Original Paper

lncRNA TUG1-Mediated Mir-142-3p Downregulation Contributes to Metastasis and the Epithelial-to-Mesenchymal Transition of Hepatocellular Carcinoma by Targeting ZEB1

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Key Words

lncrna TUG1 • Hepatocellular carcinoma • MiR-142-3p • ZEB1 • EMT

Abstract

Background/Aims: MicroRNA-142-3p (miR-142-3p) is dysregulated in many malignancies and may function as a tumor suppressor or oncogene in tumorigenesis and tumor development. However, few studies have investigated the clinical significance and biological function of miR-142-3p in hepatocellular carcinoma (HCC). **Methods:** The expression levels of taurine upregulated gene 1 (TUG1), miR-142-3p, and zinc finger E-box-binding homeobox 1 (ZEB1) were evaluated in HCC tissues and cell lines by quantitative real-time PCR. MTT and colony formation assays were used to detect cell proliferation ability, transwell assays were used to assess cell migration and invasion, and luciferase reporter assays were used to examine the interaction between the long noncoding RNA TUG1 and miR-142-3p. Tumor formation was evaluated through *in vivo* experiments. **Results:** miR-142-3p was significantly downregulated in HCC tissues, but TUG1 was upregulated in HCC tissues. Knockdown of TUG1 and upregulation of miR-142-3p inhibited cell proliferation, cell migration, cell invasion, and the epithelial-mesenchymal transition (EMT). miR-142-3p was found to be a prognostic factor of HCC, and the mechanism by which TUG1 upregulated ZEB1 was via direct binding to miR-142-3p. *In vivo* assays showed that TUG1 knockdown suppressed cell proliferation and the EMT in nude mice. **Conclusion:** The results of this study suggest that the TUG1/miR-142-3p/ZEB1 axis contributes to the formation of malignant behaviors in HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the second highest cause of cancer-related mortality worldwide due to late tumor presentation and rapid progression Siegel *et al.* [1, 2]. It has distinct clinical, pathological and molecular features, but the molecular mechanisms by which the biological behaviors of HCC are regulated are not fully understood.

Recently, emerging evidence has indicated that noncoding RNAs (ncRNAs) may participate in HCC pathogenesis Xiong *et al.* [3-7]. Integrative genomic studies have shown that about 98% of human genome transcripts are ncRNAs Mattick *et al.* [8-10]. As endogenous and single-stranded RNAs, microRNAs (miRNAs) have been found in diverse diseases, and it has been demonstrated that they can negatively regulate their target messenger RNAs (mRNAs) through translational suppression or mRNA degradation Ohtsuka *et al.* [11-13]. The regulatory role of miR-142-3p has been reported in several types of cancer. For example, miR-142-3p overexpression increases the chemosensitivity of non-small cell lung cancer (NSCLC) by inhibiting high-mobility group box 1-mediated autophagy Chen *et al.* [14]. In addition, it can modulate osteosarcoma progression by interacting with metastasis-associated lung adenocarcinoma transcript-1 Liu *et al.* [15]. Some studies have examined the biological role of miR-142-3p in HCC. Xue *et al.* [16], demonstrated that miR-142-3p directly downregulates Ras-related C3 botulinum toxin substrate 1 in HCC cells; Wang *et al.* [17], showed that miR-142-3p and miR-142-5p synergistically suppress cell motility in HCC. However, the effects of miR-142-3p on the epithelial-mesenchymal transition (EMT) process in HCC are still unknown. This study explored the clinical significance, biological role, and underlying molecular mechanism of miR-142-3p in the development and progression of HCC.

