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Original Articles

The long intergenic non-protein coding RNA 707 promotes proliferation and metastasis of gastric cancer by interacting with mRNA stabilizing protein HuR

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ABSTRACT

Multiple studies have revealed that long non-coding RNAs (lncRNAs) extensively participate in human cancer malignant progression. The long intergenic non-protein coding RNA 707 (LINC00707), 3087 bp in length, was recently reported to be an essential oncogene in promoting lung adenocarcinoma cell proliferation and metastasis. However, its role in gastric cancer (GC) remains unclear. In this study, we identified that LINC00707 was excessively expressed in GC tissues and correlated with advanced stage, larger tumor size, lymph node metastasis and poorer prognosis in GC patients. In vitro and *in vivo* assays showed that LINC00707 promote GC cell proliferation and metastasis. Mechanistically, LINC00707 could abundantly interact with mRNA stabilizing protein HuR; "LINC00707-HuR" coalition ulteriorly combined with VAV3/F11R mRNAs and increased their stability. Taken together, our findings prove that LINC00707 may act as an oncogene in GC by regulating mRNA stability and serve as a potential target for GC diagnosis and prognosis.

1. Introduction

Gastric cancer (GC) is the most common malignancy of human digestive system [1]. In China, the incidence and mortality of GC are ranked second in cancer [2]. The majority of Chinese patients are diagnosed as advanced gastric carcinoma due to unconspicuous early symptom and absence of gastroscope detection. Disappointingly, nearly 60% of postoperative patients appear recurrence or metastasis, and their 5-year overall survival rate is unsatisfactory [3,4]. Over the past decades, research identifications have proved that GC is a multifactorial disease containing many internal and external factors, such as genetic influence, irregular diet and infection with Helicobacter pylori [5–7]. More recently, molecular mechanism studies highlight that non-coding RNAs (ncRNAs) especially long non-coding RNAs (lncRNAs) emerge to be crucial regulators in GC initiation, continuation and deterioration [8,9].

lncRNAs are greater than 200 nucleotides in length and have limited or no protein-coding capacity [10]. Compared with microRNAs (miRNAs), lncRNAs possess more prevalent functions at multiple levels, including chromatin modifcation, transcriptional and post-transcriptional regulation. For instance, plenty of lncRNAs can serve as molecular signals, decoys, guides or scaffolds in nucleus [11,12], while some others act as ceRNAs sponging for miRNAs, or sometimes interact with specific proteins to maintain mRNA stability or induce mRNA decay in

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cytoplasm [13–15]. Our previous studies confirm that lncRNA HOXA11-AS can scaffold the chromatin modification factors PRC2/LSD1/DNMT1 and promote GC proliferation and invasion [16]; LncRNA HOXA-AS2 propels GC proliferation by epigenetically silencing P21/PLK3/DDIT3 expression [17]; Additionally, lncRNA SPRY4-IT1 is also been demonstrated to downregulate in GC, and contribute to cell metastasis via affecting epithelial-mesenchymal transition (EMT) process [18]. Overall, numerous GC-associated lncRNAs have been characterized; However, their functions and molecular mechanisms are still not fully excavated.

In recent study, we identified a novel long intergenic non-protein coding RNA 707 (LINC00707), which was highlighted to be an essential oncogene in promoting lung adenocarcinoma cell proliferation and migration [19]. Given the absence of LINC00707 biological functions and molecular mechanisms in GC, we next carried out a series of studies. First of all, comprehensive analysis of Gene Expression Omnibus (GEO) database, The Cancer Genome Atlas (TCGA) database and specimens from patients demonstrated that LINC00707 was significantly upregulated in human GC tissues. Particularly, overexpression of LINC00707 predicted a poor prognosis in GC patients. Functional assays manifested that LINC00707 could regulate GC cell proliferation and migration both *in vitro* and *in vivo*. Mechanistically, LINC00707 was confirmed to interact with mRNA stabilizing protein HuR; "LINC00707-HuR" could further increase VAV3 and F11R mRNAs stability. Collectively, this study may advance our understanding of LINC00707 carcinous role and novel regulatory mode in GC malignant progression.

2. Materials and methods

2.1. Bioinformatics analysis

GC gene differential expression data were downloaded from GEO dataset. GSE58828 was included in this study. The data processing was consistent as previously described [17].

LncRNA sequencing data of LINC00707 in human GC tissues from TCGA was analyzed by Bioinformatics Tool "TANRIC" (http://ibl.mdanderson.org/tanric/_design/basic/query.html).

Noncoding prediction of LINC00707 was performed by ORF Prediction of "lncRNAtor" tool (http://lncrnator.ewha.ac.kr/InformationSearch.htm). The interaction between LINC00707 and HuR was also indicated by "lncRNAtor" tool.

Moreover, the combination scores between LINC00707/potential target mRNAs and HuR were predicted by RNA-Protein interaction prediction (RPISeq) website (http://pridb.gdcb.iastate.edu/RPISeq/).

2.2. Patient samples collection

All of GC and pericarcinomatous tissues were obtained from 60 patients who underwent surgery at the First Affiliated Hospital of Nanjing Medical University. This study was approved by the Research Ethics Committee of Nanjing Medical University, China. Written informed consent was obtained from all patients. The clinicopathological characteristics of the GC patients were summarized in Table 1.

2.3. RNA extraction and qRT-PCR assays

TRIZOL reagent (Invitrogen) was used to extract total RNA in both tissues and cells. Before qRT-PCR, RNA was reverse transcribed into cDNA by using a Reverse Transcription Kit (Takara, Dalian, China). SYBR Premix Ex Taq (Takara, Dalian, China) was used for qRT-PCR assays, which were conducted on an Applied Biosystems 7500 Real-Time PCR System. Results were normalized to GAPDH expression. All of primers used in this study were summaried in Supplementary Table S1.

Table 1
Correlation between LINC00707 expression and clinicopathological characteristics of gastric cancer patients.

Characteristics	N (%)	LINC00707		P
		High NO. cases (30)	Low NO. cases (30)	Chi-squared test P-value
Gender				0.795
Male	33 (55%)	17	16	
Female	27 (45%)	13	14	
Age				0.301
≤65	28 (46.7%)	12	16	
> 65	32 (53.3%)	18	14	
Stage				0.035*
I	15 (25%)	4	11	
II	20 (33.3%)	9	11	
III	25 (41.7%)	17	8	
Lymph node metastasis				0.003*
Negative	21 (35%)	5	16	
Positive	39 (65%)	25	14	
Tumor size				0.017*
≤5 cm	23 (38.3%)	7	16	
> 5 cm	37 (61.7%)	23	14	
HP infection				0.432
Negative	25 (41.7%)	11	14	
Positive	35 (58.3%)	19	16	

*P < 0.05 was considered significant.

2.4. Cell culture and transfection

GC cell lines (BGC-823, SGC-7901, MGC-803 and AGS) and the normal human gastric epithelium cell line (GES-1) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). BGC-823 and MGC-803 cells were cultured in RPMI-1640 (GIBCO-BRL) medium; SGC-7901, AGS and GES-1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM; GIBCO-BRL) medium supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) in humidified air at 37 °C with 5% CO_2.

LINC00707, HuR, VAV3 and F11R siRNAs and the negative control siRNA (si-NC) were transfected into GC cells with Lipofectamine 2000 (Invitrogen, USA); The nucleotide sequences of all siRNAs were shown in Supplementary Table S1. Additionally, the transfection of pcDNA-LINC00707 and empty vector required XtremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland). Cells were harvested for analysis at 48 h post-transfection.

2.5. Cell proliferation assays

After transfection with siRNAs or plasmid vectors, the BGC-823, SGC-7901 and MGC-803 cells were inoculated into 96-well plates (3000 cells/well). Cell viability was assessed every 24 h by using a Cell Proliferation Reagent Kit I (MTT; Roche Applied Science).

In the colony formation assay, a certain number of transfected cells were placed in 6-well plates and maintained in proper media containing 10% FBS for two weeks, during which the medium was replaced every 4 days. Two weeks later, the cells were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich). Those visible colonies were photographed and counted.

