Original Paper

A FUS-LATS1/2 Axis Inhibits Hepatocellular Carcinoma Progression via Activating Hippo Pathway

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Key Words

Fus LATS1/2 • Hippo • Hepatocellular carcinoma • Metastasis • Stemness

Abstract

Background/Aims: The roles and related mechanisms of RNA binding protein *FUS* (fused in sarcoma/translocated in liposarcoma) are unclear in numerous cancers, including hepatocellular carcinoma (HCC). **Methods:** Quantitative reverse transcription PCR (qRT-PCR), western blot, cell viability, transwell migration and invasion, tumor spheres formation and *in vivo* tumor formation assays were used to examine the effects of *FUS* on HCC progression in HuH7 and MHCC97 cells. Additionally, transcriptome analysis based on RNA-sequencing data, qRT-PCR, western blots, luciferase reporter and RNA binding protein immunoprecipitation (RIP) assays were used to explore the *LATS1/2* (large tumor suppressor kinases 1/2)-related mechanisms contributing to *FUS* functions. Finally, qRT-PCR and western blot analysis were used to detect the levels of *FUS* and *LATS1/2* in HCC and adjacent normal tissues, and the correlation between them in HCC tissues. **Results:** Overexpression of *FUS* decreased cell viability, migration, invasion and stemness. Moreover, *FUS* interacted and stabilized *LATS1/2* stability, and thus promoted *LATS1/2* expression and activated Hippo pathway. Finally, *FUS* and *LATS1/2* levels were positively correlated and significantly down-regulated in HCC tissues. **Conclusion:** We demonstrate that *FUS/LATS1/2* axis inhibits HCC progression via activating Hippo pathway.

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Introduction

The incidence rate of hepatocellular carcinoma (HCC) ranks in the sixth, and lethality ranks in the third place worldwide [1]. And there is no obvious symptoms in the early stages and HCC holds fast development and metastasis. Although the targeted therapy and chemotherapeutics significantly improve the overall survival probability, recurrence often happens [2]. Thus, it is an urgent need to find novel biomarkers for HCC diagnosis and treatment.

Hippo pathway is an important signaling that regulates organ growth and tissue size, which plays an important role in tumor progression [3]. Typical Hippo pathway consists of *Mst1/2* (mammalian sterile 20-like kinases 1/2), *LATS1/2* (large tumor suppressor kinases 1/2), *YAP* (Yes-associated protein)/*TAZ* (transcriptional coactivator with PDZ-binding motif), which forms a three step kinase cascade [4], in which *LATS1/2* could phosphorylate *YAP/TAZ*, promote their nucleus-cytoplasm translocation and inhibit their transcriptional activity. Therefore, activating Hippo pathway or inactivating *YAP/TAZ* has been regarded to be a potential way to repress cancer progression. For example, verteporfin, as an inhibitor of *YAP*, could attenuated the resistance of *BRAF* (B-Raf Proto-Oncogene, Serine/Threonine Kinase) inhibitor in melanoma stem cells [5], and verteporfin could induce cell apoptosis in melanoma stem cells [6]. However, efforts in this direction are frustrated by the fact that the Hippo cascade is largely undruggable [7]. While the core components of the Hippo signaling (*Mst1/2/LATS1/2/YAP/TAZ*) are well established, numerous additional upstream regulators of *YAP* activity are emerging. Such as, *H-ras* (HRas Proto-Oncogene, GTPase) could inhibit Hippo pathway by promoting *Mst1/Mst2* heterodimerization [8], and SnoN antagonizes the Hippo kinase complex to promote *TAZ* activity during breast cancer progression [9]. Therefore, it is necessary to find novel modulators of Hippo pathway, which could facilitate the development of targeting this signaling.

RNA binding proteins are a kind of protein that regulates transcripts stability and expression via directly binding to them [10]. A plenty of evidences have demonstrated the critical roles of RNA binding proteins in tumor progression. Such as, Cifdaloz et al. indicated that RNA binding protein *CELF1* (CUGBP Elav-Like Family Member 1) could control a melanoma-enriched pro-oncogenic networks by a systems analysis [11]. RNA binding protein *Lin28A* (Lin-28 Homolog A) promotes osteocarcinoma cells progression by associating with the long noncoding RNA *MALAT1* (Metastasis Associated Lung Adenocarcinoma Transcript 1 (Non-Protein Coding)) [12]. And RNA binding proteins could be potential targets in prostate cancer [13]. RNA binding protein *FUS* has been shown to interact with *HSP60* (Heat Shock Protein Family 60) to promote mitochondrial damage [14]. *FUS-CHOP* promotes invasion in myxoid liposarcoma through a *SRC* (SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase)/*FAK* (Focal Adhesion Kinase)/*RHO* (Rhodopsin)/*ROCK* (Rho Associated Coiled-Coil Containing Protein Kinase)-dependent pathway [15]. And *FUS* regulates AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor function and *FTLD/ALS*-associated behaviour via stabilizing *GluA1* mRNA [16]. In HCC, miR-378 could promote the migration of HCC cells by down-regulating *FUS* expression [17], which indicates the inhibitory roles of *FUS* in HCC progression. However, the expression level, roles and related mechanisms of *FUS* in HCC progression are still unclear.

Here, we aim to explore the roles and related mechanisms of *FUS* in HCC progression. Firstly, we found that *FUS* expression was significantly decreased in HCC tissues compared with normal tissues, and consistent results were acquired in HCC and normal hepatic cells. Further *in vitro* and *in vivo* experiments elucidated the inhibitory roles of *FUS* in HCC proliferation, migration and stemness. Mechanistically, *LATS1/2* was identified as the direct target of *FUS* in HCC cells and the downstream effectors *YAP/TAZ* transcriptional activity was also reduced via *FUS* overexpression, and knockdown of *LATS1/2* attenuated or even reversed the inhibition of *FUS* overexpression on HCC cells progression. Notably, *FUS* and *LATS1/2* expression was positively correlated in HCC tissues. Therefore, our results indicate that *FUS* inhibits HCC progression via binding to *LATS1/2* and thus activating Hippo pathway.