As a group of ncRNAs, long noncoding RNAs (lncRNAs) act as competing endogenous RNAs (ceRNAs) by binding with miRNAs Yang *et al.* [18, 19]. For instance, lncRNA CCAT1 promotes gallbladder cancer development by negatively modulating miRNA-218-5p Ma *et al.* [20]; lncRNA growth-arrest specific 5 (GAS5) inhibits tumorigenesis and enhances radiosensitivity in NSCLC by suppressing miR-135b expression Xue *et al.* [16].; and lncRNA H19 and highly upregulated in liver cancer (HULC) can act as ceRNAs to promote cell migration and invasion in cholangiocarcinoma Wang *et al.* [21]. The lncRNA taurine upregulated gene 1 (TUG1) plays a role in the progression of several human cancers by interacting with miRNAs Wang *et al.* [22-24]. For example, TUG1 accelerates the malignancy of pancreatic cancer through sponging miR-382 Zhao *et al.* [25], mediates methotrexate resistance in colorectal cancer (CRC) by interacting with miR-186 Li *et al.* [26], acts as an oncogene in osteosarcoma by binding with miR-335 Wang *et al.* [22], and improves gastric cancer progression through sponging miR-145-5p Ren *et al.* [24]. Thus, this study aimed to clarify the interaction between TUG1 and miR-142-3p in HCC.

In this study, the low expression level of miR-142-3p was detected in HCC tissues. Furthermore, overexpression of miR-142-3p was found to significantly repress cell metastasis and reverse the EMT formation. The results of the mechanistic experiments revealed that miR-142-3p exerted anti-oncogenic functions in HCC in a zinc finger E-box-binding homeobox 1 (ZEB1)-dependent manner. Moreover, bioinformatics analysis revealed that TUG1 could directly bind to miR-142-3p. TUG1 was found to be highly expressed in HCC tissues and was inversely correlated with miR-142-3p expression. Additional studies confirmed that TUG1 negatively regulated miR-142-3p expression by functioning as a ceRNA. Collectively, our findings suggest that the TUG1/miR-142-3p/ZEB1 pathway contributes to the progression of HCC.

Materials and Methods

Patients

A total of 92 HCC tissues and matched tumor-adjacent tissues were obtained from the Department of Gastrointestinal Surgery, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University (Shaoxing, China). Tissue specimens were conserved in liquid nitrogen for RNA isolation. No patients received immunotherapy, radiotherapy, or chemotherapy before surgery. All samples were used after informed consent was obtained.

Cell culture

For routine cell culture, HepG2, Hep3B, SMMC-7721, HCCLM3, and Bel-7402 cell lines and the L02 normal liver epithelium cell line, purchased from the Shanghai Cell Collection (Shanghai, China), were maintained in high-glucose Dulbecco's Modified Eagle's Medium (8113035; Gibco, Gaithersburg, MD, USA) medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified air at 37°C with 5% of CO₂.

qRT-PCR

Based on the manufacturer's protocols, RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using the Tianscript RT Kit (Tiangen Biotech, Beijing, China). PCR amplification for miR-142-3p, TUG1, and ZEB1 mRNA was performed with the TaqMan Human MiRNA Assay (Genecopoeia, Guangzhou, China) and SYBR Premix Ex Taq™ Kit (Takara, Shiga, Japan) using the ABI 7300 System (Applied Biosystems, Foster City, CA, USA). As reference genes, U6 was used to normalize the expression of miR-142-3p, and GAPDH was used to normalize the expression of TUG1 and ZEB1 mRNA.

Migration and invasion assay

Cell migration and invasion were measured using transwell cell culture chambers (8 µm pore size, Corning Inc., Corning, NY, USA) with and without Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA). At 48 h post-transfection, cells in serum-free media were placed in the upper chamber with and without 10 µg/mL Matrigel. Medium containing 10% bovine calf serum was added to the lower chamber. Following 48 h of incubation, cells remaining in the upper membrane were wiped off, whereas cells that had migrated were fixed in methanol, stained with 0.1% crystal violet, and counted under a microscope. Three independent experiments were conducted.