Ethynyldeoxyuridine (Edu) experiments were performed following the manufacturer's protocol of 5-ethynyl-2-deoxyuridine (Edu) labeling/detection kit (Ribobio, Guangzhou, China). Firstly, the transfected cells were treated with 50 μM Edu labeling medium and incubated for another 2 h. Next, cells were fixed with 4% paraformaldehyde and permeated by 0.5% Triton X-100. Subsequently, cells were added with anti-Edu working solution and DAPI staining solution. Lastly, Edu positive cells were observed and counted under

fluorescent microscopy. The percentage of Edu positive cells were calculated from five random fields in three wells.

2.6. Cell apoptosis assays

Flow-cytometric analysis was carried out to evaluate cell apoptosis. Firstly, cells accepted double staining with FITC-Annexin V and Propidium iodide (PI) according to the manufacturer's recommendations of the FITC Annexin V Apoptosis Detection Kit (BD Biosciences). Then the cells were analyzed with a flow cytometry (FACScan®; BD Biosciences) equipped with a CellQuest software (BD Biosciences).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (Tunel) assay was performed by using an apoptosis detection kit (KeyGEN BioTECH, China) according to the manufacturer's instructions. Tunel positive cells were observed and counted under fluorescent microscopy. The percentage of Tunel positive cells were calculated from five random fields in three wells.

2.7. Cell migration assays

Transwell assays were conducted. 24 h after transfection, 3×10^4 cells in 300 μl medium without serum were inoculated into the upper culture chamber with 8-mm membrane (Millipore, Billerica, MA, USA). The lower wells of 24-well plate was added with 700 μl medium supplemented with 10% fetal bovine serum. 24 h later, the cells on upper membrane were removed using cotton wool, whereas cells on the lower membrane surface were fixed by 4% paraformaldehyde and stained by 0.1% crystal violet. Finally, Five randomly selected views were imaged and counted in each well.

2.8. Western blot assay and antibodies

Cells protein lysates were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to 0.22 μm NC membranes (Sigma) and incubated with specifc antibodies. GAPDH antibody was used as control, and Anti-HuR, VAV3, F11R were purchased from Cell Signaling Technology, Inc (CST).

2.9. In vivo assays

Four-week-old female athymic BALB/c nude mice were maintained under specific pathogen-free conditions and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. Sh-LINC00707 and empty vector were stably transfected into SGC-7901 cells. For cell proliferation assay *in vivo*, the transfected cells were harvested, washed with PBS and re-suspended at a concentration of 1×10^7 cells/ml, then 100 μl of suspended cells were subcutaneously injected into a single side of the posterior flank of each mouse. Tumor growth was examined every 3 days, and tumor volumes were calculated using the equation $V = 0.5 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). 15 days after injection, mice were euthanized, and the subcutaneous tumors were obtained and imaged.

For cell metastasis assay *in vivo*, the transfected cells were re-suspended to 2×10^7 cells/ml, then 100 μl of suspended cells were injected into the tail veins of nine mice. 8 weeks after injection, the mice in two groups were sacrificed. Lungs of these mice were removed and photographed. Hematoxylin and Eosin (HE) Staining was performed to evaluate the metastasis nodes, and the number of metastatic nodes on the lung were counted. This study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

2.10. Subcellular fractionation location

The separation of nuclear and cytosolic fractions was performed using the PARIS Kit (Life Technologies) according to the manufacturer's instructions.

2.11. In vitro transcription and RNA pull-down assays

LINC00707 was *in vitro* transcribed by using T7 RNA polymerase (Ambio Life), purified with the RNeasy Plus Mini Kit (Qiagen), and treated with RNase-free DNase I (Qiagen). Subsequently, transcribed LINC00707 was biotin labeled with the Biotin RNA Labeling Mix (Ambio Life). Then RNA pull-down assays were performed by Pierce™ Magnetic RNA-Protein Pull-Down Kit according to the manufacturer's instructions (Pierce, Thermo).

2.12. RNA immunoprecipitation (RIP) assays

A Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was used for RIP assays according to the Manufacturer's instructions. GC cells at 80–90% confluency were scraped off, then lysed in complete RIP lysis buffer. 100 μl of whole cell extract was incubated with magnetic beads conjugated with HuR antibody or control IgG for 6 h at 4 °C. After the beads were washed, the complexes were incubated with Proteinase K to remove proteins. Purifed RNA was subjected to qRT-PCR analysis to determine the combination between HuR protein and RNAs.

2.13. RNA stability assays

After transfecting with siRNAs or plasmid vectors, GC cells were treated with actinomycin D (1 μg/ml). Then Cells were collected at different time points and the RNA was extracted using Trizol reagent (Invitrogen). mRNA levels were measured by qRT-PCR.

2.14. Statistical analysis

The Student's t-test (two-tailed), chi-square test analysis and the Mann-Whitney *U* test were used to analyze differences between groups. The survival curves were drawn by Kaplan-Meier survival plot and tested with log-rank tests. All statistical analysis were conducted by SPSS 17.0 software. P values less than 0.05 were recognized as statistically significant.

3. Results

3.1. LINC00707 expression is significantly upregulated in human gastric cancer tissues

Firstly, we downloaded and analyzed the raw human gastric cancer microarray profile (GSE58828) from GEO database online, and found that LINC00707 was approximately 6-fold increased in GC tissues compared with noncancerous tissues (Fig. 1A). Subsequently, LINC00707 expression level was evaluated in 30 paired human GC tissues and normal tissues by using the bioinformatics tool “TANRIC”, which data was downloaded from TCGA database. As a result, LINC00707 was indeed overexpressed in GC tissues (Fig. 1B). Next, LINC00707 was further confirmed as a noncoding transcript by ORF Prediction of “lncRNAtor” tool (Supplementary Fig. S1A). To validate the overexpression levels of LINC00707, 60 paired human GC tissues and corresponding non-tumor tissues were examined by qRT-PCR (Fig. 1C). Overall, these findings revealed that the high expression of LINC00707 may exert imperative clinical signifcance in GC initiation and development.

