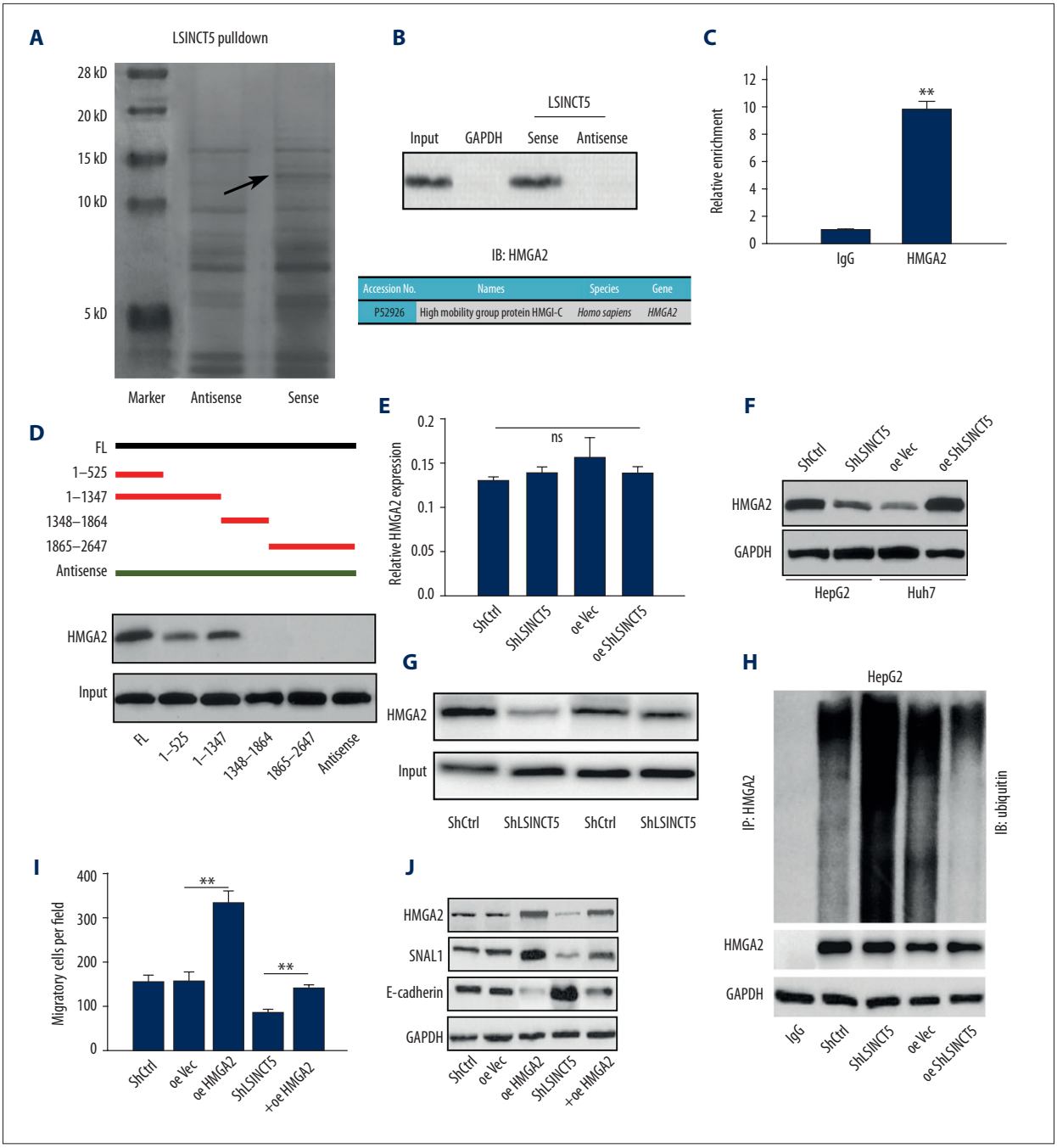


**Figure 6.** LSINCT5 interacted with HMGA2 in HCC cells. (**A**) Biotin labeled LSINCT5-sense and anti-sense probes incubated with HepG2 lysates. The ~12 kD band (black arrow) was excised and subject to mass spectrometry and verification. (**B**) Immunoblots for physical interaction between LSINCT5 and HMGA2. The table at bottom denotes the verified partner for LSINCT5. (**C**) RIP assays were performed using primary antibody against HMGA2 followed by qPCR. (**D**) Illustration of different truncated forms of LSINCT5 to identify the domain for HMGA2 interaction. (**E**) Relative *HMGA2* mRNA expression with LSINCT5 knockdown or overexpression. ns: not significant. (**F**) Western blot assay for HMGA2 protein levels by altering LSINCT5. (**G**) HepG2 cells were transfected with shCtrl or shLSINCT5 with or without MG132 (30 μM) treatment followed by immunoblot for HMGA2. (**H**) Cell lysates from HepG2 cells with shCtrl, shLSINCT5, pWPXL-Vec (oe Vec) or pWPXL-LSINCT5 (oe LSINCT5) were first immunoprecipitated with HMGA2 followed by immunoblot towards ubiquitin or HMGA2. (**I**) Migration in HepG2 cells transfected with indicated vector or plasmids. ** $P<0.01$. (**J**) The epithelial-mesenchymal transition (EMT) markers SNAIL1 and E-cadherin expression with altered LSINCT5 expression rescued by HMGA2 overexpression or not. HCC – hepatocellular carcinoma; RIP – RNA immunoprecipitation; LSINCT5 – long stress induced non-coding transcripts 5.





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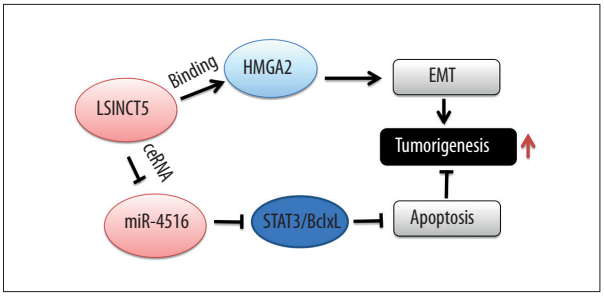


**Figure 7.** A working model for LSINCT5 mediated HCC progression. Arrows (black) indicate activation while blunt arrows denote inhibition. LSINCT5 – long stress induced non-coding transcripts 5; HCC – hepatocellular carcinoma.

partially by stabilizing and promoting the oncogenic function of HMGA2. However, it is possible that LSINCT5 could interact with distinct proteins specifically in different cells; this issue should be investigated in future.