MTT assay

Cell viability was measured using the Cell Proliferation Reagent Kit I (MTT; Roche Applied Science, Penzberg, Germany). The SMMC-7721 and HepG2 cells were added to 96-well plates at a density of 2×10^3 cells per well and then cultured in six replicate wells. Cell proliferation was evaluated at 24, 48, and 72 h; 10 µL MTT solution was added to each well in the last 4 h of the incubation period. Cell viability was defined as the mean of the absorbance of the converted dye at 450 nm.

Colony formation assay

For the colony formation assay, we added 500 cells per well to 6-well plates and cultured them in normal medium at 37°C. After 14 days, the cells were fixed in methanol and then dyed with 0.1% crystal violet. Visible colonies were counted manually.

Xenograft transplantation and immunohistochemistry

Approximately 5.0×10^6 SMMC-7721 cells stably transfected with sh-TUG1 or shRNA suspended in 100 µl phosphate-buffered saline were injected subcutaneously into the right side of the posterior flank of female BALB/c athymic nude mice (Department of Gastrointestinal Surgery, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University) at 5–6 weeks of age. Tumor growth was examined every other day with a vernier caliper. Tumor volumes were calculated using the equation: $V = A \times B^2 / 2$ (mm³), where A is the largest diameter and B is the perpendicular diameter. After 5 weeks, all mice were sacrificed and

necropsies were performed. The primary tumors were excised, paraffin-embedded, and formalin-fixed, followed by hematoxylin and eosin staining and immunostaining for Ki-67 protein expression according to the manufacturer's instructions. All animal experiments were strictly conducted in accordance with ethical standards and performed in accordance with the institutional guidelines.

Western blotting

Total proteins were collected with RIPA lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and protein concentrations were measured with the BCA method (Beyotime, Jiangsu, China). A total of 40 µg protein was subjected to 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Sigma St. Louis, MO, USA), after which the proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Roche, Indianapolis, IN, USA). Then, the membranes were blocked in 5% milk, followed by incubation with primary antibodies for ZEB1 (Abcam, Cambridge, MA, USA), E-cadherin (Abcam), N-cadherin (Abcam), α-SMA (Abcam), or Vimentin (Abcam) and subsequent incubation with the appropriate secondary antibodies (Cell Signaling Technology, Beverly, MA, USA). GAPDH (US Biological, Swampscott, MA, USA) was used as a loading control.

Immunofluorescence

HCC cells transfected with the appropriate miRNA vectors were seeded onto chamber slides and fixed in 4% paraformaldehyde for 10 min at room temperature. Then, cells were incubated with antibodies against E-cadherin (Abcam) or N-cadherin (Abcam) at 4°C overnight. Slides were incubated with matched secondary antibodies (Invitrogen) at room temperature for 1 h. The nucleus of HCC cells were stained with DAPI (Sigma) at room temperature for 10 min. Fluorescence confocal images were captured using the LSM 5 Pascal Laser Scanning Microscope (Zeiss Germany, Oberkochen, Germany).