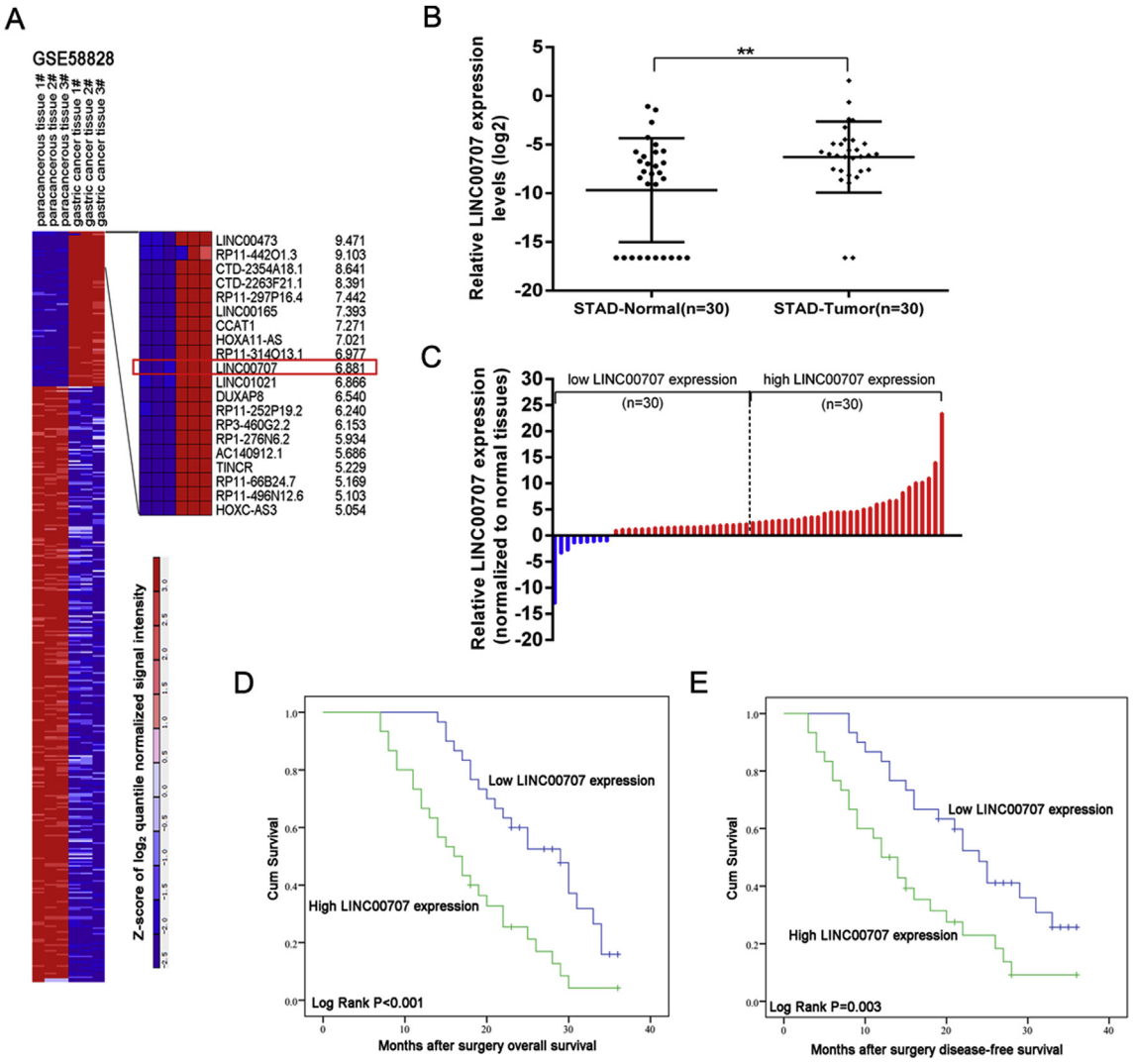


Fig. 1. Relative LINC00707 expression in gastric cancer tissues and its clinical significance. (A) Relative expression of LINC00707 in human gastric cancerous tissues compared with noncancerous tissues via GSE58828 data analysis. (B) LINC00707 expression levels were analyzed in 30 paired human gastric cancer tissues and normal tissues from TCGA RNA sequencing data. (C) LINC00707 expression was examined by qRT -PCR and normalized to GAPDH expression in 60 paired human gastric tissues compared with corresponding non-tumor tissues. (D, E) Kaplan-Meier overall survival and disease-free survival curves according to LINC00707 expression levels. **P < 0.01.

3.2. Overexpression of LINC00707 is correlated with gastric cancer malignant progression and poor prognosis

To further investigate the important role of LINC00707 clinically, we explored the correlation between LINC00707 expression and clinicopathological characteristics of GC patients. 60 patients were classified into two groups: low LINC00707 expression (n = 30) and high LINC00707 expression (n = 30) groups according to the median value (Fig. 1C). Noticeably, the incresed LINC00707 expression was significantly correlated with advanced stage (P = 0.035), lymph node metastasis (P = 0.003) and larger tumor size (P = 0.017), but not associated with patients' gender (P = 0.795), age (P = 0.301) or whether there is HP infection (P = 0.432) (Table 1). Furthermore, overall survival (OS) and disease-free survival (DFS) curves were plotted to evaluate the relationship between LINC00707 expression level and outcome of GC patients after gastrectomy. As shown in Fig. 1D and E, overexpression of LINC00707 indicated a poorer prognosis in GC patients.

3.3. LINC00707 promotes GC cell proliferation and migration in vitro

To measure the biological features of LINC00707 in GC malignant progression, we detected LINC00707 expression levels in diverse human GC cell lines, and performed gain-of-function and loss-of-function experiments. Fig. 2A showed that LINC00707 was higher upregulated in BGC-823 and SGC-7901 cells compared with MGC-803 and AGS cell lines. Next, three independent siRNAs 1#, 2#, 3# were transfected into BGC-823 and SGC-7901 cells to knock down LINC00707 expression levels. It was satisfactory that LINC00707 was more efficiently diminished by siRNAs 1# and 2# (Fig. 2B), which were selected for the next functional experiments. Additionally, LINC00707 was ectopically overexpressed with pcDNA-LINC00707 vector in MGC-803 cells (Fig. 2C).

Apparently, MTT and colony formation assays *in vitro* showed that cell proliferative vitality was impaired after LINC00707 down-regulation (Fig. 2D, E and 2G); Inversely, increased LINC00707 expression could dramatically enhance cell growing ability (Fig. 2F and H). Moreover, Edu staining revealed that the proportion of Edu positive cells in interference group was significantly reduced compared with the matched control group (Fig. 2I and J). Taken together, LINC00707

