

Long noncoding RNA TSLNC8 Enhances Pancreatic Cancer Aggressiveness by Regulating CTNNB1 Expression via Association with HuR

Wei Chai

Cangzhou central hospital

Ruhai Liu

cangzhou central hospital

Fengshan Li

cangzhou chentral hospital

Zhiquan Zhang

cangzhou central hospital

Bao Lei (✉ baolei7808@tom.com)

Cangzhou central hospital

Research

Keywords: HuR, mRNA stability, posttranscriptional regulation, Wnt/ β -catenin signaling pathway

Posted Date: June 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-32087/v1>

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Version of Record: A version of this preprint was published at Human Cell on September 20th, 2020. See the published version at <https://doi.org/10.1007/s13577-020-00429-4>.

Abstract

Background

Pancreatic cancer (PC) is one of the most lethal malignancies worldwide. Tumor suppressor long noncoding RNA on chromosome 8p12 (TSLNC8) is a newly identified long noncoding RNA (lncRNA) and play an important role in human cancers. However, the function and molecular mechanism of TSLNC8 in PC progression remains to be elucidated.

Methods

qRT-PCR was performed to examine the expression pattern of TSLNC8 in PC tissues and cell lines. Overexpression and knockdown experiments were conducted to detect the function of TSLNC8 in PC. The interaction between TSLNC8 and HuR was tested by RNA immunoprecipitation assay.

Results

Our results showed a significant increase of TSLNC8 expression in PC tissues and cell lines. Upregulation of TSLNC8 expression in PC tissues was closely correlated with TNM stage, distant and lymph node metastasis, and poor prognosis of PC patients. Functional experiments demonstrated that TSLNC8 promoted PC cells proliferation and invasion in vitro, and enhanced PC growth and metastasis in vivo. Mechanistically, TSLNC8 associated with HuR, promoted the binding of HuR with CTNNB1 mRNA and increased the stability of CTNNB1 mRNA, thus activating WNT/ β -catenin signaling pathway.

Conclusion

Our present study revealed that oncogenic lncRNA TSLNC8 positively regulate PC growth and metastasis via HuR-mediated mRNA stability of CTNNB1, extending the understanding of PC pathogenesis regulated by lncRNAs.

Background

Pancreatic cancer (PC) is one of the most lethal malignancies worldwide, which causes more than 331,000 deaths per year (1). The risk factors of PC includes smoking, obesity, genetics, diabetes mellitus, alcohol use and physical inactivity (2). Due to the atypical symptoms and limitation of diagnostic techniques of PC, most of patients were diagnosed at an advanced stage. Despite the great improvement of comprehensive therapy of PC over the past decades, the 5-year survival rate of PC patients remains only approximately 7% (3, 4). Therefore, revealing the underlying mechanism of PC progression are urgently needed for the identification of novel diagnostic and therapeutic targets for PC.

Long noncoding RNAs (lncRNAs) belong to a subfamily of noncoding RNAs, whose length are larger than 200 nucleotides. Increasing evidence demonstrated a closely relationship between lncRNAs and cancer occurrence and progression. lncRNAs regulated malignant phenotypes of cancer cells, such as proliferation, invasion, metabolism, autophagy, drug resistance, via association with chromatin modifiers, proteins, miRNAs and mRNAs (5–8). To date, numbers of lncRNAs have been annotated, but only a handful of lncRNAs have been functionally identified. Some lncRNAs function as tumor suppressors or oncogenes in PC. For example, overexpression of lncRNA-BX111887 (BX111) in PC tissues indicates short overall survival time in patients. BX111 contributes to the hypoxia-induced epithelia-mesenchymal transition (EMT) of PC cells by activation of ZEB1 transcription via recruiting transcriptional factor Y-box protein (YB1) to its promoter region (9). lncRNA LINC01111 is significantly downregulated in PC tissues and positively correlated with the survival of PC patients. LINC01111 sequesters miR-3924 to upregulate DUSP1 and inactivate the SAPK/JNK signaling pathway, thus suppressing PC tumorigenesis and metastasis (10). Tumor suppressor long noncoding RNA on chromosome 8p12 (termed TSLNC8) is a newly identified lncRNA. TSLNC8 was firstly found to be downregulated in hepatocellular carcinoma (HCC) tissues and significantly suppress the proliferation and metastasis of HCC cells by competitively interacting with transketolase and STAT3 and inactivating IL-6/STAT3 pathway (11). Downregulation of TSLNC8 expression was also observed in breast cancer, glioma, and non-small cell lung cancer (12–14). However, the functional role and molecular mechanism of TSLNC8 in PC progression remain to be elucidated.

In the present study, we aim to analyze role of TSLNC8 in PC progression. The differential expression of TSLNC8 in PC and normal pancreatic tissues was detected. The correlations between TSLNC8 expression and clinicopathological features and survival of PC patients were evaluated. The function of TSLNC8 in PC proliferation was analyzed through gain-of-function and loss-off function experiments. Mechanistic investigation revealed that TSLNC8 stabilized CTNNB1 mRNA via association with RNA binding protein HuR. Our findings represent a novel therapeutic target for PC therapy.

Materials And Methods

Tissue collection

Seventy patients with PC were included in the present study, and the PC and corresponding adjacent normal pancreatic tissues were obtained from these PC patients in Cangzhou Central Hospital. The tissues were immediately frozen in liquid nitrogen and stored at -80 °C until used. This research was approved by the Ethics Committee of Cangzhou Central Hospital and carried out in accordance with the Declaration of Helsinki. All the patients provided the informed consents. Tumor stage was evaluated by two experienced pathologists according to the TNM staging of the International Union against Cancer/American Joint Committee on Cancer system (15).

Cell Culture

A normal human pancreatic cell line (HPDE) and six different PC cell lines (AsPC-1, Capan-2, SW1990, PANC-1, PaCa-2, and BxPC-3) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco) in a humidified incubator containing 5% CO₂ at 37 °C. All cell lines were authenticated and tested to be mycoplasma-free.

Construction Of Stable Cells And Cell Transfection

To generate cells stably overexpressing TSLNC8, cells were infected with a lentiviral vector containing full-length TSLNC8 or an empty vector control (GenePharm, Shanghai). To generate cells stably silencing TSLNC8, cells were infected with a lentiviral vector expressing TSLNC8 shRNA or negative control scramble shRNA (GenePharm, Shanghai). The target sequence of TSLNC8 shRNA (shTSLNC8) was shown as follow: GCTGAACTCTCTGCCAAA. Stably clones were selected for 2 weeks using puromycin. Cell transfection was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. pLKO.1 containing HuR shRNA was constructed by GenePharm (Shanghai). The target sequence of indicated shRNA was shown as follow: shHuR: TCGGGAGAACGAATTTGATCGTCAA.