Plasmid construction and dual luciferase reporter assay

Full-length (537 base pairs) human TUG1 WT with a potential miR-142-3p binding site or MUT site was generated and fused to the luciferase reporter vector psi-CHECK-2 (Promega, Madison, WI, USA), designated pTUG1-WT or pTUG1-MUT, respectively, and amplified using the following primers: TUG1-WT forward: 5'-TTTAGGGTTATTGGCCAAGACGGTTCCG-3' and reverse: 5'-GGTTTGAATTATATGAATACCGGTTATATAG-3'; TUG1-MUT forward: 5'-CTTGTGAGGCTAAGGTCCTTAGGGCTG-3' and reverse: 5'-TTCTTGCATTAATGGTTAGGCCTTATGGTTTA-3'. Full-length human WT ZEB1 with a potential miR-142-3p binding site or MUT site was generated and fused to the luciferase reporter vector psi-CHECK-2 (Promega), designated pZEB1-WT or pZEB1-MUT, respectively, and amplified with the following primers: forward: 5'-AGTAGATATACAGAATATTAACATATGTG-3' and reverse primer: 5'-TCTAGGCTTATGTTTCTCTGTCTC-3'; ZEB1-MUT forward: 5'-ATTAGATATGAGTTATAACGATGATG-3' and reverse: 5'-TAGTACCATTAGCGGTATACCATGTC-3'. Luciferase reporter assay were performed in 96-well plates using a Dual Luciferase Reporter Assay kit (Dual-Glo Luciferase Assay System; Promega) and SpectraMax M5 instrument software (Molecular Devices, Sunnyvale, CA, USA) was used to analyze the results. Cells were co-transfected with 100 ng pZEB1-miR-142-3p-UTR-WT or pZEB1-miR-142-3p-UTR-MUT and pTUG1-miR-142-3p-UTR-WT or pTUG1-miR-142-3p-UTR-MUT with 50 nM Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). After 48 h, the luciferase assay was performed using a Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions.

RIP

RIP was performed using a RIP Kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol. Cells at 80–90% confluency were scraped off and lysed in complete RIP lysis buffer. Then, 100 µL whole cell extract was incubated with RIP buffer containing magnetic beads conjugated to human anti-Ago2 antibody (Cell Signaling Technology); the negative control was normal mouse IgG (Millipore) and SNRNP70 (Millipore, Stafford, VA, USA) binding to U1 was used as the positive control. The co-precipitated RNAs were detected by reverse transcription PCR. Total RNAs (input controls) and IgG were assayed simultaneously to test whether the detected signals resulted from RNAs specifically bound to Ago2.

RNA pull-down

SMMC-7721 cells were transfected with biotinylated miRNA, and were collected at 48 h after transfection. The cell lysates were incubated with M-280 streptavidin magnetic beads (Invitrogen, San Diego, CA, USA). The bound RNAs were purified using TRIzol reagent (Invitrogen) for further qRT-PCR analysis.

Statistical analysis

SPSS 17.0 software was used for all statistical analyses. The significance of the differences between two groups was estimated with the Student's *t*-test. Multiple group comparisons were made and analyzed with one-way analysis of variance. The overall survival probability was analyzed and evaluated by Kaplan-Meier analyses and the log-rank test. Cox proportional hazards model was used to identify factors associated with the overall survival rate of HCC patients through multivariate analysis. The statistically significant correlation between TUG1 and miR-142-3p as well as that between miR-142-3p and ZEB1 in 92 cases of HCC tissues was analyzed with Spearman's correlation analysis. P values less than 0.05 were considered statistically significant.

Results

Downregulation of miR-142-3p is closely correlated with poor prognosis of HCC patients

To explore the biological role of miR-142-3p in HCC, the levels of miR-142-3p in 92 HCC tissues were measured by quantitative real-time PCR (qRT-PCR). As illustrated in Fig. 1A, the expression level of miR-142-3p was significantly lower in HCC tissues than in corresponding normal tissues ($P < 0.001$). Next, we analyzed the correlation between miR-142-3p and the clinical pathological parameters of HCC patients. According to the mean level of miR-142-3p, 92 HCC patient samples were divided into two groups: miR-142-3p high-expression group ($n = 45$) and miR-142-3p

Table 1. Correlation between miR-142-3p expression and clinical Features. (n=92). Low/high by the sample mean. Pearson χ^2 test. * $P < 0.05$, ** $P < 0.01$ was considered statistically significant

Variable	miR-142-3p expression		P-value
	low	high	
Age			
<60	35	41	0.278
≥60	10	6	
Sex			
Male	26	18	0.094
Female	19	29	
HbsAg			
Negative	17	20	0.675
Positive	28	27	
ALT			
≤45	31	39	0.145
>45	14	8	
AFP			
≤13.6	34	33	0.642
>13.6	11	14	
Vascular invasion			
Absent	13	25	0.021*
Present	32	22	
Cirrhosis			
Absent	20	39	<0.001**
Present	25	8	
Tumor size			
≤5	17	36	<0.001**
>5	28	11	
Edmondson grade			
I-II	20	34	0.011*
III-IV	25	13	