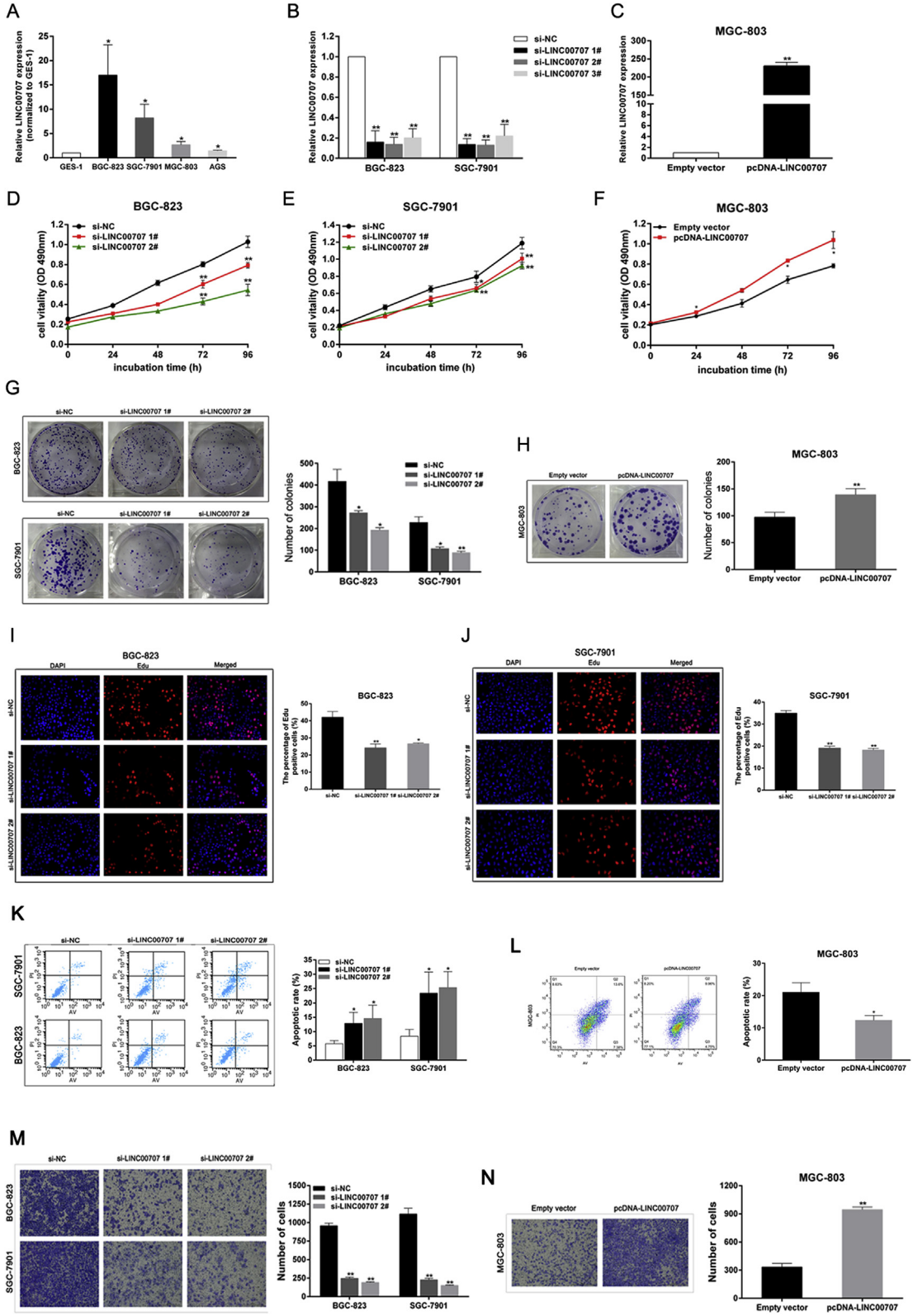


Fig. 2. The effect of LINC00707 on GC cell proliferation and migration *in vitro*. (A) QRT-PCR analysis of LINC00707 expression in the normal human gastric epithelium cell line (GES-1) and gastric cancer cells. (B) Relative expression levels of LINC00707 in BGC-823 and SGC-7901 cells transfected with si-NC or si-LINC00707 1#, 2#, 3#, was tested by qRT-PCR. (C) Relative expression levels of LINC00707 in MGC-803 cells transfected with empty vector or pcDNA-LINC00707, was tested by qRT-PCR. (D, E) MTT assays were used to determine the viability of si-LINC00707-transfected cells. (F) MTT assays in MGC-803 cells transfected with pcDNA-LINC00707 or Empty vector. (G, H) Colony formation assays were performed to determine the proliferation of si-LINC00707-transfected BGC-823 and SGC-7901 cells or overexpression plasmid-transfected MGC-803 cells. (I, J) Proliferating BGC-823 and SGC-7901 cells were labeled with Edu (red); Cell nucleus were stained with DAPI (blue). (K) Flow cytometry was performed to detect the apoptotic rates of BGC-823 and SGC-7901 cells after LINC00707 knockdown. (L) Flow cytometry was performed to detect the apoptotic rates of MGC-803 cells after LINC00707 overexpression. (M, N) Transwell assays were performed to investigate the changes in migratory abilities of si-LINC00707-transfected BGC-823 and SGC-7901 cells or pcDNA-LINC00707-transfected MGC-803 cells. *P < 0.05, **P < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

appeared to be an oncogene promoting GC cell proliferation. To explore whether apoptosis and cell cycle regulation were involved in LINC00707-propelled GC cell proliferation, we performed flow cytometric analysis. The results proved that decreased LINC00707 expression could induce GC cell apoptosis (Fig. 2K), whereas excessive LINC00707 weakened cell apoptosis (Fig. 2L). Tunel staining analysis in SGC-7901 cells verified the anti-apoptotic character of LINC00707 (Supplementary Fig. S1B). However, LINC00707 had no obvious effect on cell cycle progression (Data not shown).

Cancer cell migration also plays an important role in tumor malignant progression. To estimate whether excessive expression of LINC00707 could propel GC cell migration, transwell assays were performed. Interestingly, the number of migrational cells was strongly reduced after LINC00707 depletion in BGC-823 and SGC-7901 cells, while observably increased once overexpression of LINC00707 in MGC-803 cells (Fig. 2M and 2N).

3.4. LINC00707 promotes GC cell tumorigenesis and metastasis in vivo

To further determine whether LINC00707 could promote GC cell tumorigenesis *in vivo*, SGC-7901 cells stably transfected with sh-LINC00707 or control empty vector were inoculated into nude mice. In the process of xenograft tumors growth, we measured and calculated tumor volumes, and found that tumor growth in sh-LINC00707 group was obviously slower than that in the empty vector group (Fig. 3B). Fifteen days after injection, xenograft tumors were obtained and photographed. The picture showed that the tumors formed in the control group were generally larger than those in the sh-LINC00707 group (Fig. 3A). Apparently, the mean tumor weight of interfering group cut down compared to the matched group (Fig. 3C). Ulteriore analysis found that LINC00707 expression levels significantly lessened in tumor tissues formed from sh-LINC00707 cells (Fig. 3D). Immunohistochemical (IHC) analysis exhibited fewer Ki67-positive cells in tumor tissues collected from LINC00707 knockdown group (Fig. 3E).

Metastasis is sustained development and deterioration of caner cell proliferation, which is also recognized as the most terrible feature of advanced malignant neoplasm and the major cause of death in cancer patients. To explore LINC00707 regulation on GC cell metastasis *in vivo*, we established a nude mice lung metastasis model. As requested, SGC-7901 cells stably transfected with sh-LINC00707 or control empty vector were inoculated into the tail veins of nude mice. The entire lungs were acquired after 8 weeks, and the metastatic nodules on the lung surface were counted (Fig. 3F). The result displayed that LINC00707 knockdown could significantly reduce the number of metastatic nodules compared with the control group (Fig. 3G). Recent theoretical and practical research stated that EMT can elucidate patial reasons of cancer invasion and migration. During the entire EMT process, epithelial cell characteristics decreased, such as E-cadherin molecule; On the contrary, the mesenchymal markers increased including Snail, Slug, N-cadherin, Vimentin [20,21]. IHC staining of lung sections confirmed that sh-LINC00707 cells-metastatic lung displayed higher E-cadherin staining and lower N-cadherin, Vimentin staining (Fig. 3H). To confirm the involvement of EMT in LINC00707-mediated GC malignant progression, we continued detecting the expression levels of E-cadherin, N-cadherin and Vimentin in GC cells after LINC00707 downregulation or upregulation. QRT-PCR analysis displayed the same conclusions with above results *in vivo* (Fig. 3I and J).

Collectively, these *in vivo* data demonstrated that LINC00707 may also act as an oncogene promoting GC cell proliferation and metastasis, which is consistent with those functional results *in vitro*.

3.5. LINC00707 interacts with mRNA stabilizing protein HuR

To further explore the potential molecular mechanisms of LINC00707 in GC cells, subcellular localization was primarily detected. We found that LINC00707 RNA were mostly located in the cytoplasm versus the nucleus, which indicated that LINC00707 may exert regulatory function at post-transcriptional level. U6 was used as a nucleus marker and GAPDH was a cytosol marker (Fig. 4A). As reported, cytoplasmic lncRNAs are extensively known to cooperate with RNA-binding proteins [22]. Coincidentally, LINC00707 was proved to combine with mRNA stabilizing protein HuR by “lncRNAtor” tool (Supplementary Fig. S1C). Then the interaction between LINC00707 with HuR was further predicted by RNA-Protein interaction prediction (RPISeq) website. The score of RF Classifier and SVM Classifier are both over 0.8, intensively manifesting that LINC00707 is highly integrated with HuR (Fig. 4B). In particular, RNA pull-down and RIP analysis validated such abundant binding (Fig. 4C and D). Dramatically, it was noted that HuR expression levels remained unaltered after knockdown or overexpression of LINC00707 (Fig. 4E); LINC00707 also did not exhibit any changement with HuR depletion (Fig. 4F). Taken together, these data suggested that HuR and LINC00707 were not downstream regulators by each other, supporting their interation with each other. It is well known that HuR is one of the most common mRNA stabilizing proteins, we further explored whether HuR could regulate LINC00707 stability. Actinomycin D was used to inhibit total RNA transcript, and LINC00707 levels were detected every 3 h. The results showed that decreased HuR did not exert any influence on decay rate of LINC00707 (Supplementary Fig. S1D).

3.6. HuR functions as an oncogene in promoting GC cell proliferation and migration

Recent studies have reported that HuR is pervasively overexpressed in a variety of tumors, including breast cancer, colorectal cancer, and gastric cancer [23–25]. Our study found that HuR was higher upregulated in BGC-823 and SGC-7901 cells compared with MGC-803 and AGS cell lines (Fig. 4G). To validate its carcinous feature in GC, we synthesized two independent siRNAs for HuR. Si-HuR 2# was selected for the next functional experiments (Fig. 4H and I). MTT and colony formation assays showed that downregulated HuR weakened GC cell viability (Fig. 4J and K). Additionally, the transfer ability of GC cells was also strongly decreased due to HuR knockdown (Fig. 4L). These findings summarized the oncogenic role of HuR in promoting GC cell malignant proliferation and migration, which corresponds with carcinogenesis of LINC00707 in GC. Furthermore, we detected HuR expression levels in 60 paired human GC tissues and corresponding non-tumor tissues used in Fig. 1C, and found that HuR was indeed overexpressed in GC (Fig. 4M).