Cell Proliferation Assay

Cell proliferation was detected by Cell Counting Kit-8 (CCK-8). 1×10^3 cells/well was seeded into 96-well plates. At indicated time point, 10 μ L of CCK-8 reagent (Dojindo) was added to each well and incubated for additional 1 hour at 37 °C. Then, the absorbance was measured at 450 nm in a microplate reader (Bio-rad).

Colony Formation Assay

2000 cells/well were seeded into 6-well plates and cultured for one week. The cells were fixed with 4% paraformaldehyde and then stained with 0.5% crystal violet. The number of colonies was counted.

Transwell Assay

Cell invasion was tested by transwell assay. Briefly, 2×10^5 cells in 200 μ L serum-free medium were added into the upper chamber with matrigel, while medium with 10% FBS was added into the lower chamber. 24 hours later, the cells in the upper chamber were carefully removed with a cotton swab, and the cells in the lower surface of the membrane was fixed with 4% paraformaldehyde and then stained with 0.5% crystal violet.

Quantitative Real-time PCR (qRT-PCR)

Total RNA from cells or tissue samples was isolated using RNeasy mini kit (Qiagen) and reverse transcribed using Reverse Transcription System (Promega). qRT-PCR was performed using the SYBR Green MasterMix (Takara) on ABI StepOne Plus system. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of indicated genes. GAPDH was used as internal reference. The primer sequences were provided as follow:

TSLNC8-forward: CCCAAGAGTGTCCAGATGATAC

TSLNC8-reverse: GGAAGGGTCTCAGTGCTTATT

CTNNB1-forward: CTTACCTGACAGATCCAAGTC

CTNNB1-reverse: CCTTCCATCCCTTCCTGTTTAG

HuR-forward: GGCTACGGCTTTGTGAACTA

HuR-reverse: GCGAGCATACGACACCTTAAT

GAPDH-forward: GTCAACGGATTTGGTCGTATTG

GAPDH-reverse: TGTAGTTGAGGTCAATGAAGGG

c-myc-forward: AAGCTGAGGCACACAAAGA

c-myc-reverse: GCTTGGACAGGTTAGGAGTAAA

c-jun-forward: CACAGAGAGACAGACTTGAGAAC

c-jun-reverse: ACTTGGATACCCTTGGCTTTAG

MMP7-forward: CACTGTTCCCTCCACTCCATTTA

MMP7-reverse: GACATCTACCCACTGCAAGTATAG

CD44-forward: GAAATGGCACCCTGCTTATG

CD44-reverse: CTACTAGGAGTTGCCTGGATTG

AXIN2-forward: CTTATCGTGTGGGCAGTAAGA

AXIN2-reverse: GTTCTCGGGAAATGAGGTAGAG.

Immunoblotting

Cells were lysed using RIPA buffer (Beyotime). Equal amounts of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto PVDF

membrane (Millipore). The membranes were incubated with antibodies against HuR (Abcam), β -catenin (Cell signaling) and GAPDH (Proteintech) overnight at 4 °C and then cultured with corresponding HRP-conjugated secondary antibody (Jackson) for 1 hour. Finally, signals were detected using an enhanced chemiluminescence (ECL) system (Millipore).

RNA Immunoprecipitation (RIP) Assay

RIP assay was performed using an EZ-Magna RIP Kit (Millipore) according to the manufacturer's instructions. The anti-HuR antibody (Abcam) and negative control IgG (Cell Signaling) was used. The RNA in the immunoprecipitate complex was analyzed by qRT-PCR.

RNA Pull-down Assay

RNA pull-down assay was carried out using Magnetic RNA-Protein Pull-Down Kit (Thermo) according to the manufacturer's instructions. The protein pulled down by TSLNC8 or negative control antisense TSLNC8 was subjected to western blot analysis.

Subcellular Localization Of TSLNC8

The PARIS Kit (Life Technologies) was used to isolate the cytoplasmic and nuclear RNA according to the manufacturer's instructions. Then, the subcellular localization of TSLNC8 was detected by qRT-PCR. The GAPDH and U6 transcripts were used as internal reference of cytoplasmic and nuclear RNA, respectively.

Luciferase Reporter Assay

To detect the activity of Wnt signaling, TOPflash or FOPflash (Promega) with pRL-TK plasmid were transfected into cells. The luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega). The relative ratio of firefly luciferase activity to Renilla luciferase activity was determined as the TOPflash reporter activity. FOPflash was taken as a negative control. To detect the luciferase activity of CTNNB1 promoter, full-length CTNNB1 promoter was cloned into pGL3 plasmid (pGL3-CTNNB1). pGL3 or pGL3-CTNNB1 with pRL-TK was transfected into stable PC cells. After 48 hours, the luciferase activities were measured using a dual-luciferase reporter gene assay system (Promega). The relative ratio of firefly luciferase activity to Renilla luciferase activity was measured.

Animal Experiments

Animal experiments were carried out following the protocol approved by the Ethics Committee of Cangzhou Central Hospital. To investigate tumor growth *in vivo*, 1×10^7 control and TSLNC8-knockdown

PaCa-2 cells were subcutaneously inoculated into the right flank of nude mice. Tumor growth was monitored and volume was calculated as the formula $1/2 \times \text{length} \times \text{width}^2$. The mice were sacrificed after 30 days and tumors were excised and weighted. To investigate tumor metastasis *in vivo*, 1×10^6 control and TSLNC8-knockdown PaCa-2 cells were injected into tail vein of nude mice. After 60 days, the mice were sacrificed, and the lung tissues were excised and subjected to H&E staining according to the standard procedure. The pulmonary metastasis nodule was calculated under a microscope.

Statistical Analysis

All the experiments were repeated at least three times. Comparisons between groups were performed by Student's t test or ANOVA method followed by Tukey's test. Chi-square test was used to analyze the relationship between TSLNC8 expression and clinicopathological features of PC patients. Kaplan–Meier method and log-rank tests were used to evaluate survival curves. $p < 0.05$ was considered as statistically significant difference.