Materials and Methods

HCC clinical samples and cells culture

Forty-three pairs of HCC paraffin-embedded tissue samples were randomly selected from the Department of Intervention, Xuzhou Cancer Hospital between October 2015 and November 2017. Written informed consent from all patients and approval of the Hospital Ethic Review Committees was obtained. Isogenic HCC tumorigenic line SMMC7721, Bel-7402, MHCC97, HepG2, Hep3B, Huh-7, PLC/PRF/5, and normal hepatic cell line L02 were purchased from the Chinese Academy of Sciences Cell Bank. HCC cell lines were cultured in Dulbecco's Minimum Essential Medium (DMEM) medium (Gibco, USA), and L02 cells were cultured in 1640 medium (Gibco). Both of the media were supplemented with 10% FBS (fetal bovine serum) (Gibco), 80 U/ml penicillin and 0.08 mg/ml streptomycin, and cells were cultured at 37°C under humidified atmosphere with 5% CO₂.

Lentivirus plasmids and stable-infected cell lines construction

Lentiviral shRNAs targeting human *LATS1/2* were obtained from Santa Cruz (Cat # sc-35797 and Cat # sc-37444). A scramble non-targeting shRNA was used as control. *FUS* CDS sequences were inserted into pLVX-IRES-ZsGreen1, named as Lenti-FUS-CDS. Lentiviral particles were packaged referred to the previous study [18]. For infection, virus supernatants with 2 µg/ml Polybrene were added to Huh7 and HepG2 cells for 72 h. After two rounds infection, the infected cells were selected with puromycin (Sigma, 2 µg/ml) for 2 weeks, followed by qRT-PCR and western blot verification. Fluorescent cell sorting was used to select cells infected with Lenti-FUS-CDS, named as HepG2^{FUS-CDS} and Huh7^{FUS-CDS}.

Cell viability assay

Cell Counting Kit-8 (CCK8, Cat No: HY-K0301, MedchemExpression) was used to examine cell viability. Cells with different treatment were seeded in 96-well plates at 3000 cells/well. After 24, 48 h, 72 h, the growth rates were determined via measuring the absorbance at 450 nm using a microplate reader. Triplicate experiments were performed for each assay.

Cell migration and invasion assays

Transwell migration was carried out by using 24-well MILLIcell Hanging Cell Culture inserts 8 mm PET (MILLIPORE), invasion assay was analyzed by the same cell culture insert 8 mm PET pre-coated with Matrigel matrix gel (BD Biosciences). Briefly, a total of 6 – 8 × 10⁴ cells/200 µl in serum-free medium were added to the upper chamber. Eight hundreds microlitre (µl) medium containing 20 % FBS was used as a chemo-attractant in the bottom chamber. Cells were allowed to migrate for 24 h, and invade for 48 h. Cells migrating and invading into the underside were washed with PBS, fixed in methanol for 20 min, and stained with 0.1 % viola crystalline solution for 20 min. Five random fields from each of the triplicate were counted by using phase contrast microscopy. Quantification was performed by measuring with Microplate Reader (OD 570 nm) after being destained with glacial acetic acid.

Cell sphere formation assay

Cells with different treatment were cultured in ultra-low attachment 24-well plates (Corning, Union City, CA) at 500 cells/well with MammoCult™ Human Medium Kit (Cat#05620, Stemcell Technologies, Vancouver, BC, Canada). After 10 days culture, mammospheres number and size were detected and counted using a microscope fitted with a ruler.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using TRIeasy™ Total RNA Extraction Reagent TRIeasy™ (YEASEN, Shanghai, China) following the manufacturer's protocols. Then total RNA was reverse transcribed into cDNA using Hifair™ II 1st Strand cDNA Synthesis Kit (gDNA digester plus) (YEASEN) according to the standard protocols. Afterwards, mRNA expression was examined following the protocols of Hieff™ qPCR SYBR® Green Master Mix (High Rox Plus) (YEASEN) and performed on an ABI Prism 7500 Detection System (Applied Biosystems, Inc., USA). *GAPDH* was used as an internal reference. 2^{-ΔΔCt} method was used to calculate each transcript expression. qRT-PCR primers were mentioned in Table 1 [19].

RNA-sequencing and data analysis

RNA from Huh7 and Huh7^{FUS-CDS} cells was extracted using TRIeasy™ Total RNA Extraction Reagent TRIeasy™. RNA-seq libraries were acquired using TruSeq PE Cluster Kit V4 and sequenced by HiSeq 2000 sequencer. We used Cuff-diff to estimate fragments per kilobase of transcript per million (FPKM) values for known transcripts, and analyze differentially expressed transcripts. $P < 0.05$ was considered as significant. Heatmap of gene expression was generated based on log2 (FPKM) using Heml 1.0.3.7 (<http://hemi.biocuckoo.org/down.php>). KEGG rich analysis was carried out according to Rich factor, Qvalue and numbers of enriched genes. The richer factor is bigger, the degree of enrichment is bigger; The Qvalue is smaller, the degree of enrichment is more significant.

Western blotting

Detailed procedure was described elsewhere [20]. The antibodies against *FUS* (ab70381), *E-cadherin* (ab1416), *Vimentin* (ab92547), *YAP* (ab52771), *TAZ* (ab224239), *Caspase-3* (ab18347, Abcam), *cleaved-caspase3* (ab32042, Abcam), *PARP* (ab32138, Abcam), *Ki67* (ab15580), *cleaved-PARP* (ab32064, Abcam), *Oct4* (ab19857) and *Nanog* (ab21624) were purchased from Abcam, and *LATS1* (sc-12494) and *LATS2* (sc-23065) were purchased from Santa Cruz. *p-YAP* (cat # 13008) and *p-TAZ* (cat #59971) antibodies were purchased from Cell Signaling Technology. Primary antibody against β -actin (Cat # TA-09) and Secondary peroxidase-conjugated goat anti-rabbit (Cat # ZB-2306) were purchased from ZSGB-BIO, and Peroxidase-conjugated goat anti-mouse (Cat # HS201-01) was purchased from TRANSGEN BIOTECH (Beijing, China). Chemiluminescence was detected using an enhanced chemiluminescence kit (Tanon, China) followed by exposure with Tanon 5200.