3.7. VAV3 and F11R are potential downstream target mRNAs of “LINC00707-HuR”

To investigate associated pathways and potential targets involved in “LINC00707-HuR” regulation on an unbiased basis in GC, we performed high-solution transcriptome microarray after LINC00707 or HuR knockdown in BGC-823 cells. Data analysis identified that 603 genes were differentially expressed after depletion of LINC00707, while 1353 genes altered when HuR was blocked (fold-change > 2, $p < 0.05$). Noticeably, 276 genes appeared in the intersection of these two gene sets, which were suggested as the common target genes regulated by LINC00707 and HuR (Fig. 5A, Supplementary Table S2). Gene Ontology analysis and KEGG pathway analysis implicated that cell adhesion, cell migration, cell growth and cell apoptosis were prominent pathways involved in “LINC00707-HuR” modulatory biological processes (Fig. 5B and C). Subsequently, qRT-PCR assay was conducted to validate relevant differentiated genes included in these pathways. As shown in Fig. 5D, the mRNA levels of VAV3, F11R, TNFRSF10C, WNT6, PDGFRB, GLI1 and NRXN2 were all significantly diminished due to LINC00707 or HuR knockdown in BGC-823 and SGC-7901 cells.

Recent studies have reported that mRNA stabilizing protein HuR could pervasively interact to the 3′UTR region of numerous target

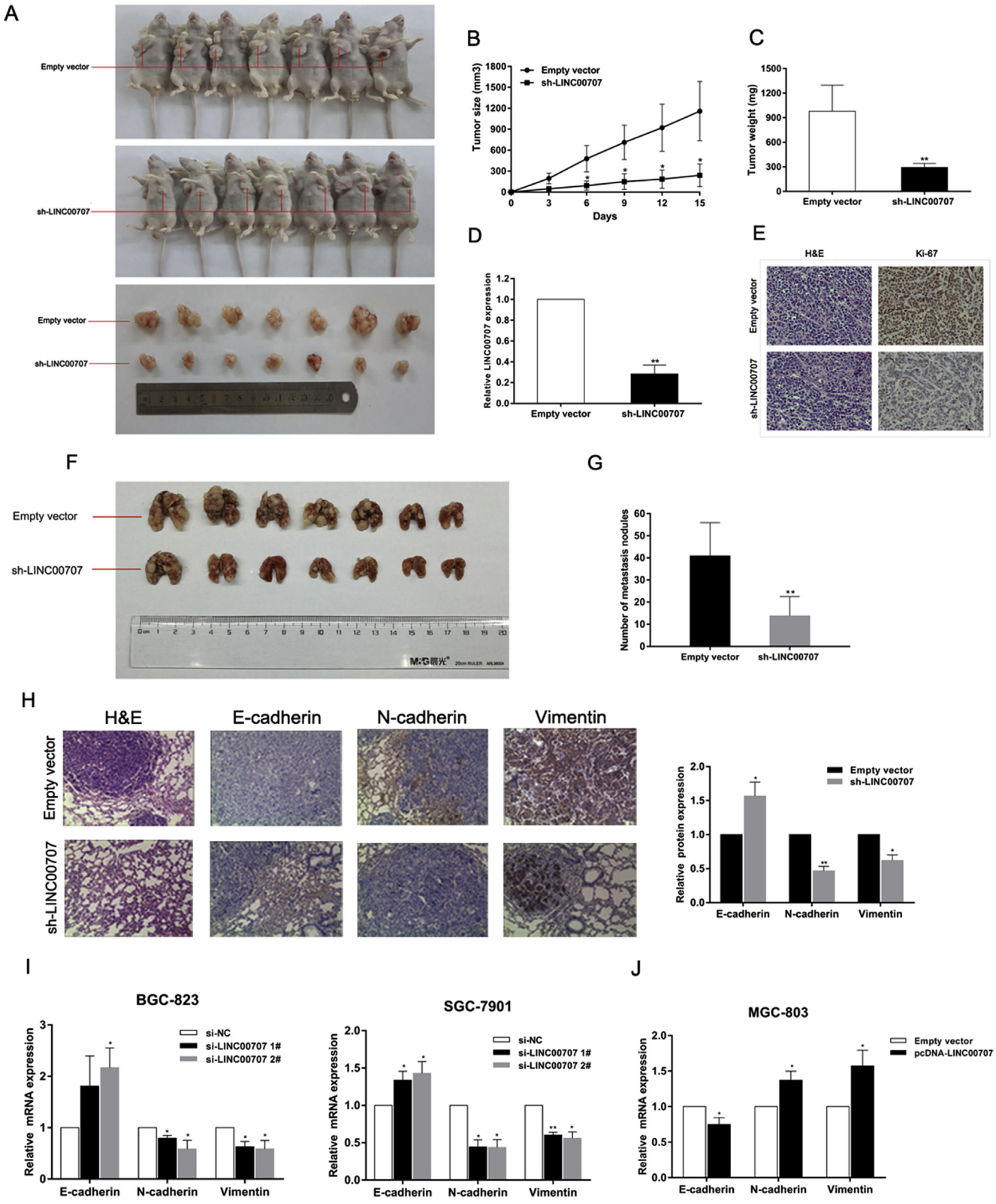


Fig. 3. The effect of LINC00707 on GC cell tumorigenesis and metastasis *in vivo*. (A) Empty vector or sh-LINC00707 were transfected into SGC-7901 cells, which were injected into the nude mice (n = 7), respectively. Tumors formed in sh-LINC00707 group were smaller than the control group. (B) Tumor volumes were calculated after injection every three days. (C) Tumor weights were represented as means of tumor weights ± SD. (D) The expression levels of LINC00707 were detected by qRT-PCR in xenograft tumors (n = 7). (E) The tumor sections were under H&E staining and IHC staining using antibodies against ki-67. (F) The entire lungs were acquired from all mice in each group. (G) The numbers of metastasis nodules on the lung surfaces were counted. (H) The lung sections were under H&E staining and IHC staining with antibodies of E-cadherin, N-cadherin, Vimentin. (I) The mRNA levels of E-cadherin, N-cadherin, Vimentin were detected after LINC00707 inhibition. (J) The mRNA levels of E-cadherin, N-cadherin, Vimentin were detected after overexpression of LINC00707. *P < 0.05, **P < 0.01.