Results

Overexpression of TSLNC8 indicates poor prognosis of PC patients

We first explored the expression pattern of TSLNC8 in PC tissues via performing qRT-PCR analysis. As shown in Fig. 1A, TSLNC8 expression was significantly upregulated in PC tissues compared with paired corresponding normal pancreatic tissues. Consistently, PC cell lines expressed higher TSLNC8 expression than did in the normal pancreatic cell line HPDE (Fig. 1B). To clarify the clinical significance of TSLNC8 expression, patients were divided into high and low TSLNC8 group with the median expression level in PC tissues as the cutoff values. The correlation analysis between TSLNC8 expression and clinicopathological features of patients with PC showed that TSLNC8 was markedly associated with TNM stage, distant and lymph node metastasis (Table 1). However, we did not observe significant relationship of TSLNC8 with other factors, including age, gender, tumor size and differentiation. Moreover, the Kaplan–Meier survival analysis demonstrated that PC patients with high-level TSLNC8 showed shorter overall survival compared with those with low-level TSLNC8 (Fig. 1C).

Table 1
The correlation analysis between TSLNC8 expression and clinicopathological features of pancreatic cancer patients

Features	TSLNC8 expression		p value
	High	Low	
Gender			
Male	20	18	0.631
Female	15	17	
Age			
<60	21	19	0.629
≥ 60	14	16	
Tumor Size (cm)			
<2	18	22	0.334
≥ 2	17	13	
Differentiation			
Well/moderate	17	24	0.089
Poor	18	11	
TNM Stage			
I-II	9	23	0.001
III-IV	26	12	
Vascular infiltration			
Yes	16	18	0.632
No	19	17	
Distant metastasis			
Yes	21	9	0.004
No	14	26	
Lymph node metastasis			
Yes	20	11	0.030
No	15	24	

TSLNC8 promotes proliferation of PC cells in vitro and in vivo

Then, the biological function of TSLNC8 was investigated via performing gain- and loss-of-function experiments. Based on the endogenous expression level of TSLNC8 of different PC cell lines (Fig. 1B), lentiviral expressing system was used to establish stable PaCa-2 cells with TSLNC8 knockdown and PANC-1 cells with TSLNC8 overexpression (Fig. 2A-2B). The results of CCK-8 and colony formation assays showed that knockdown of TSLNC8 obviously suppressed the proliferative ability of PaCa-2 cells compared with that of control cells (Fig. 2C-2D), while PANC-1 cell proliferation was elevated by TSLNC8 overexpression (Fig. 2E-2F).

To further confirm the effect of TSLNC8 on tumorigenic capability of PC cells *in vivo*, the control and TSLNC8-knockdown PaCa-2 cells were injected subcutaneously into the right flanks of nude mice. The results showed that tumor growth was significantly decreased in mice injected with TSLNC8-knockdown PaCa-2 cells compared to control group (Fig. 2G). Moreover, xenograft tumors formed from TSLNC8-knockdown cells showed smaller weights than tumors grown from control cells (Fig. 2H).

TSLNC8 enhances PC cell invasion *in vitro* and metastasis *in vivo*

The significant correlation between TSLNC8 and PC metastasis prompted us to investigate whether TSLNC8 has an effect on PC cell invasion and metastasis. Transwell assay showed that the invasive ability was obviously attenuated by knockdown of TSLNC8 in PaCa-2 cells (Fig. 3A), while overexpression of TSLNC8 significantly enhanced the invasion of PANC-1 cells (Fig. 3B).

To further validate the function of TSLNC8 on metastasis capability of PC cells *in vivo*, the control and TSLNC8-knockdown PaCa-2 cells were injected into the tail vein of nude mice. After 60 days, the pulmonary metastasis was detected and the results revealed that TSLNC8-knockdown group had less pulmonary metastatic nodules than control group (Fig. 3C-3D).

TSLNC8 Increases CTNNB1 Expression

To further uncover the underlying mechanism of TSLNC8 in PC growth and metastasis, the expression profile difference in TSLNC8-knockdown PaCa-2 cells was examined by RNA-sequencing. KEGG pathway analysis showed that the genes regulated by TSLNC8 were significantly enriched in WNT/ β -catenin signaling pathway which is crucial for cancer occurrence and progression (Fig. 4A). Among the downstream genes of TSLNC8, CTNNB1 (encoded β -catenin) is of particular interest because of its remarkable expression fold change upon TSLNC8 knockdown and its important contribution to WNT/ β -catenin signaling pathway. We found that knockdown of TSLNC8 inhibited both mRNA and protein levels of CTNNB1 (Fig. 4B-4C). Conversely, overexpression of TSLNC8 upregulated CTNNB1 expression (Fig. 4D-4E).

To clarify the mechanism by which TSLNC8 increased CTNNB1 expression, we constructed the luciferase reporter containing CTNNB1 promoter and detect whether TSLNC8 affected the CTNNB1 transcription. Neither knockdown nor overexpression of TSLNC8 influenced the luciferase activity of CTNNB1 promoter

(Fig. 4F-4G). Moreover, TSLNC8 mainly distributed in cytoplasm (Fig. 4H), indicating that TSLNC8 might suppress the degradation of CTNNB1 mRNA. To validate this, stable PC cells with TSLNC8 alteration were treated with Actinomycin D to block RNA synthesis, and the degradation of CTNNB1 mRNA was examined by using qRT-PCR assay. Knockdown of TSLNC8 shortened the half-life of CTNNB1 mRNA (Fig. 4I), while overexpression of TSLNC8 elongated the half-life of CTNNB1 mRNA (Fig. 4J).

TSLNC8 Activates WNT/ β -catenin Signaling Pathway

We then tested whether TSLNC8 regulated Wnt/ β -catenin signaling, and found that luciferase activity of Topflash reporter was suppressed after knockdown of TSLNC8 in PaCa-2 cells (Fig. 5A), but enhanced by overexpression of TSLNC8 in PANC-1 cells (Fig. 5B). Similarly, TSLNC8 was capable to modulate the expression of Wnt/ β -catenin signaling target genes, including c-myc, c-jun, MMP7, CD44, and AXIN-2 (Fig. 5C-5D).