RIP (RNA immunoprecipitation) assay

Cells were lysed with 25 mM Tris-HCl buffer (pH 7.5) and 100 U/ml RNase inhibitor (Sigma), and then incubated with protein-A Sepharose beads precoated with 3 μ g anti-FUS antibody or control rabbit IgG for 1.5 h at 4 °C. The RNA-protein complexes were pulled-down by protein A/G agarose beads, and RNA extraction and abundance in IP were performed and detected via qRT-PCR.

mRNA stability assay

HCC cells with *FUS* overexpression or not were cultured, and de novo RNA synthesis was blocked with 5 μ g/ml of ActD (actinomycin D) (Apexbio, USA) treatment. Then total RNA was harvested at indicated time points and mRNA expression was detected by qRT-PCR. The mRNA half-life was evaluated by comparing to the mRNA levels before adding ActD.

Immunofluorescent assay

The detailed procedure was referred to the previous study [21]. Briefly, cells were cultured on glass bottom plates, washed with PBS, and then fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized in 0.1% Triton X-100 for 15 min and blocked with 5% BSA in PBS for 1.5 h at room temperature. Then cells were incubated with anti-YAP antibody overnight at 4 °C, and washed with PBS and incubated with FITC-

Table 1. Primer sequences used for PCR

Gene	Sequences (5' to 3')
FUS of qRT-PCR forward	GGGAATGATAAGTGATAAGAGGGG
FUS of qRT-PCR reverse	CTAAATAACTGGTTGGCAGGTACG
LATS1 of qRT-PCR forward	GTGGAGTGTTGGTGTTATTCTTTT
LATS1 of qRT-PCR reverse	TATCTGTTGGGTGTGTGATTTTAG
LATS2 of qRT-PCR forward	ACTTTTCCTGCCACGACTTATTCT
LATS2 of qRT-PCR reverse	TGGTGGTAGGACGCAACGAATCG
Bcl2l1 qRT-PCR forward	GGCACTGTGCGTGGAAGCGTAGA
Bcl2l1 qRT-PCR reverse	GTGGAGCAGAGAAGGGGGTGGGAG
Bcl3 qRT-PCR forward	TCCACTCTCTACCAGATAACTGAG
Bcl3 qRT-PCR reverse	TATTACAAAAGAAATCCAACAAA
Birc3 qRT-PCR forward	AGGTGTGGTGGTATGTGCCTGTAG
Birc3 qRT-PCR reverse	TATGATGTGTTGACCCCGTGTCT
Bcl2a1 qRT-PCR forward	AGGTGTGGTGGTATGTGCCTGTAG
Bcl2a1 qRT-PCR reverse	TATGATGTGTTGACCCCGTGTCT
Ki67 of qRT-PCR forward	GAACACCTACAAAATGACTTCTCT
Ki67 of qRT-PCR reverse	GTTTCTTCACTCTTACTTTCCACA
E-cadherin of qRT-PCR forward	ATGGCTTCCCTCTTTCATCTCCTG
E-cadherin of qRT-PCR reverse	CATAGTTCCGCTCTGTCTTTGGCT
Vimentin of qRT-PCR forward	AGGAACCAATGAGTCCCTGGAACG
Vimentin of qRT-PCR reverse	CTGCAGAAAGGCACCTGAAAGCTG
Nanog of qRT-PCR forward	GGGGTATTCTTTTGTATGTGTCG
Nanog of qRT-PCR reverse	TGTCCTTGGTAGTGTGTACTGGG
Oct4 of qRT-PCR forward	TGGAGGAAGCTGACAACATGAAA
Oct4 of qRT-PCR reverse	GAGAGCCCAGAGTGGTGACGGAGA
GAPDH of qRT-PCR forward	AGGTCGGTGTGAACGGATTTGR
GAPDH of qRT-PCR reverse	GGGGTCGTTGATGGCAACA

conjugated secondary antibody (Cat # F-2765, ThermoFisher Scientific) for 1 h at room temperature, and washed with PBS for three times again and observed under the confocal microscopy.

Luciferase reporter assay

Luciferase reporter assay was performed to detect *YAP* transcriptional and *LATS1/2* promoter activity. *LATS1/2* promoter sequences were introduced into the pGL3 vector (Promega), named as pGL-LATS1 and pGL-LATS2, respectively. Briefly, 8xGT10C-luciferase plasmid (Plasmid # 34615), a *YAP*-responsive synthetic promoter driving luciferase expression plasmid, and pGL-LATS1/2 were co-transfected into HCC cells with FUS overexpression or not plus with β -gal (Ambion, USA) plasmid using Lipofectamine^{TM2000} according to the manufacturer's recommendation. After 72 h, the luciferase activity was examined. Transfection efficiency was determined using a Luciferase Reporter Assay Kit (cat. no. K801-200, BioVision, Inc., Milpitas, CA, USA). β -gal activity was determined using a β -Galactosidase Enzyme Assay System with Reporter Lysis Buffer (cat. no. E2000, Promega Corporation) for normalization following the manufacturer's protocols.

In vivo tumor initiation and doxorubicin sensitivity assays

All animal experiments were performed with the approval of Ethics Committee for Animal Experimentation of Xuzhou Cancer Hospital. Four – six week male athymic BALB/c nude mice were purchased from Model Animal Research Center of Nanjing University, were housed and fed in standard pathogen-free conditions. For tumor-limiting dilution assays, tumor cells were mixed 1:1 with Matrigel matrix (BD Biosciences) and orthotopically implanted in the inguinal mammary gland of mice. On day 8, all mice were killed and tumor tissues were collected, weighed and fixed in 10% formalin at room temperature and embedded in paraffin for immunohistochemistry assay.