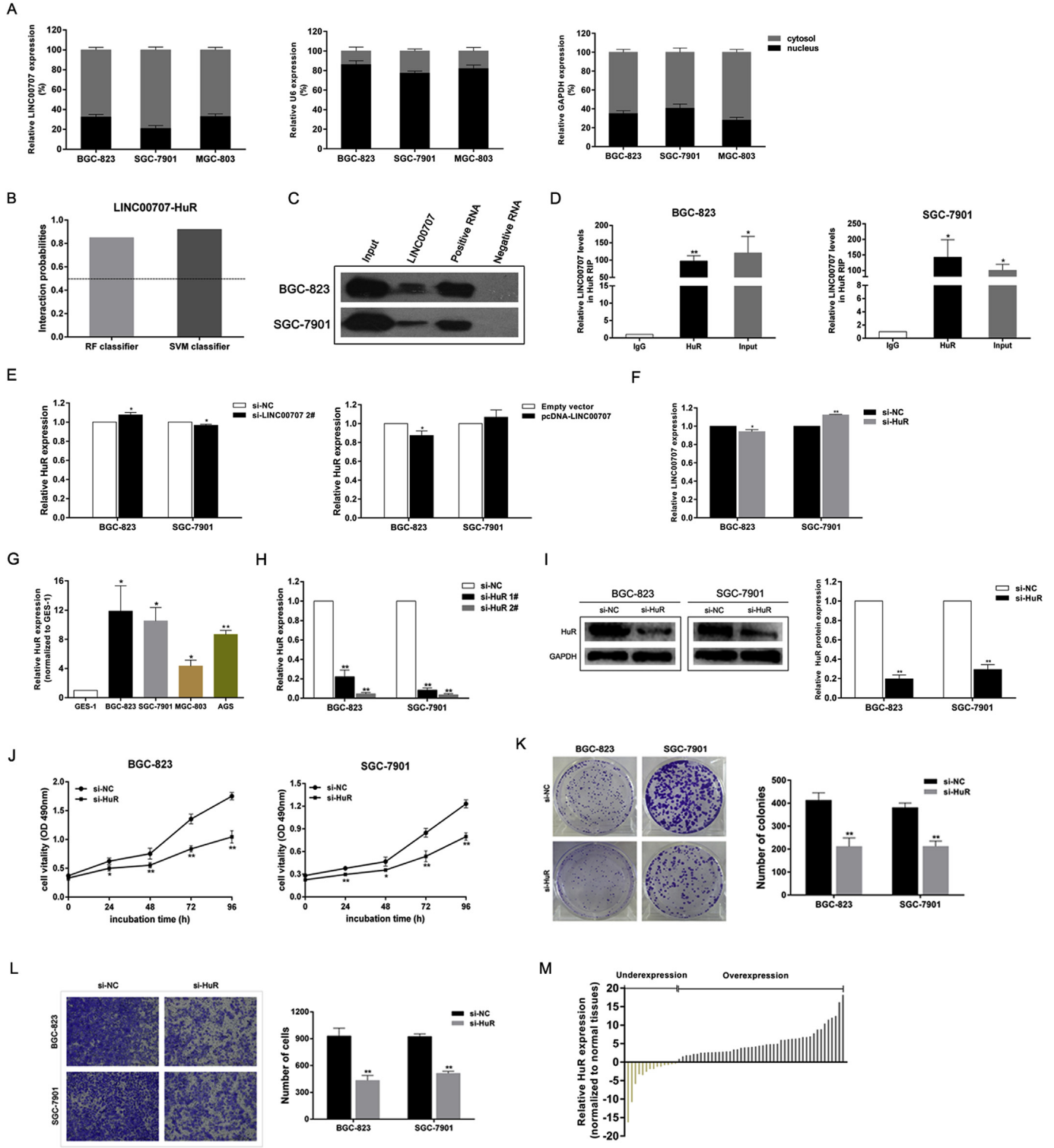


Fig. 4. LINC00707 interacts with HuR; HuR functions as an oncogene in GC. (A) LINC00707 expression levels in cell nucleus or cytoplasm of BGC-823, SGC-7901 and MGC-803 cells were detected by qRT-PCR. U6 was used as a nucleus marker and GAPDH was a cytosol marker. (B) The interaction probabilities between LINC00707 and HuR were predicted by RNA-Protein interaction prediction (RPISeq) website. Predictions with probabilities > 0.5 were considered "positive", indicating that the corresponding RNA and protein are likely to interact. (C) Biotinylated LINC00707 RNA pulled down the HuR protein, detected by western blot analysis. HuR also as a positive control. (D) RIP experiments were performed in BGC-823 and SGC-7901 cells, and the coprecipitated RNA was subjected to qRT-PCR for LINC00707. The fold enrichment of LINC00707 in HuR RIP is relative to its matching IgG control. (E) The mRNA levels of HuR were detected after LINC00707 downregulation or overexpression in BGC-823 and SGC-7901 cells. (F) The levels of LINC00707 were detected after HuR downregulation in BGC-823 and SGC-7901 cells. (G) QRT-PCR analysis of HuR expression in the normal human gastric epithelium cell line (GES-1) and gastric cancer cells. (H) Relative expression levels of HuR in BGC-823 and SGC-7901 cells transfected with si-NC or si-HuR 1#, 2#, was tested by qRT-PCR. (I) Western blot analysis of HuR after si-NC or si-HuR transfection in BGC-823 and SGC-7901 cells. GAPDH protein was used as an internal control. (J) MTT assays were used to determine the viability of si-HuR-transfected gastric cancer cells. (K) Colony formation assays were performed to determine the proliferation ability of si-HuR-transfected BGC-823 and SGC-7901 cells. (L) Transwell assays were performed to investigate the changes in migratory abilities of si-HuR-transfected BGC-823 and SGC-7901 cells. (M) HuR expression was examined by qRT-PCR and normalized to GAPDH expression in 60 paired human gastric tissues compared with corresponding non-tumor tissues. *P < 0.05,