TSLNC8 Associates With HuR To Stabilize CTNNB1 mRNA

Recently, RNA binding protein HuR has been found to stabilize CTNNB1 mRNA, which is regulated by some lncRNAs (16–18). We suspected that TSLNC8 might regulate CTNNB1 in this manner. To confirm this hypothesis, we performed a RIP assay using an anti-HuR antibody and found that TSLNC8 was significantly enriched by HuR (Fig. 6A). To further validate the interaction between TSLNC8 and HuR, TSLNC8-pull-down protein samples were subjected to immunoblot with HuR antibody. The HuR could be significantly pulled down by TSLNC8 (Fig. 6B). Moreover, silence of TSLNC8 attenuated the interaction between HuR and CTNNB1 mRNA in PaCa-2 cells (Fig. 6C), whereas HuR-CTNNB1 association was enhanced by TSLNC8 overexpression in PANC-1 cells (Fig. 6D).

We then determined the role of HuR in TSLNC8-mediated CTNNB1 upregulation and PC aggressiveness. shRNA targeting HuR was transfected into TSLNC8-overexpressing PANC-1 cells. We found that depletion of HuR abolished the CTNNB1 increase by TSLNC8 overexpression (Fig. 6E), and weaken the proliferative and invasive capacity of TSLNC8-overexpressing PANC-1 cells (Fig. 6F-6H).

Discussion

To the best of our knowledge, the present study is the first to reveal the oncogenic role of lncRNA TSLNC8 in PC cells. Here, we found a significant increase of TSLNC8 expression in PC tissues and cell lines. Overexpression of TSLNC8 in PC tissues was closely correlated with TNM stage, distant and lymph node metastasis, and poor prognosis of PC patients. Functional experiments demonstrated that TSLNC8 promoted PC cell proliferation and invasion in vitro, and enhanced PC growth and metastasis in vivo. Mechanistically, TSLNC8 associated with HuR, promoted the binding of HuR with CTNNB1 mRNA and increased the stability of CTNNB1 mRNA, thus activating WNT/ β -catenin signaling pathway. Together, our

findings indicated that lncRNA TSLNC8 exerts its function as an oncogene in PC and may be used as a potential prognostic indicator and therapeutic target PC.

Previous studies demonstrated that TSLNC8 acted as a tumor suppressor in some cancer. TSLNC8 inactivates IL-6/STAT3 pathway to inhibit HCC progression (11). In breast cancer cells, TSLNC8 increased FOXP2 expression through sponging miR-214-3p, repressing cellular proliferation (19). TSLNC8 also downregulated HIF-1 α expression to inhibit proliferation and migration and accelerate apoptosis of lung cancer cells (13). However, here, we found that TSLNC8 was upregulated in PC tissues and cells and functioned as an oncogene. lncRNAs generally show tissue- or disease-specific expression patterns (20, 21). Consistent with our finding, some lncRNAs have been found to exert opposite effects in different types of human cancers. For example, lncRNA FTX upregulates FOXA2 expression to suppress non-small-cell lung cancer progression (22). Nevertheless, lncRNA FTX inhibits phosphorylation of vimentin for promoting colorectal cancer metastasis (23). The specific expression pattern and function of some lncRNAs may be useful for diagnosis and treatment for specific cancer. Why the function of some lncRNAs in different cancers is opposite needs further investigation.

Here, CTNNB1 was identified as a downstream gene of TSLNC8. Our results showed a cytoplasmic distribution of TSLNC8 in PC cells, suggesting that TSLNC8 may regulate CTNNB1 expression in a post-transcriptional manner. HuR is an RNA binding protein and increased in PC (24). The target mRNA of HuR in PC includes PARG, IDH1, WEE1 and PIM1 (25–27). Previous research reported that HuR physically interacts with CTNNB1 mRNA and represses its degradation (28). Some lncRNAs, such as lncARSR, UFC1 and DUXAP10, associates with HuR and promotes HuR-induced stability of CTNNB1 mRNA (16–18). TSLNC8 also regulated CTNNB1 expression in this manner. Knockdown of HuR expression abolished the oncogenic effects of TSLNC8 in PC, indicating that TSLNC8 functions in a HuR-dependent manner. Additionally, we demonstrated that TSLNC8 activated WNT/ β -catenin signaling pathway, indicating that TSLNC8-HuR-WNT/ β -catenin axis may be crucial for PC progression.

Conclusion

In conclusion, our present study revealed that oncogenic lncRNA TSLNC8 positively regulate PC growth and metastasis via HuR-mediated mRNA stability of CTNNB1, extending the understanding of PC pathogenesis regulated by lncRNAs.

List Of Abbreviations

Pancreatic cancer (PC)

Long noncoding RNAs (lncRNAs)

epithelia-mesenchymal transition (EMT)

Y-box protein (YB1)

hepatocellular carcinoma (HCC)

Tumor suppressor long noncoding RNA on chromosome 8p12 (termed TSLNC8)

Cell Counting Kit-8 (CCK-8)

Quantitative real-time PCR (qRT-PCR)

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

enhanced chemiluminescence (ECL)

RNA immunoprecipitation (RIP)

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Cangzhou Central Hospital. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients provided written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during this research are available.

Competing interests

None.

Funding

None.

Authors' contributions

Wei Chai designed the study, wrote and revised the manuscript. Wei Chai, Ruhai Liu, Fengshan Li, Zhiquan Zhang and Bao Lei: performed all the experiments and analyzed the data. All of the authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