Statistical analysis

All data were presented as the mean $\pm$ SD (standard deviation). Datasets with only two groups were analyzed using a student's *t*-test. The differences between the groups were analyzed using One-way ANOVA with the Tukey–Kramer post-test, and *P* < 0.05 was considered significant.

Results

FUS expression is significantly decreased in HCC cells and tissues, and negatively correlated with HCC progression

Firstly, we examined FUS expression in HCC and adjacent normal tissues via qRT-PCR and western blot assays. qRT-PCR and western blot results showed that FUS expression is significantly decreased in HCC tissues compared with that in normal adjacent tissues (Fig. 1A and 1B). Additionally, statistical analysis indicated that FUS expression was not correlated with sex, age, but negatively correlated with clinical stage, tumor size, lymph node metastasis (Table 2). FUS expression was further detected in HCC (SMMC7721, Bel-7402, MHCC97, HepG2, Hep3B, Huh-7, PLC/PRF/5) and normal hepatic (L02) cells. qRT-PCR and western blot results indicated that FUS expression was decreased in HCC cells compared with that in L02 cells (Fig. 1C and 1D). Especially in Huh7 and HepG2 cells which were used for the following experiments. These results suggest that FUS might inhibit HCC progression.

FUS overexpression inhibits HCC cells proliferation and promotes cells apoptosis

Given the comparatively low level of FUS in Huh7 and HepG2 cells, we constructed Huh7 and HepG2 cells with FUS stable overexpression by lentivirus infection. qRT-PCR and western blot confirmed the overexpression efficiency of FUS in Huh7 and HepG2 cells (Fig. 2A and 2B). CCK8 assay showed that FUS overexpression significantly suppressed cell proliferation in Huh7 and HepG2 cells (Fig. 2C and 2D). The expression of *Ki67*, a proliferation marker, was remarkably decreased in Huh7 and HepG2 cells with FUS overexpression (Fig. 2E and 2F). Additionally, the effect of FUS on cell apoptosis was determined. As shown in Fig. 2G, FUS overexpression enhanced cell apoptosis in Huh7 and HepG2 cells characterized as the increase of *cleaved caspase3* and *cleaved PARP* (Poly(ADP-Ribose) Polymerase) expression.

Fig. 1. FUS expression is negatively correlated with HCC progression. (a) FUS mRNA level was detected in HCC and normal adjacent tissues via qRT-PCR. (b) FUS protein level was measured in HCC and normal adjacent tissues via western blot. N represents normal, T represents tumor. (c and d) FUS mRNA and protein levels were examined in HCC and normal hepatic cells. Data were presented as the mean $\pm$ s.d.; * $P < 0.05$, ** $P < 0.01$ vs. Normal tissues or L02 cells.

Notably, the mRNA levels of anti-apoptotic genes (*bcl2l1*, *bcl3*, *birc3* and *bcl2a1*) were also measured via qRT-PCR. Consistent result was acquired (Fig. 2H).

FUS overexpression inhibits HCC cells migration, invasion and epithelial-mesenchymal transition (EMT) process

We continue investigating the effects of *FUS* on HCC cells migration and invasion. Transwell migration and invasion

analysis indicated that *FUS* overexpression significantly inhibited HCC cells migration and invasion (Fig. 3A and 3B). As EMT plays promotive roles in tumor metastasis, we further explored *FUS* roles in HCC EMT process. As expected, the expression of epithelial marker *E-cadherin* was increased in HCC cells with *FUS* overexpression, while the expression of mesenchymal marker *Vimentin* was decreased (Fig. 3C and 3D).

FUS overexpression attenuates the stemness of HCC cells

Since *FUS* expression was negatively correlated with tumor size and clinical degree, and EMT process is linked with the stemness of cancer cells [22], we speculated that *FUS* could also attenuate the stemness of HCC cells. Firstly, *FUS* expression was examined in HCC cells spheres and wild-type cells. As expected, *FUS* expression was remarkably decreased in HCC cells spheres compared with that in wild-type cells (Fig. 4A and 4B). And the expression of cancer stemness markers *OCT4* (POU Class 5 Homeobox 1) and *Nanog* (Nanog Homeobox) was indeed significantly increased in HCC cells spheres (Fig. 4B). The expression of *OCT4* and *Nanog* was further examined in HCC cells with *FUS* overexpression or not.

Table 2. Correlation between FUS expression and clinical pathological characteristics of 43 HCC patients

Parameter	n	Low FUS	High FUS	P Value
Age				0.638
>50	20	18	2	
≤50	23	19	4	
Sex				
Male	23	16	7	
Femal	20	17	3	
T grade of tumor				0.012
T1	16	10 (62.5%)	6 (37.5%)	
T2	8	6 (75%)	2 (25%)	
T3	9	8 (98.9%)	1 (11.1%)	
T4	10	9 (90%)	1 (10%)	
Tumor size				0.006
<6 cm	19	16(68.4%)	3(31.6%)	
>6 cm	24	22(91.7%)	2(8.3%)	
Lymph node metastasis				0.025
Negative	17	11(64.7%)	6(35.3%)	
Positive	26	21(80.8%)	5(19.2%)

Fig. 2. Overexpression of FUS inhibits HCC cells proliferation and promotes cells apoptosis. (a and b) The infection efficiency of Lenti-FUS-CDS was evaluated in HepG2 and Huh7 cells via qRT-PCR (a) and western blot (b) analyses. (c and d) Cell viability of HepG2 and Huh7 cells with FUS overexpression or not was examined at 24 h, 48 h and 72 h by CCK8 assay. (e and f) The expression of proliferation marker Ki67 was detected in HepG2 and Huh7 cells with FUS overexpression or not. (g) The apoptotic executors (Cleaved caspase3 and Cleaved PARP) expression was measured in HepG2 and Huh7 cells with FUS overexpression or not. (h) The anti-apoptotic markers (Bcl2l1, Bcl3, Birc3 and Bcl2a1) expression was examined in HepG2 and Huh7 cells with FUS overexpression or not. Data were presented as the mean $\pm$ s.d; * $P < 0.05$, ** $P < 0.01$ vs. HepG2 or Huh7.