Fig. 1. Downregulation of miR-142-3p was closely correlated with poor prognosis in HCC patients. A. The level of miR-142-3p in HCC tissues was measured by qRT-PCR. B. The association between miR-142-3p and overall survival rate of HCC patients was analyzed by Kaplan-Meier analysis (log-rank test). C. The level of miR-142-3p in HCC cell lines was measured by qRT-PCR. Data are represented as the mean $\pm$ standard deviation (SD) of three independent experiments. The p-value represents the comparison between groups (* $p < 0.05$, ** $p < 0.01$).

low-expression group (n=47). As shown in Table 1, low miR-142-3p levels were significantly correlated with vascular invasion (P=0.021), cirrhosis (P<0.001), tumor size (P<0.001), and Edmondson grade (P=0.011), but not with age, sex, hepatitis B surface antigen, alanine aminotransferase, and alpha fetoprotein levels (P>0.05). Moreover, Kaplan-Meier analysis (log-rank test) revealed that a low level of miR-142-3p was associated with poor prognosis (P<0.001, log-rank test; Fig. 1B). Additionally, Cox regression analysis showed that a low level of miR-142-3p could be an independent prognostic factor (P<0.001), as well as a predictor of vascular invasion and Edmondson grade (Table 2). We evaluated the level of miR-142-3p in a panel of HCC cell lines (SMMC7721, Hep3B, HepG2, Bel-7402, HCCLM3), and found that miR-142-3p was notably downregulated in HCC cells compared with the L02 human normal liver epithelial cell line; the lowest miR-142-3p expression levels were found in SMMC-7721 and HepG2 cells (Fig. 1C). Collectively, these findings indicate that miR-142-3p acts as a tumor suppressor in HCC progression and might be a promising prognostic marker for HCC patients.

miR-142-3p suppresses the proliferation, migration, and invasion of HCC cells

According to abovementioned findings, we postulated that miR-142-3p might be associated with tumor metastasis. Because the lowest

Table 2. Multivariate and univariate analyses of prognostic parameters in patients with HCC by Cox regression analysis. Cox regression analysis showed the positive, independent prognostic importance of miR-142-3p expression (P = 0.013*). *P < 0.05 and **P<0.01 were considered statistically significant

Variable	Univariate analysis		Multivariate analysis	
	Category	P-value	P-value	
Age	<60	0.307		
	≥60			
Sex	Male	0.364		
	Female			
HbsAg	Negative	0.352		
ALT	≤45	0.688		
AFP	≤13.6	0.267		
	>13.6			
Vascular invasion	Absent	0.014*	0.029*	
	Present			
Cirrhosis	Absent	0.245		
	Present			
Tumor size	≤5	0.318		
	>5			
Edmondson grade	I-II	0.015*	0.030*	
	III-IV			
miR-142-3p expression	Low	0.013*	<0.001**	
	High			

Fig. 2. miR-142-3p suppressed the proliferation, migration, and invasion of HCC cell. A. SMMC-7721 and HepG2 cells were transfected with miR-142-3p mimics, and transfection efficiency was determined with RT-qPCR. B–C. Wound healing and transwell assays were performed to measure the effect of miR-142-3p on the migration and invasion of HCC cells. D–E. The effect of miR-142-3p on cell proliferative ability was evaluated by MTT and colony formation assays. Data are represented as the mean ± SD of three independent experiments. The p-value represents the comparison between groups (*p<0.05, **p<0.01).