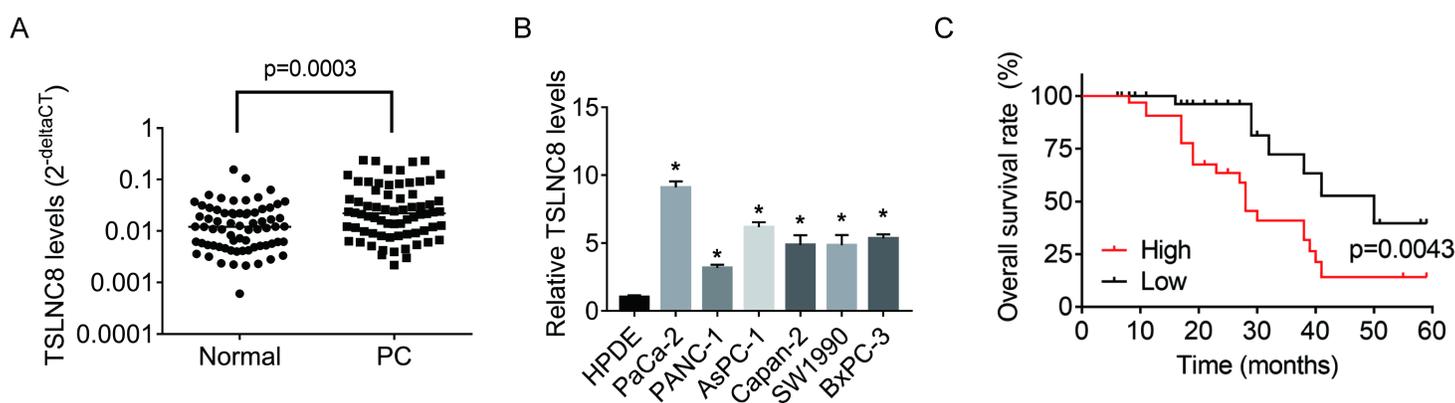


Figure 1

TSLNC8 expression is upregulated in PC tissues and indicates poor prognosis. A. The expression of TSLNC8 in the PC and corresponding adjacent normal pancreatic tissues from seventy patients with PC was examined by qRT-PCR. B. The expression of TSLNC8 in a normal human pancreatic cell line (HPDE)

and six different PC cell lines (AsPC-1, Capan-2, SW1990, PANC-1, PaCa-2, and BxPC-3) was detected by qRT-PCR. * $p < 0.05$. C. The associations of TSLNC8 expression with overall survival of PC patients were analyzed using Kaplan-Meier survival analysis.

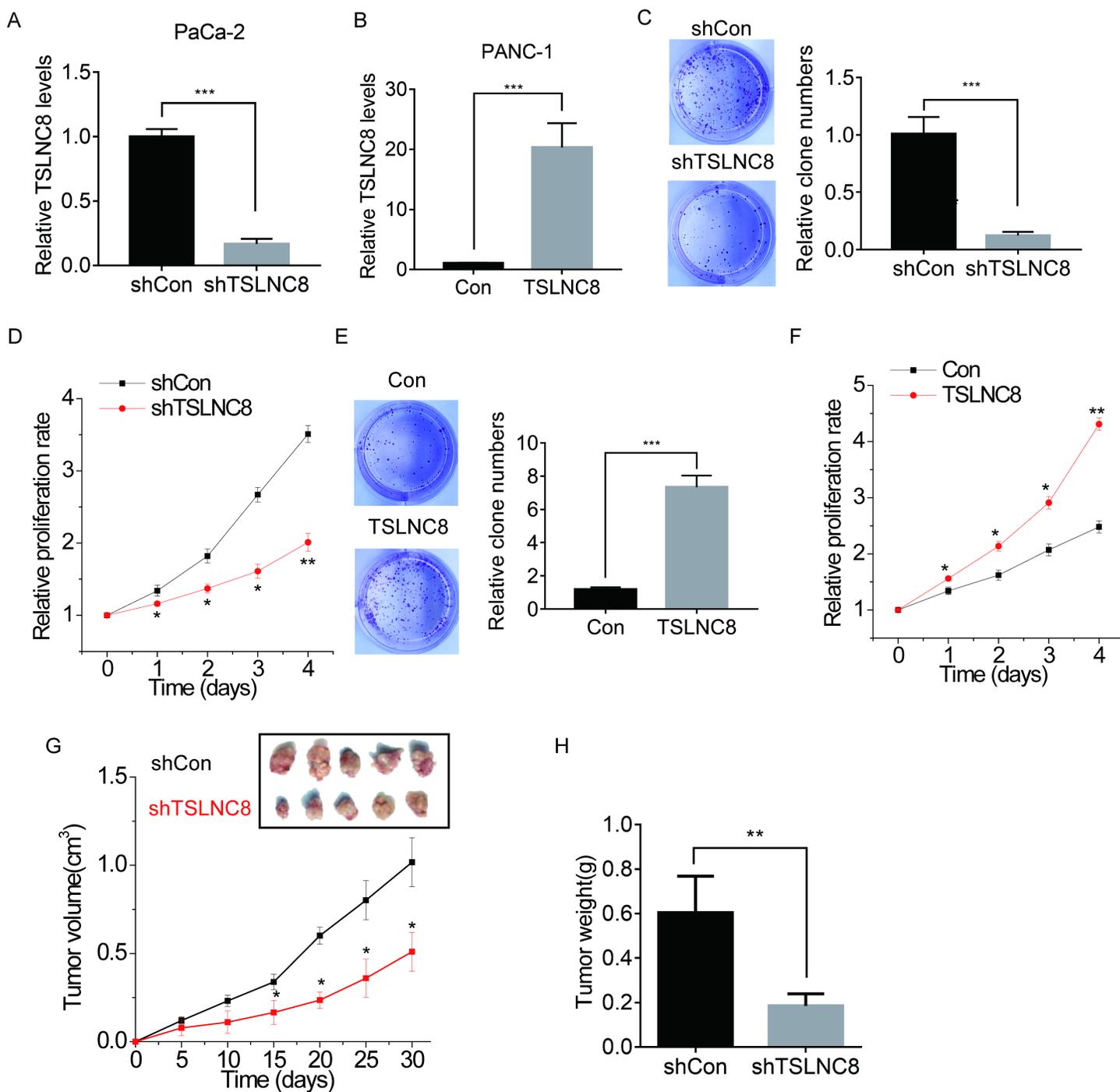


Figure 2

TSLNC8 promotes PC cell proliferation in vitro and in vivo. A. The TSLNC8 expression was knocked down by infection of lentiviral particles expressing TSLNC8 shRNA (shTSLNC8) in PaCa-2 cells. B. The TSLNC8 expression was overexpressed by infection of lentiviral particles expressing TSLNC8 in PANC-1 cells. C. The relative clone numbers of control and TSLNC8-knockdown PaCa-2 cells. D. The proliferation of

control and TSLNC8-knockdown PaCa-2 cells was detected by CCK-8 assay. E. The relative clone numbers of control and TSLNC8-overexpressed PANC-1 cells. F. The proliferation of control and TSLNC8-overexpressed PANC-1 cells was detected by CCK-8 assay. G. The growth curve of xenografts formed by control and TSLNC8-knockdown PaCa-2 cells. H. The weight of tumors formed by control and TSLNC8-knockdown PaCa-2 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