As expected, *OCT4* and *Nanog* expression was decreased in *FUS*-overexpressed cells (Fig. 4C and 4D). Additionally, the ability of cell sphere formation was attenuated in HCC cells with *FUS* overexpression characterized as decreased size and numbers of cell spheres (Fig. 4E). Therefore, our results indicate that *FUS* overexpression could attenuate HCC cells stemness.

FUS directly binds to LATS1/2 and enhance LATS1/2 mRNA stability

To explore the related mechanisms contributing to *FUS* in prohibiting HCC progression, we tried to characterize pathways regulated by *FUS*. Firstly, we analyzed transcriptome of Huh7 cells with *FUS* stable overexpression or not based on RNA-sequencing data. Hippo signaling was shown on the top of the most upregulated pathways by *FUS* overexpression (Fig. 5A), and *LATS1/2* expression was markedly increased in *FUS*-overexpressed cells (Fig. 5B). As *FUS* belongs to an RNA binding protein, bioinformatics analysis (<http://starbase.sysu.edu.cn/index.php>) predicted that *LATS1/2* was the direct targets of *FUS*. *LATS1/2* expression was measured in HCC (SMMC7721, Bel-7402, MHCC97, HepG2, Hep3B, Huh-7, PLC/PRF/5) and normal hepatic (L02) cells, similar results with *FUS* expression were obtained (Fig. 5C and 5D). Furthermore, overexpression of *FUS* enhanced *LATS1/2* expression (Fig. 5E and 5F). When *FUS* was overexpressed and then de novo synthesis was blocked with ActD, the decay

Fig. 3. Overexpression of FUS suppresses HCC cells migration, invasion and EMT process. (a and b) Effects of FUS overexpression on HCC cells migration and invasion were evaluated via transwell migration and invasion assays. (c and d) Expression of EMT markers (E-cadherin and Vimentin) was examined in HepG2 and Huh7 cells with FUS overexpression or not. Data were presented as the mean $\pm$ s.d.; * $P < 0.05$, ** $P < 0.01$ vs. HepG2 or Huh7.

rate of *LATS1/2* was slower in the *FUS*-overexpressed cells ($t_{1/2} = 5.3 \pm 0.2$ h and $t_{1/2} = 5.7 \pm 0.3$ h) than in control cells (Fig. 5G - J). Notably, the luciferase reporter assay showed that *LATS1/2* promoter transcriptional activity was not affected by *FUS* overexpression (Fig. 5K), demonstrating that *FUS* could not alter the transcriptional activity of *LATS1/2*. In addition, the *FUS*-binding complex was pulled down with *FUS* antibody, followed by examination of the bound mRNAs by qRT-PCR. As shown in Fig. 5L, RIP assays indicated that *FUS* associated with *LATS1/2* more specifically than in the control. Therefore, these results indicated that *FUS* interacts with and stabilizes *LATS1/2*.

FUS overexpression activates Hippo pathway in a *LATS1/2*-dependent manner

We further investigated whether *FUS* overexpression indeed activated Hippo pathway through *LATS1/2* expression. As expected, the expression level of *p-YAP* and *p-TAZ* was significantly increased in HCC cells with *FUS* overexpression, which was attenuated by *LATS1/2* knockdown (Fig. 6A). Additionally, transcriptome analysis based on RNA-sequencing showed that overexpression of *FUS* decreased the expression of the most targets of *YAP* (Fig. 6B). Furthermore, the promotion of *FUS* overexpression on *YAP* nucleus-cytoplasm translocation was repressed by *LATS1/2* knockdown (Fig. 6C and 6D). And luciferase reporter assay showed that *FUS* overexpression decreased *YAP* transcriptional activity characterized as the decrease of 8xGTIIC-luciferase activity, a *YAP*-responsive synthetic promoter driving luciferase expression plasmid (Fig. 6E), this effect was also attenuated by *LATS1/2* knockdown. Notably, *FUS* and *LATS1/2* expression was positively correlated in HCC tissues (Fig. 6F and 6G).

Fig. 4. Overexpression of FUS attenuates the stemness of HCC cells. (a) FUS mRNA level was measured in HepG2 and Huh7 cells mammosphere cultures and monolayer cultures. (b) Expression of tumor stemness markers (Oct4 and Nanog) was detected in HepG2 and Huh7 cells mammosphere cultures and monolayer cultures via western blot analysis. (c and d) Expression of tumor stemness markers (Oct4 and Nanog) was detected in HepG2 and Huh7 cells with FUS overexpression or not via qRT-PCR (c) and western blot (d) assays. (e) The ability of cell sphere formation was examined in HepG2 and Huh7 cells with FUS overexpression or not. Data were presented as the mean $\pm$ s.d.; * P <0.05, ** P <0.01 vs. HepG2 or Huh7.

The inhibitory effects of FUS overexpression are partly dependent on LATS1/2 expression

We tried to explore whether *FUS* exerts its inhibition on HCC cells progression dependent on *LATS1/2* expression. As expected, the decrease of HCC cells viability, migration and invasion induced by *FUS* overexpression was attenuated by *LATS1/2* knockdown (Fig. 7A – 7C), and consistent results were acquired in EMT process (Fig. 7D). Furthermore, *FUS* overexpression-mediated attenuation on HCC cells stemness was rescued by *LATS1/2* knockdown (Fig. 7E and 7F). Thus, our results suggest that *LATS1/2* is necessary for *FUS* function on HCC cells progression.