Fig. 3. Ectopic expression of miR-142-3p reversed the phenotype from EMT to MET in HCC cells. A–B. Western blot analysis was utilized to determine the effect of miR-142-3p on the expression levels of EMT markers. C–D. Immunofluorescence was performed to assess the levels of EMT markers in HCC cells. Data are represented as the mean $\pm$ SD of three independent experiments. The p-value represents the comparison between groups (* $p < 0.05$, ** $p < 0.01$).

Fig. 4. ZEB1 was identified as a target of miR-142-3p. A. The binding sites between miR-142-3p and ZEB1 was predicted. B. The level of ZEB1 in HCC cells was measured. C. The effect of miR-142-3p on the mRNA and protein levels of ZEB1 was analyzed and investigated by qRT-PCR and western blot analysis. D. Luciferase reporter assays were applied to detect the interaction between miR-142-3p and ZEB1. E. The level of ZEB1 in HCC tissues and corresponding normal tissues was detected by qRT-PCR. F. The correlation between miR-142-3p and ZEB1 was analyzed with Spearman's correlation analysis ($r = -0.653$, $P < 0.001$). Data are represented as the mean $\pm$ SD of three independent experiments. The p-value represents the comparison between groups (* $p < 0.05$, ** $p < 0.01$).

miR-142-3p expression was found in SMMC-7721 and HepG2 cells, they were chosen for subsequent experiments. To assess the biological effects of miR-142-3p on cell migration and invasion, we transfected SMMC-7721 and HepG2 cells with miR-142-3p mimics; the transfection efficiency was determined by RT-qPCR (Fig. 2A). Wound healing and transwell assays revealed that miR-142-3p mimics suppressed the migratory and invasive capacity of SMMC-7721 and HepG2 cells (Fig. 2B, C). Upregulation of miR-142-3p weakened the proliferative ability of HCC cells (Fig. 2D, E). These results showed that miR-142-3p inhibited the metastasis of HCC by regulating cell migration and invasion.

Ectopic expression of miR-142-3p reverses the phenotype from the EMT to the mesenchymal-epithelial transition in HCC cells

The EMT process is crucial for malignant transformation; thus, we evaluated the effects of miR-142-3p on the EMT process. Western blot analysis showed that after transfection with miR-142-3p mimics, levels of the epithelial marker (E-cadherin) in HCC cells were significantly increased, whereas those of the mesenchymal markers N-cadherin, vimentin, and α -smooth muscle actin (α -SMA) were decreased (Fig. 3A, B). Consistent with these data, immunofluorescence experiments confirmed the effect of miR-142-3p on EMT progress of HCC cells (Fig. 3C, D). These results indicated that miR-142-3p restrained acquisition of the EMT phenotype in HCC cells, which helped suppress the metastatic ability of HCC cells.

ZEB1 is a target of miR-142-3p

To explore the downstream molecular mechanism of miR-142-3p in HCC cells, we utilized three publicly available databases (TargetScan, starBase, and miRanda). ZEB1, a key regulator of the EMT progress, was found to be a target of miR-142-3p (Fig. 4A). High ZEB1 levels were found in HCC cell lines (SMMC7721, Hep3B, HepG2, Bel-7402, HCCLM3) compared with L02 cells (Fig. 4B). We measured the effects of miR-142-3p on the mRNA and protein levels of ZEB1, and found that miR-142-3p overexpression decreased these levels (Fig. 4C).

Fig. 5. Overexpression of ZEB1 reversed the effects of miR-142-3p in HCC cells. A–C. Overexpressed ZEB1 partially abrogated the inhibitory effects of miR-142-3p on the cell metastatic abilities and EMT progress. Data are represented as the mean $\pm$ SD of three independent experiments. The p-value represents the comparison between groups (* $p < 0.05$, ** $p < 0.01$).