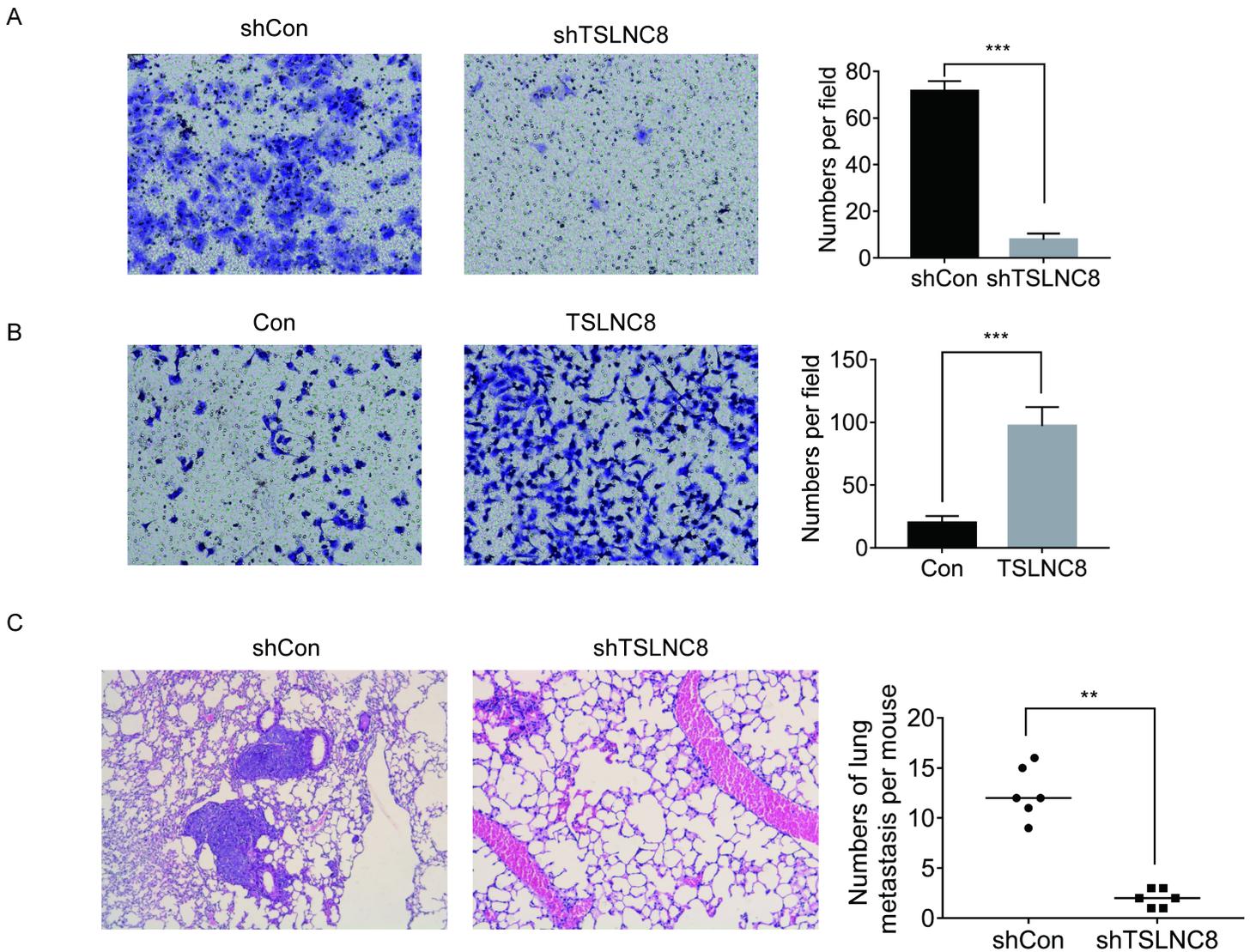


Figure 3

TSLNC8 enhances PC cell invasion in vitro and metastasis in vivo. A. The invasion ability of control and TSLNC8-knockdown PaCa-2 cells was detected by transwell assay. B. The invasion ability of control and TSLNC8-overexpressed PANC-1 cells was detected by transwell assay. C. The represent images of pulmonary metastatic nodules formed by control and TSLNC8-knockdown PaCa-2 cells. D. The numbers of pulmonary metastatic nodules formed by control and TSLNC8-knockdown PaCa-2 cells. ** $p < 0.01$, *** $p < 0.001$.