FUS overexpression inhibits HCC cells progression in vivo

Finally, we evaluated whether *FUS* regulates HCC cells progression *in vivo*. We compared the capacity of Huh7 cells with *FUS* overexpression or not to seed tumors at limiting dilutions. Although all cell lines could form tumors at the density of 1×10^5 cells, *FUS* overexpressed cells showed a decrease of tumor size and weight (Fig. 8A and 8B). Notably, the tumor-seeding ability of *FUS*-overexpressed cells was significantly decreased at the density of 1×10^4 (5/6 vs 3/6) and 1×10^3 (4/6 vs 2/6) cells. Concordantly, the staining intensity and number of *Ki67*, which is required for maintenance of proliferation and stemness [23], were decreased in tumors derived from *FUS*-overexpressed cells (Fig. 8C and 4D). Taken together, these results indicate that *FUS* overexpression inhibits HCC cells progression *in vivo*.

Fig. 5. FUS directly binds to LATS1/2 and enhances LATS1/2 expression. (a and b) Enrichment of signaling signatures differentially expressed (a) and identification of LATS1/2 expression (b) between Huh7 and Huh7^{FUS-CDS} cells based on RNA-sequencing analysis. (c and d) LATS1/2 mRNA and protein levels were examined in HCC and normal hepatic cells. Data were presented as the mean $\pm$ s.d.; * P <0.05, ** P <0.01 vs. L02. (e and f) LATS1/2 mRNA and protein levels were detected in HepG2 and Huh7 cells with FUS overexpression or not. (g-j) HepG2 and Huh7 cells with FUS overexpression or not were treated with actinomycin D (2.5 μ g/ml) for the indicated times. LATS1/2 mRNA level was measured by qRT-PCR and the percentage of mRNA that remained was plotted. (k) Cells depicted in (g-j) were transiently transfected with pGL-LATS1 and pGL-LATS2 for 72 h. Luciferase activity in these cells was measured. (l) qRT-PCR was used to measure the abundance of LATS1/2 mRNA that was present in the FUS-IP materials after the RIP assay. Data were presented as the mean $\pm$ s.d.; * P <0.05, ** P <0.01 vs. HepG2 or Huh7.

Fig. 6. Overexpression of FUS activates Hippo pathway in LATS1/2-dependent manner. (a) The phosphorylation and total levels of YAP/TAZ were measured in HepG2, Huh7, HepG2^{FUS-CDS} and Huh7^{FUS-CDS} cells with LATS1/2 knockdown or not. (b) Expression of YAP targeting genes in Huh7 and Huh7^{FUS-CDS} cells based on RNA-sequencing analysis. (c and d) Confocal images showing localization of YAP in HepG2, HepG2^{FUS-CDS} with LATS1/2 knockdown or not, which is quantified in Fig. 6d. (e) The luciferase activity of 8xGTIC-luciferase plasmid was detected in cells depicted in (a). (f and g) Correlation between the levels of FUS and LATS1/2 in HCC tissues. Data were presented as the mean $\pm$ s.d.; * $P < 0.05$, ** $P < 0.01$ vs. HepG2 or Huh7.

Fig. 7. The inhibitory effects of FUS overexpression are partly through LATS1/2. (a) Cells viability was measured in HepG2, Huh7, HepG2^{FUS-CDS} and Huh7^{FUS-CDS} cells with LATS1/2 knockdown or not by CCK8 assay at 48 h after culture. Data were presented as the mean $\pm$ s.d; *P<0.05, **P<0.01 vs. HepG2 or Huh7. (b and c) Cell migration ability was detected in HepG2^{FUS-CDS} and Huh7^{FUS-CDS} cells with LATS1/2 knockdown or not by transwell migration analysis, which was quantified in Fig. 7c. (d) Expression of EMT markers (E-cadherin and Vimentin) was examined in cells depicted in (b). (e) Tumor stemness markers (Oct4 and Nanog) expression was detected in cells depicted in (b). (f) The ability of cell sphere formation was evaluated in cells depicted in (b). Data were presented as the mean $\pm$ s.d; *P<0.05, **P<0.01 vs. HepG2^{FUS-CDS} or Huh7^{FUS-CDS}.

Discussion

Using a combination of clinical samples, biochemical, functional and bioinformatics approaches, we identified RNA binding protein *FUS* as a suppressor of HCC progression *in vitro* and *in vivo*. We also elucidated a mechanism through which *FUS* mediated, in part, its suppressive effects. We found that, in HCC cells, *FUS* directly bound to *LATS1/2* and enhanced their mRNA stability, which inhibited the downstream effectors *YAP/TAZ* transcriptional activity. Notably, knockdown of *LATS1/2* attenuated the suppression of *FUS* overexpression on HCC progression, and *LATS1/2* expression was positively correlated with *FUS* expression in HCC tissues. To the best of knowledge, this is the first study showing the roles of *FUS* in HCC progression.

RNA binding proteins are abundant with > 800 RNA proteins reported in human cells [24]. Given their ability to co-ordinately regulate sets of transcripts, RNA binding proteins have the potential to confer the progression of cancer cells, and their functions always depend on their targets mRNA [25]. Indeed, various studies have noted such effects on HCC progression of RNA binding proteins, such as knockdown of anillin actin binding protein

Fig. 8. Overexpression of FUS represses HCC cells progression in vivo. (A and B) Images (A) and weight (B) of tumors harvested when serially diluted Huh7 cells with FUS overexpression or not were planted. (C) Represented images of Ki67 staining of tumors harvested when 1,000 cells were injected in (A). (D) Quantification of Ki67 positive cells in (C). Data were presented as the mean $\pm$ s.d.; *P<0.05, **P<0.01 vs. Huh7 cells.

blocks cytokinesis in hepatocytes and reduces liver tumor development in mice [26]. *ANGPTL1* (Angiopoietin-related protein 1) interacts with *Integrin $\alpha 1 \beta 1$* to suppress HCC angiogenesis and metastasis by inhibiting *JAK2* (*Janus Kinase 2*)/*STAT3* (*Signal Transducer And Activator Of Transcription 3*) signaling [27]. The effects of RNA binding proteins were shown to arise through diverse mechanisms, including direct modulation of transcripts stability as well as regulation of alternative splicing patterns [28, 29]. In the present study, knockdown of *LATS1/2* could attenuate but not reverse the suppression of *FUS* overexpression, this might be due to the fact that one RNA binding protein might bind to various transcripts, and one transcript could be bound by various RNA binding protein. Thus, there might other targets bound by *FUS* in HCC cells, and *FUS* binding would not be expected to impart the same effect on entire set of RNAs with which *FUS* interacts, this effect should be further explored.