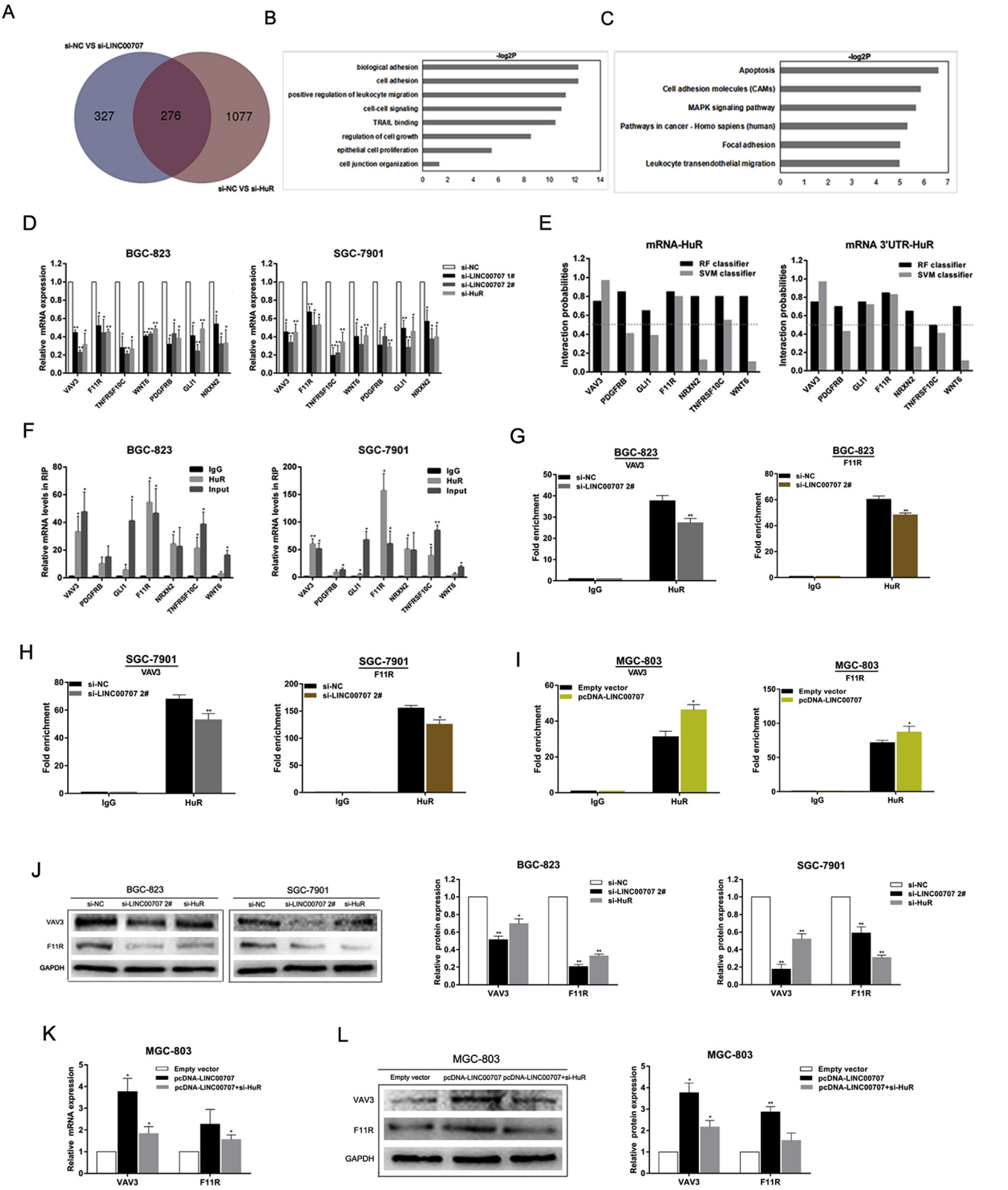


Fig. 5. VAV3 and F11R are potential downstream target mRNAs of "LINC00707-HuR". (A) 276 transcripts altered simultaneously in si-LINC00707-treated cells and si-HuR-treated cells (fold-change > 2, p < 0.05). (B) Gene Ontology analysis for 276 genes with altered expressions in Fig. 5A. (C) KEGG pathway analysis for 276 genes with altered expressions in Fig. 5A. (D) QRT-PCR was used to validate the changes of several mRNAs involved in cell adhesion, cell migration, cell growth and cell apoptosis. (E) The prediction of the interaction probabilities of HuR with those target mRNAs selected in Fig. 5D or those mRNA 3'UTR regions by RPISeq. (F) RIP experiments were performed in BGC-823 and SGC-7901 cells, and the coprecipitated RNA was subjected to qRT-PCR for those mRNAs selected in Fig. 5D. The fold enrichment of these mRNAs in HuR RIP is relative to its matching IgG control. (G, H) The fold enrichment of VAV3 and F11R were analyzed in HuR RIP after LINC00707 knockdown in BGC-823 and SGC-7901 cells. (I) The fold enrichment of VAV3 and F11R were analyzed in HuR RIP after LINC00707 overexpression in MGC-803 cells. (J) Western blot analysis of VAV3 and F11R after LINC00707 or HuR downregulation in BGC-823 and SGC-7901 cells. GAPDH protein was used as an internal control. (K) mRNA levels of VAV3 and F11R were detected for pcDNA-LINC00707 and si-HuR co-transfected MGC-803 cells. (L) Western blot analysis of VAV3 and F11R for pcDNA-LINC00707 and si-HuR co-transfected MGC-803 cells. GAPDH protein was used as an internal control. *P < 0.05, **P < 0.01.

mRNAs and enhance mRNA stability [26,27]. To probe whether analogical mechanism exists in screened target mRNAs in Fig. 5D, we summarized the prediction scores of the interaction probabilities between HuR and these mRNAs or their 3′UTR regions by RPISeq website. As a result, only VAV3, F11R mRNAs and their respective 3′UTR sequence were recognized as interacted positively with HuR protein (The scores of RF Classifier and SVM Classifier are both over 0.7) (Fig. 5E). RIP analysis in BGC-823 and SGC-7901 cells further manifested that VAV3, F11R mRNAs possess higher binding capacity with HuR protein (Fig. 5F). Moreover, the interaction of HuR and VAV3/F11R was weakened when LINC00707 was depleted (Fig. 5G and H); On the contrary, the combination was increased after overexpression of LINC00707 (Fig. 5I). In addition, the protein levels of VAV3 and F11R were significantly reduced when LINC00707 or HuR was knockdown (Fig. 5J). We also found that HuR silencing could significantly cut down accelerated VAV3/F11R levels by LINC00707 (Fig. 5K and L), which further supported “LINC00707-HuR” coalition and their co-regulation. The expression levels of LINC00707 and HuR were unaltered when VAV3 and F11R was separately inhibited, suggesting that VAV3 and F11R were potential downstream target mRNAs of “LINC00707-HuR” (Supplementary Fig. S1E).

3.8. “LINC00707-HuR” could increase VAV3 and F11R mRNAs stability

More importantly, to further inspect whether “LINC00707-HuR″ could regulate VAV3 and F11R mRNAs stability, BGC-823 and SGC-7901 cells were treated with actinomycin D, which measures the decay of pre-existing mRNA. As exhibited in Fig. 6A and C, downregulation of LINC00707 or HuR could both decrease VAV3 and F11R mRNA half-life. However, the protein levels of VAV3 and F11R in BGC-823 cells were nearly unchanged at these points (0 h, 3 h, 6 h, 9 h), which may be attributed to this short period of time and longer half-life of these proteins (Fig. 6B). Furthermore, rescue assays were carried out to illustrate the function of LINC00707 depending on HuR. MGC-803 cells were co-transfected with pcDNA-LINC00707 vector and HuR siRNAs. As expected, HuR silencing could significantly reduce LINC00707-increased VAV3/F11R mRNAs half-life (Fig. 6D). In summary, these data revealed that LINC00707 and HuR could separately increase VAV3 and F11R mRNAs stability, and this function of LINC00707 was dependent on HuR regulation.

3.9. Regulation of VAV3 and F11R is potentially involved in the oncogene function of LINC00707

The biological functions of VAV3 and F11R were explored in BGC-823 and SGC-7901 cells afterwards. Fig. 6E showed that siRNAs 2#, 3# of VAV3 and F11R possessed more effective inhibition than siRNA 1#, thus 2# and 3# siRNAs were used for the following functional assays. Moreover, the proteins of VAV3/F11R were respectively reduced after 2# siRNAs silencing (Fig. 6F). Colony formation assays found that the number of cell colonies significantly decreased in siRNAs-transfected group compared to the control group (Fig. 6G). Similarly, Edu staining in BGC-823 and SGC-7901 cells further demonstrated that cell proliferation ability impaired after suppression of VAV3 and F11R (Fig. 6I). In addition, VAV3 and F11R were confirmed to promote GC cell migration by transwell assays (Fig. 6H). Overall, these findings indicated that VAV3 and F11R may function as oncogenes in GC malignant process, corresponding to the carcinogenesis of LINC00707 and HuR in GC.

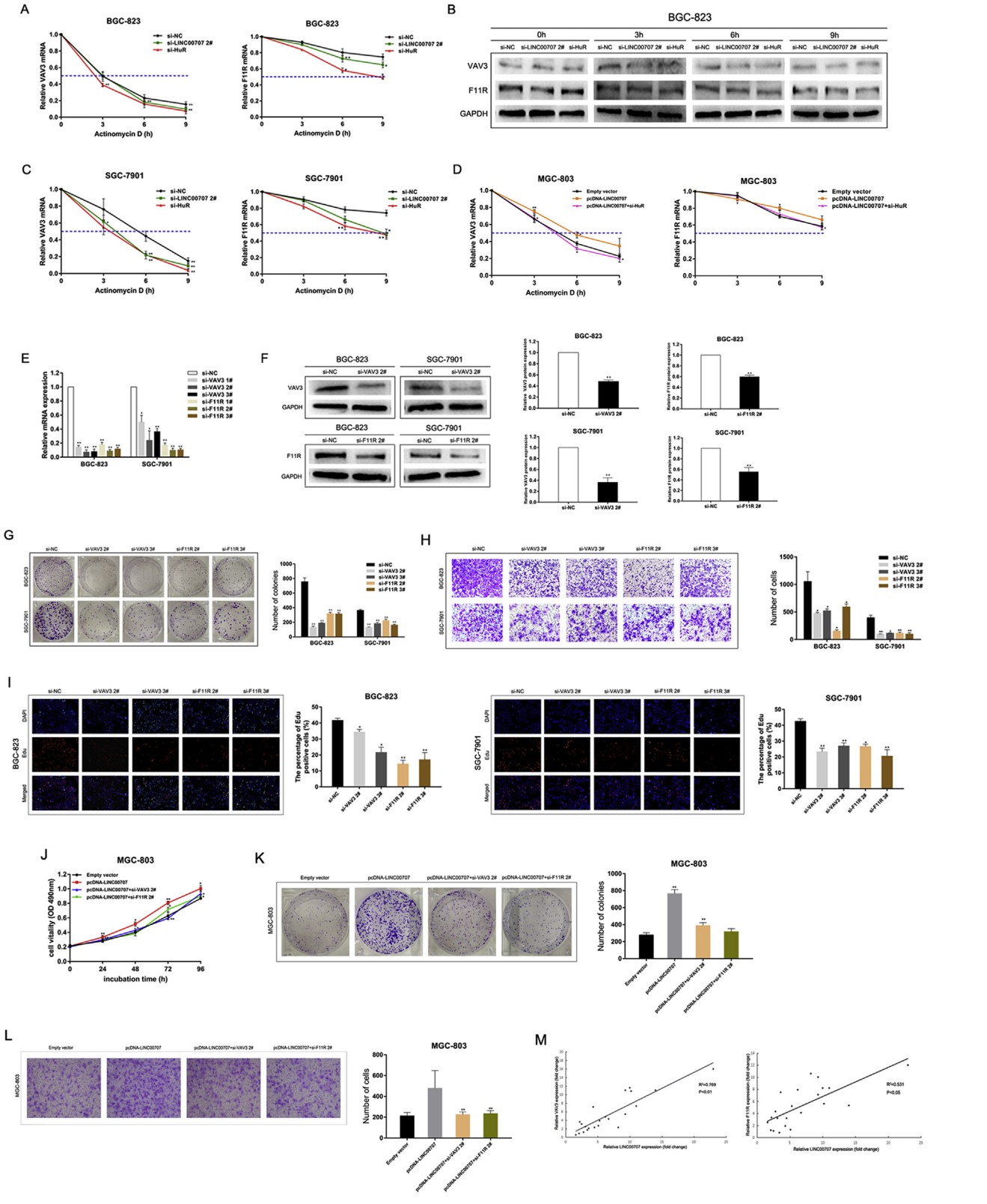
To investigate whether VAV3 and F11R were involved in the LINC00707-induced GC cell proliferation and metastasis, we carried out rescue experiments. MGC-803 cells were co-transfected with pcDNA-LINC00707 and VAV3/F11R siRNAs. MTT and colony formation assay results indicated that co-transfection could partially cut down LINC00707-induced GC cell proliferation (Fig. 6J and K); Metastatic cells were significantly decreased after co-transfection (Fig. 6L). Further analysis turned out that the expression levels of VAV3 and F11R were both positively correlated with LINC00707 expression levels in GC tissues (Fig. 6M).