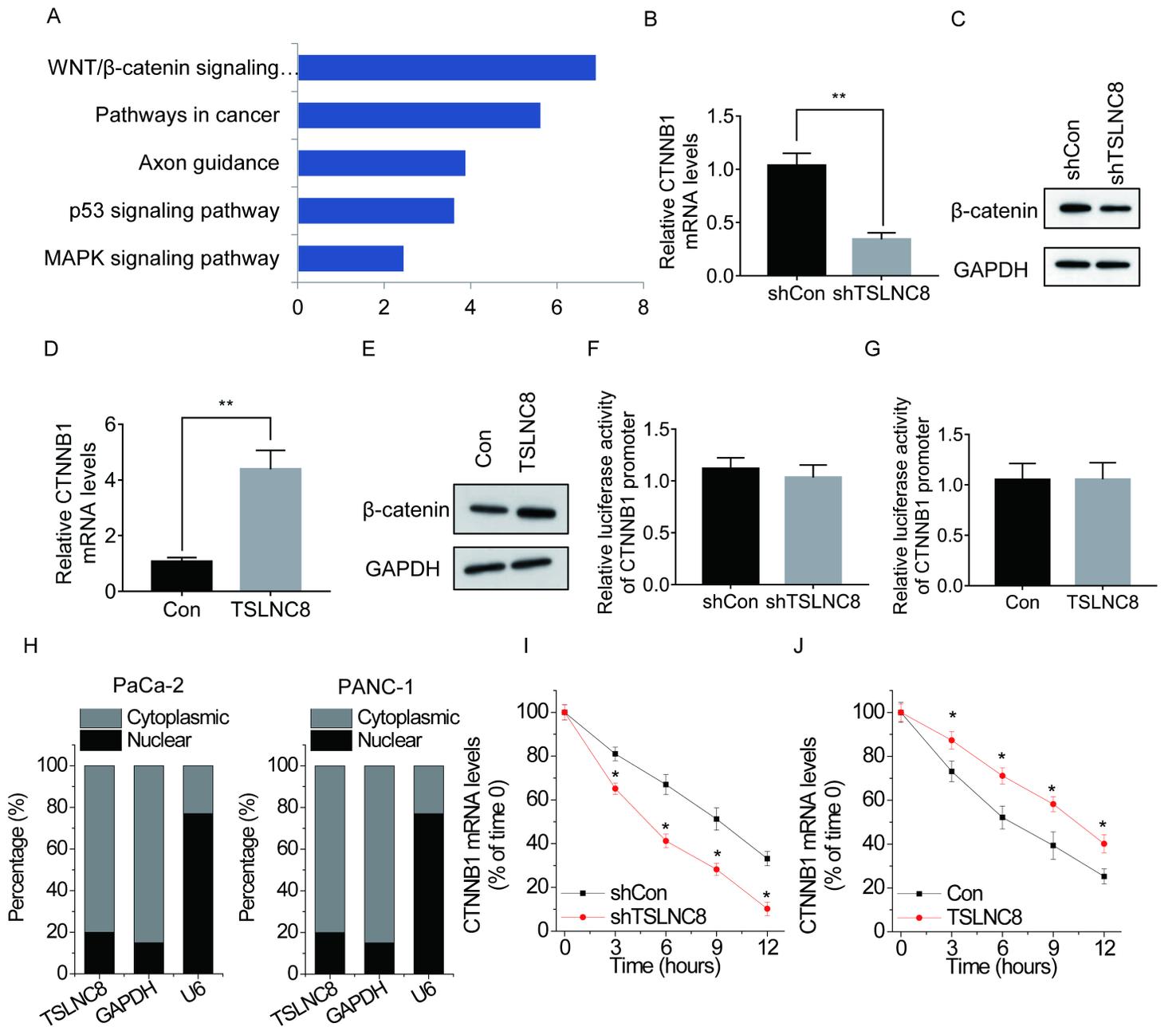


Figure 4

TSLNC8 increases CTNNB1 expression A. The top 5 pathways regulated by TSLNC8 knockdown in PaCa-2 cells by KEGG analysis. B. The CTNNB1 mRNA levels in control and TSLNC8-knockdown PaCa-2 cells was detected by qRT-PCR. C. The β-catenin levels in control and TSLNC8-knockdown PaCa-2 cells was detected by western blot. D. The CTNNB1 mRNA levels in control and TSLNC8-overexpressed PANC-1 cells was detected by qRT-PCR. E. The β-catenin levels in control and TSLNC8-overexpressed PANC-1 cells was detected by western blot. F. The luciferase activity of CTNNB1 promoter in control and TSLNC8-knockdown PaCa-2 cells. G. The luciferase activity of CTNNB1 promoter in control and TSLNC8-overexpressed PANC-1 cells. H. The cellular distribution of TSLNC8 in PC cells. GAPDH and U6 was used as cytoplasmic and nuclear internal reference, respectively. I. control and TSLNC8-knockdown PaCa-2 cells were treated with Actinomycin D, and the loss of CTNNB1 mRNA was detected by qRT-PCR. J.

Control and TSLNC8-overexpressed PANC-1 cells were treated with Actinomycin D, and the loss of CTNNB1 mRNA was detected by qRT-PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