Previous study has shown that *FUS*-binding sites are rich in GU contents and are highly degenerative, and *FUS*-binding motifs of GGU, GGUG, GUGGU and CGCGC have been previously reported [30], however, the detailed binding sites of *FUS* on *LATS1/2* are still unclear, which would be solved in our future work. It is interesting to note that *miR-378* promotes HCC cells migration by down-regulating *FUS* expression [17], which suggests the inhibitory roles of *FUS* in HCC migration and this effect is consistent with our results. And since the stemness of cancer cells could confer chemoresistance [31], and *FUS* expression has been shown to be correlated with cisplatin chemosensitivity in neuroblastoma cell lines [32], future studies could focus on the effects of *FUS* on chemotherapeutics sensitivity in HCC cells. In light of our findings concerning a suppressive role for *FUS* in HCC progression, future studies into therapeutics methods promoting *FUS* expression are warranted.

Disclosure Statement

The authors declare no conflict of interests.

References

- 1 Pu W, Li J, Zheng Y, Shen X, Fan X, Zhou JK, He J, Deng Y, Liu X, Wang C, Yang S, Chen Q, Liu L, Zhang G, Wei YQ, Peng Y: Targeting Pin1 by inhibitor API-1 regulates microRNA biogenesis and suppresses hepatocellular carcinoma development. *Hepatology* 2018;68:547-560.
- 2 Nishioka ST, Sato MM, Wong LL, Tiirikainen M, Kwee SA: Clinical and molecular sub-classification of hepatocellular carcinoma relative to alpha-fetoprotein level in an Asia-Pacific island cohort. *Hepatoma Res* 2018;4:pii: 1 doi: 10.20517/2394-5079.2017.46.
- 3 Zhou Z, Hu T, Xu Z, Lin Z, Zhang Z, Feng T, Zhu L, Rong Y, Shen H, Luk JM, Zhang X, Qin N: Targeting Hippo pathway by specific interruption of YAP-TEAD interaction using cyclic YAP-like peptides. *FASEB J* 2015;29:724-732.
- 4 Cheng Y, Feng Y, Jansson L, Sato Y, Deguchi M, Kawamura K, Hsueh AJ: Actin polymerization-enhancing drugs promote ovarian follicle growth mediated by the Hippo signaling effector YAP. *FASEB J* 2015;29:2423-2430.
- 5 Fisher ML, Grun D, Adhikary G, Xu W, Eckert RL: Inhibition of YAP function overcomes BRAF inhibitor resistance in melanoma cancer stem cells. *Oncotarget* 2017;8:110257-110272.
- 6 Ma YW, Liu YZ, Pan JX: Verteporfin induces apoptosis and eliminates cancer stem-like cells in uveal melanoma in the absence of light activation. *Am J Cancer Res* 2016;6:2816-2830.
- 7 Sorrentino G, Ruggeri N, Specchia V, Cordenonsi M, Mano M, Dupont S, Manfrin A, Ingallina E, Sommaggio R, Piazza S, Rosato A, Piccolo S, Del Sal G: Metabolic control of YAP and TAZ by the mevalonate pathway. *Nature Cell Biol* 2014;16:357-366.
- 8 Rawat SJ, Araiza-Olivera D, Arias-Romero LE, Villamar-Cruz O, Prudnikova TY, Roder H, Chernoff J: H-ras Inhibits the Hippo Pathway by Promoting Mst1/Mst2 Heterodimerization. *Curr Biol* 2016;26:1556-1563.
- 9 Zhu Q, Le Scolan E, Jahchan N, Ji X, Xu A, Luo K: SnoN Antagonizes the Hippo Kinase Complex to Promote TAZ Signaling during Breast Carcinogenesis. *Dev Cell* 2016;37:399-412.
- 10 Brennan CM, Steitz JA: HuR and mRNA stability. *Cell Mol Life Sci* 2001;58:266-277.
- 11 Cifdaloz M, Osterloh L, Grana O, Riveiro-Falkenbach E, Ximenez-Embun P, Munoz J, Tejedo C, Calvo TG, Karras P, Olmeda D, Miñana B, Gómez-López G, Cañon E, Eyraes E, Guo H, Kappes F, Ortiz-Romero PL, Rodríguez-Peralto JL, Megías D et al.: Systems analysis identifies melanoma-enriched pro-oncogenic networks controlled by the RNA binding protein CELF1. *Nat Commun* 2017;8:2249.
- 12 Wang Z, Pang J, Ji B, Zhang S, Cheng Y, Yu L, Pan W: RNA binding protein Lin28A promotes osteocarcinoma cells progression by associating with the long noncoding RNA MALAT1. *Biotechnol Lett* 2018;40:493-500.
- 13 Fei T: Targeting RNA binding protein in prostate cancer. *Mol Cell Oncol* 2017;4:e1353855.
- 14 Deng J, Yang M, Chen Y, Chen X, Liu J, Sun S, Cheng H, Li Y, Bigio EH, Mesulam M, Xu Q, Du S, Fushimi K, Zhu L, Wu JY: FUS Interacts with HSP60 to Promote Mitochondrial Damage. *PLoS Genet* 2015;11:e1005357.
- 15 Tornin J, Hermida-Prado F, Padda RS, Gonzalez MV, Alvarez-Fernandez C, Rey V, Martinez-Cruzado L, Estupinan O, Menendez ST, Fernandez-Nevado L, Astudillo A, Rodrigo JP, Lucien F, Kim Y, Leong HS, Garcia-Pedrero JM, Rodriguez R: FUS-CHOP Promotes Invasion in Myxoid Liposarcoma through a SRC/FAK/RHO/ROCK-Dependent Pathway. *Neoplasia* 2018;20:44-56.
- 16 Udagawa T, Fujioka Y, Tanaka M, Honda D, Yokoi S, Riku Y, Ibi D, Nagai T, Yamada K, Watanabe H, Katsuno M, Inada T, Ohno K, Sokabe M, Okado H, Ishigaki S, Sobue G: FUS regulates AMPA receptor function and FTL/ALS-associated behaviour via GluA1 mRNA stabilization. *Nat Commun* 2015;6:7098.
- 17 Ma J, Lin J, Qian J, Qian W, Yin J, Yang B, Tang Q, Chen X, Wen X, Guo H, Deng Z: MiR-378 promotes the migration of liver cancer cells by down-regulating Fus expression. *Cell Physiol Biochem* 2014;34:2266-2274.
- 18 Xu CZ, Jiang C, Wu Q, Liu L, Yan X, Shi R: A Feed-Forward Regulatory Loop between HuR and the Long Noncoding RNA HOTAIR Promotes Head and Neck Squamous Cell Carcinoma Progression and Metastasis. *Cell Physiol Biochem* 2016;40:1039-1051.