4. Discussion

The terrible proliferation, metastasis and anti-apoptosis are the most critical and intrinsic biology characteristics of GC, which are also the major causes of death in GC patients. The process of GC development is extremely complicated, and a large body of oncogenes or tumor suppressor genes are involved in GC carcinogenesis [28–30]. LncRNAs can act as essential mediators for GC malignant progression. Several lncRNAs have been identified as biomarkers for GC diagnosis and therapy [31–33]. To date, LINC00707-associated functions and mechanisms in GC have not been publicly reported. In this study, we primarily displayed that LINC00707 was observably overexpressed in GC tissues by bioinformatics analysis (GEO and TCGA database) and qRT-PCR verification. Clinicopathological evidences suggested that high LINC00707 expression was significantly correlated with advanced stage, larger tumor size, lymph node metastasis and poorer prognosis in GC patients. Gain or loss of function assays ulteriorly verified that excessive LINC00707 could promote GC cell proliferation and migration *in vitro* and *in vivo*. These findings indicate that LINC00707 act as an oncogene in GC development, and turn to be a GC-related biomarker.

It is prevalent for lncRNAs cooperating with mRNA-binding proteins to maintain mRNA stability these years [14,34,35]. In this article, bioinformatics analysis, RNA pull-down and RIP assays together emphasized the abundant binding relationship between LINC00707 and mRNA stabilizing protein HuR. According to literature reports, HuR (ELAVL1) is one ubiquitous mRNA-binding protein of drosophila embryonic lethal abnormal vision (ELAV) family members. It is necessary for cell growth and differentiation normally, while its abnormal expression can induce tumor occurance [36]. In Kang's study, the mRNA and protein levels of HuR in GC tissues were both twofold than that in normal gastric tissues; In addition, its high expression was closely related to TNM stage [25]. HuR protein is widely known to post-transcriptionally interact with 3‘UTR regions of target mRNAs, thus enhancing mRNAs stability [37,38]. Systematically, VAV3 and F11R mRNAs were gradually demonstrated as the common targets of LINC00707 and HuR. Furthermore, LINC00707 and HuR could separately increase VAV3/F11R mRNAs stability; Particularly, such positive capacity of LINC00707 was dependent on HuR regulation.

VAV3 belongs to VAV family proteins, which is a guanine nucleotide exchange factor (GEF) for Rho family GTPases [39,40]. A large body of evidences implicate that VAV3 plays an important role in cell adhesion, angiogenesis and cell differentiation [41,42]. Strikingly, VAV3 appears to abnormally overexpress in a variety of tumors, including gastric cancer, ovarian cancer and colorectal cancer et al. [43–45]. On the basis of previous studies, high expression of VAV3 can promote GC cell growth and metastasis [46]. Notably, VAV3 can be an independent therapeutic and prognostic marker for GC clinically [43]. F11R is also known as junctional adhesion molecule A (JAM-A), which is located in tight junction (TJ) and affects epithelial cell morphology and migration [47,48]. Oncology researches confirm that the depletion of F11R can impair cellular adhesion and polarity, then inducing tumor initiation and metastasis [49]. Ikeo et al. showed that overexpression of F11R promoted GC cell proliferation and inhibited GC cell apoptosis [50]. Tian's team pointed out that high expression of F11R could be an EMT inducer, which was correlated with metastasis and poor prognosis in human nasopharyngeal carcinoma [51]. Our manuscript further verified VAV3/F11R carcinogenesis and their involvement in the LINC00707-induced GC cell proliferation and metastasis. In addition, the expression levels of VAV3 and F11R were both positively correlated with LINC00707 expression level in GC tissues. However, whether LINC00707 directly associates with VAV3/F11R mRNAs or enhances the physical interaction between HuR protein and VAV3/F11R mRNAs deserves further investigation.



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In summary, this study determined that upregulated LINC00707 could promote GC growth and metastasis both *in vitro* and *in vivo*. In addition, it is recognized as a potential target for GC diagnosis and prognosis. Mechanistically, LINC00707 could abundantly interact with mRNA stabilizing protein HuR; "LINC00707-HuR" coalition ulteriorly combined with VAV3/F11R mRNAs and increased their stability. Our findings may provide a new sight of LINC00707 regulating mRNA stability, and advance our understanding of lncRNA-regulatory characteristics in GC malignant progression.

Fig. 6. "LINC00707-HuR" could increase VAV3 and F11R mRNAs stability. (A) RNA stability assays were performed to measure degradation rates of VAV3 and F11R mRNA in BGC-823 cells with LINC00707 or HuR knockdown. (B) Western blot was used to analyze the protein stability of VAV3 and F11R in BGC-823 cells. GAPDH protein was used as an internal control. (C) RNA stability assays were performed to measure degradation rates of VAV3 and F11R mRNA in SGC-7901 cells with LINC00707 or HuR knockdown. (D) Rescue assays were carried out to illustrate that HuR silencing could significantly reduce LINC00707-increased VAV3/F11R mRNAs half-life in MGC-803 cells. (E) Relative expression levels of VAV3/F11R in BGC-823 and SGC-7901 cells transfected with si-NC or siRNA 1#, 2#, 3#, were tested by qRT-PCR. (F) Protein levels of VAV3 and F11R were separately analyzed in si-VAV3 2# and si-F11R 2# transfected cells. GAPDH protein was used as an internal control. (G) Colony formation assays were performed to determine the proliferation ability of VAV3/F11R-inhibited BGC-823 and SGC-7901 cells. (H) Transwell assays were performed to investigate the changes in migratory abilities of VAV3/F11R-knockdown BGC-823 and SGC-7901 cells. (I) Proliferating BGC-823 and SGC-7901 cells were labeled with Edu (red); Cell nucleus were stained with DAPI (blue). (J, K) MTT and colony formation assays were conducted to determine the cell viability of pcDNA-LINC007 and si-VAV3 2#, si-F11R 2# co-transfected MGC-803 cells. (L) Transwell assays were performed to investigate the changes in migratory abilities of pcDNA-LINC007 and si-VAV3 2#, si-F11R 2# co-transfected MGC-803 cells. (M) Analysis of the relationship between VAV3/F11R and LINC00707 expression. *P < 0.05, **P < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Authors' contributions

MX designed the whole study and wrote this manuscript. TM and HM performed cell culture, transfection, cell proliferation and metastasis assays. JX performed the database analysis from GEO,TCGA, and carried out Western blot assays. RNA pull-down and RIP experiments were carried out by MS. ZZ was responsible for collecting GC tissues and analyzing clinicopathological characteristics of GC patients. ML and YL conducted RNA extraction, qRT-PCR assays. ZW and WD collected, evaluated experimental data, and made the pictures. All authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare that they have no conflicts of interest.

Acknowledgements

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.032.

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