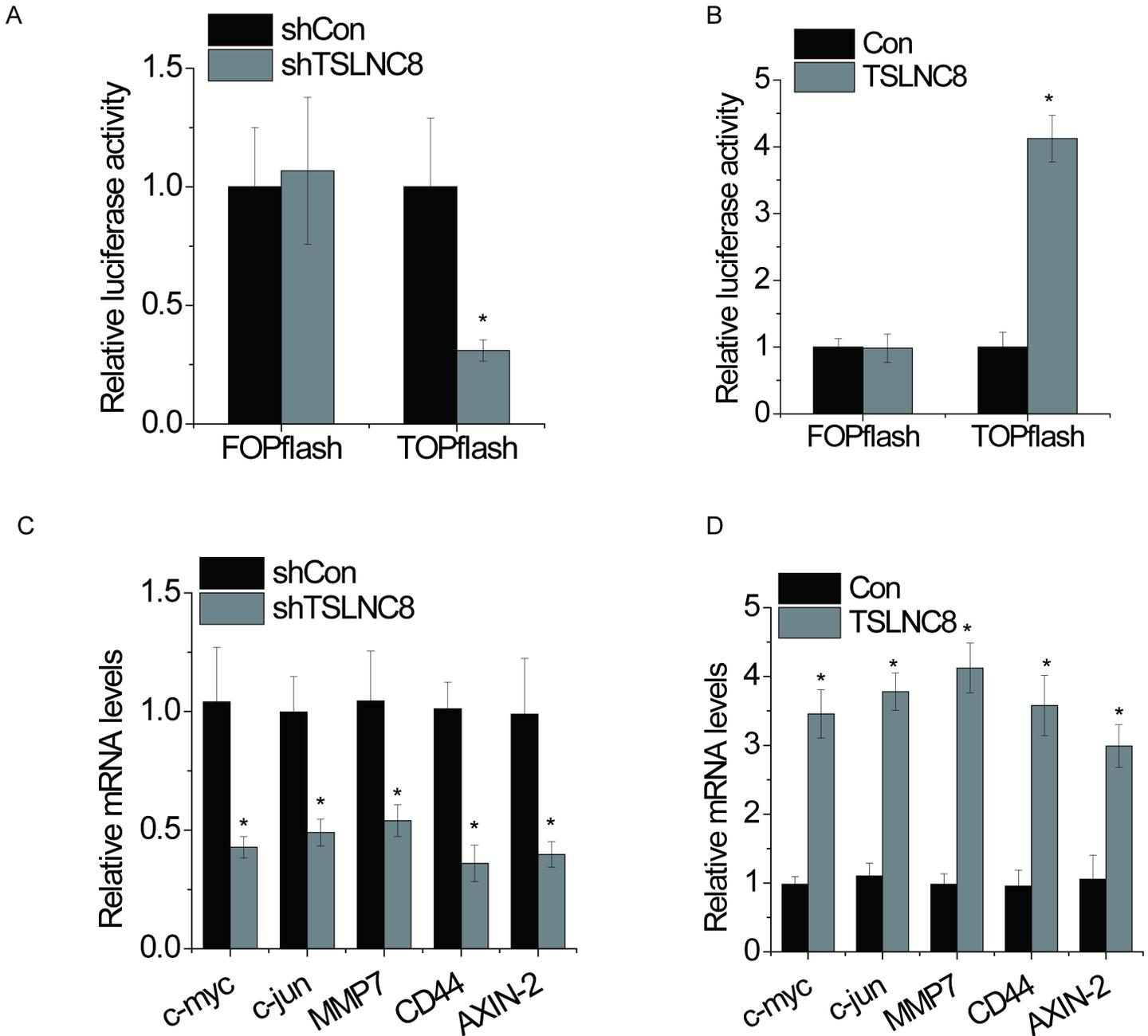


Figure 5

TSLNC8 activates WNT/ β -catenin signaling pathway A. The luciferase activity of WNT/ β -catenin signaling pathway was detected using TOPflash in control and TSLNC8-knockdown PaCa-2 cells. FOPflash was taken as a negative control. B. The luciferase activity of WNT/ β -catenin signaling pathway was detected using TOPflash in control and TSLNC8-overexpressed PANC-1 cells. FOPflash was taken as a negative control. C. The downstream genes of WNT/ β -catenin signaling pathway was tested by qRT-PCR in control and TSLNC8-knockdown PaCa-2 cells. D. The downstream genes of WNT/ β -catenin signaling pathway was tested by qRT-PCR in control and TSLNC8-overexpressed PANC-1 cells. * $p < 0.05$.

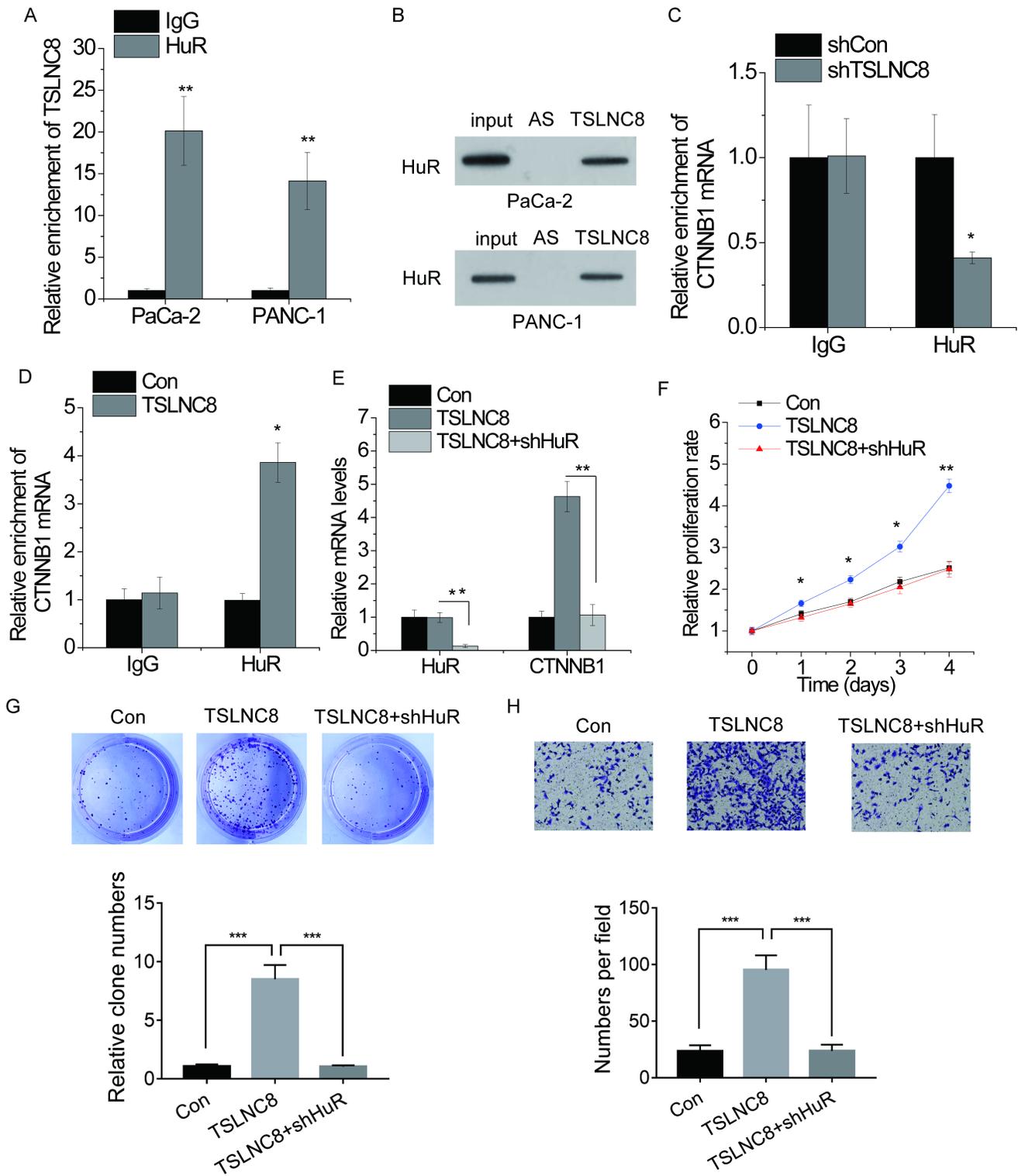


Figure 6

TSLNC8 associates with HuR to stabilize CTNNB1 mRNA A. The interaction between TSLNC8 and HuR was tested by RIP assay in PaCa-2 and PANC-1 cells. IgG was taken as negative control. B. The TSLNC8-pull-down or antisense-TSLNC8 (AS) protein samples were subjected to western blot with HuR antibody. Antisense-TSLNC8 (AS) was taken as a negative control. C. The interaction between CTNNB1 mRNA and HuR was tested by RIP assay in control and TSLNC8-knockdown PaCa-2 cells. D. The interaction between

CTNNB1 mRNA and HuR was tested by RIP assay in control and TSLNC8-overexpressed PANC-1 cells. E. The HuR shRNA was transfected into TSLNC8-overexpressed PANC-1 cells, and the CTNNB1 mRNA was determined by qRT-PCR. F. The HuR shRNA was transfected into TSLNC8-overexpressed PANC-1 cells, and the cell proliferation was determined by CCK-8 assay. G. The HuR shRNA was transfected into TSLNC8-overexpressed PANC-1 cells, and then colony formation assay was carried out. H. The HuR shRNA was transfected into TSLNC8-overexpressed PANC-1 cells, and then transwell assay was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.