LncRNAs can function as ceRNAs to modulate miRNAs [16,17]. For instance, lncRNA-ATB can promote HCC invasion and metastasis by competitively binding to miR-200s to alter ZEB1/2 expression [27]. The lncRNA-UCA1 could also serve as an endogenous sponge through direct binding to miR-216b, enhancing FGFR1-ERK signaling and promoting HCC progression [28]. In our current research, by computational prediction, RNA pulldown and RIP verification, we identified miR-4516 as the target for LSINCT5 binding. Previous work has demonstrated that miR-4516 can target *STAT3* leading to STAT3/BclxL downregulation and inhibition of apoptosis [18]. BclxL is a transcriptional target of STAT3 and is critically involved in antagonizing apoptosis [29]. The anti-apoptotic member BclxL in BCL-2 family may create a threshold for apoptotic initiation [30]. Our data suggested that LSINCT5 overexpression might increase BclxL abundance at the protein level and this effect is possibly mediated by acting as a ceRNA for miR-4516.

Collectively, we have provided a working model in which LSINCT5 can promote HCC progression (Figure 7). There are 2 routes to HCC tumorigenesis mediated by lncRNA LSINCT5: 1) LSINCT5 physically interacts with HMGA2 to stabilize HMGA2. HMGA2 functions as a master activator of EMT and therefore promotes EMT progression; and 2) LSINCT5 interacts with miR-4516 and acts as a ceRNA. Lowing miR-4516 by LSINCT5 may inhibit miR-4516 induced apoptosis by upregulating STAT3 activation and Bclxl expression. Therefore, LSINCT5 might coordinately promote HCC tumorigenesis by increasing EMT and decreasing apoptosis.

## Conclusions

Our findings suggest that LSINCT5 serves as a novel oncogenic factor in HCC progression. LSINCT5 can interact with HMGA2 and miR-4516 to facilitate EMT and inhibit apoptosis, respectively. We cannot rule out the possibility that other undetermined factors might also participate in physical interaction among LSINCT5, HMGA2 and miR-4516. Therefore, it will be of general interest to unravel the hidden layer of complex during LSINCT5 mediated HCC progression in future studies.

### Acknowledgements

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### Conflict of interest

None.

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