- 19 Zheng L, Xiang C, Li X, Guo Q, Gao L, Ni H, Xia Y, Xi T: STARD13-correlated ceRNA network-directed inhibition on YAP/TAZ activity suppresses stemness of breast cancer via co-regulating Hippo and Rho-GTPase/F-actin signaling. *J Hematol Oncol* 2018;11:72.
- 20 Zheng L, Meng X, Li X, Zhang Y, Li C, Xiang C, Xing Y, Xia Y, Xi T: miR-125a-3p inhibits ERalpha transactivation and overrides tamoxifen resistance by targeting CDK3 in estrogen receptor-positive breast cancer. *FASEB J* 2017;32:588-600.
- 21 Hu J, Li X, Guo X, Guo Q, Xiang C, Zhang Z, Xing Y, Xi T, Zheng L: The CCR2 3'UTR functions as a competing endogenous RNA to inhibit breast cancer metastasis. *J Cell Sci* 2017;130:3399-3413.
- 22 Mukherjee S, Manna A, Bhattacharjee P, Mazumdar M, Saha S, Chakraborty S, Guha D, Adhikary A, Jana D, Gorain M et al: Non-migratory tumorigenic intrinsic cancer stem cells ensure breast cancer metastasis by generation of CXCR4 migrating cancer stem cells. *Oncogene* 2016;35:4937-4948.
- 23 Cidado J, Wong HY, Rosen DM, Cimino-Mathews A, Garay JP, Fessler AG, Rasheed ZA, Hicks J, Cochran RL, Croessmann S, Zabransky DJ, Mohseni M, Beaver JA, Chu D, Cravero K, Christenson ES, Medford A, Mattox A, De Marzo AM, Argani P et al: Ki-67 is required for maintenance of cancer stem cells but not cell proliferation. *Oncotarget* 2016;7:6281-6293.
- 24 Castello A, Fischer B, Eichelbaum K, Horos R, Beckmann BM, Strein C, Davey NE, Humphreys DT, Preiss T, Steinmetz LM, Krijgsvelde J, Hentze MW: Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* 2012;149:1393-1406.
- 25 Gerstberger S, Hafner M, Tuschl T: A census of human RNA-binding proteins. *Nat Rev Genet* 2014;15:829-845.
- 26 Zhang S, Nguyen LH, Zhou K, Tu HC, Sehgal A, Nassour I, Li L, Gopal P, Goodman J, Singal AG, Yopp A, Zhang Y, Siegwart DJ, Zhu H: Knockdown of Anillin Actin Binding Protein Blocks Cytokinesis in Hepatocytes and Reduces Liver Tumor Development in Mice without Affecting Regeneration. *Gastroenterology* 2018;154:1421-1434.
- 27 Yan Q, Jiang L, Liu M, Yu D, Zhang Y, Li Y, Fang S, Zhu YH, Yuan YF, Guan XY: ANGPTL1 Interacts with Integrin alpha1beta1 to Suppress HCC Angiogenesis and Metastasis by Inhibiting JAK2/STAT3 Signaling. *Cancer Res* 2017;77:5831-5845.
- 28 Huang YH, Peng W, Furuuchi N, Gerhart J, Rhodes K, Mukherjee N, Jimbo M, Gonye GE, Brody JR, Getts RC, Sawicki JA: Delivery of Therapeutics Targeting the mRNA-Binding Protein HuR Using 3DNA Nanocarriers Suppresses Ovarian Tumor Growth. *Cancer Res* 2016;76:1549-1559.
- 29 Yu TX, Gu BL, Yan JK, Zhu J, Yan WH, Chen J, Qian LX, Cai W: CUGBP1 and HuR regulate E-cadherin translation by altering recruitment of E-cadherin mRNA to processing bodies and modulate epithelial barrier function. *Am J Physiol Cell Physiol* 2016;310:C54-65.
- 30 Takeda JI, Masuda A, Ohno K: Six GU-rich (6GUR) FUS-binding motifs detected by normalization of CLIP-seq by Nascent-seq. *Gene* 2017;618:57-64.
- 31 Jia Y, Peng L, Rao Q, Xing H, Huai L, Yu P, Chen Y, Wang C, Wang M, Mi Y, Wang J: Oncogene iASPP enhances self-renewal of hematopoietic stem cells and facilitates their resistance to chemotherapy and irradiation. *FASEB J* 2014;28:2816-2827.
- 32 Wang Z, Lei H, Sun Q: MicroRNA-141 and its associated gene FUS modulate proliferation, migration and cisplatin chemosensitivity in neuroblastoma cell lines. *Oncol Rep* 2016;35:2943-2951.