Fig. 6. TUG1 acted as an oncogene and negatively regulated miR-142-3p. A. The level of TUG1 in HCC tissues and corresponding normal tissues was detected by qRT-PCR. B. SMMC-7721 and HepG2 cells were transfected with si-TUG1 or siRNA to silence TUG1. C–D. MTT and colony formation assays were conducted to detect the effect of TUG1 on the proliferative ability of HCC cells. E. Transwell assays were performed to evaluate the effect of TUG1 on cell migration and invasion. F. The effect of TUG1 on miR-142-3p expression was detected by qRT-PCR. G. The correlation between miR-142-3p and TUG1 was analyzed by Spearman's correlation analysis ($r = -0.741$, $P < 0.001$). Data are represented as the mean $\pm$ SD of three independent experiments. The p-value represents the comparison between groups (* $p < 0.05$, ** $p < 0.01$).

Luciferase reporter assays revealed that miR-142-3p overexpression notably reduced the luciferase activity of the wild-type 3' untranslated region (WT 3'-UTR) of ZEB1 but not that of the mutant (MUT) 3'-UTR of ZEB1 (Fig. 4D). Furthermore, qRT-PCR experiments showed that ZEB1 was upregulated in HCC tissues (Fig. 4E), and Spearman's correlation analysis showed that miR-142-3p expression was inversely correlated with that of ZEB1 ($r = -0.653$, $P < 0.001$, Fig. 4F). Therefore, we conclude that ZEB1 is a direct target of miR-142-3p in HCC.

Overexpression of ZEB1 reverses the effects of miR-142-3p in HCC cells

Rescue assays were performed to further confirm the impact of ZEB1 on miR-142-3p-mediated function in HCC cells. As expected, ZEB1 overexpression partially abrogated the inhibitory effects of miR-142-3p on the migration and invasion of SMMC7721 cells (Fig. 5A). Additionally, overexpressed ZEB1 partially reversed the MET to EMT in SMMC7721 cells (Fig. 5B, C). These findings suggest that ZEB1 is a target of miR-142-3p and mediates the function of miR-142-3p in HCC.

TUG1 acts as an oncogene and negatively regulates miR-142-3p

To determine the function of TUG1, we measured its level in HCC tissues and corresponding normal tissues, and found that TUG1 was upregulated in HCC tissues (Fig. 6A). Subsequently, TUG1 was knocked down by transfection with specific small interfering RNAs (siRNAs); the transfection efficiency was determined by qRT-PCR (Fig. 6B). Knockdown of TUG1 repressed the proliferation, migration, and invasion of HCC cells compared with the negative controls (Fig. 6C–E). The functions of TUG1 in HCC cells were different from those of miR-142-3p. Accordingly, we hypothesized that TUG1 is inversely correlated with miR-142-3p in HCC. To test our hypothesis, qRT-PCR was conducted to determine the effect of TUG1 on the expression level of miR-142-3p. Silenced TUG1 clearly increased the level of miR-142-3p (Fig. 6F), and Spearman's correlation analysis confirmed the inverse relationship between TUG1 and miR-142-3p (Fig. 6G). Taken together, we conclude that lncRNA TUG1 plays an oncogenic role in HCC and is inversely correlated with miR-142-3p.

Fig. 7. TUG1 regulated ZEB1 by competitively binding with miR-142-3p. A. The binding sites for miR-142-3p harboring the TUG1 are presented. B. Ago2-RIP assays were applied to verify binding between TUG1 and miR-142-3p. IgG was used as the negative control, and SNRNP70 binding to U1 was used as the positive control. C. RNA pulldown assay was used to confirm the combination between miR-142-3p and TUG1. D. Luciferase reporter assay was conducted to verify the interaction between TUG1 and miR-142-3p. E. qRT-PCR was performed to measure the effect of silenced TUG1 on the level of ZEB1. Immunofluorescence and western blot assays were performed to measure the effect of silenced TUG1 on EMT acquisition. F. The correlation between TUG1 and ZEB1 in HCC tissues was analyzed. Data are represented as the mean $\pm$ SD of three independent experiments. The p-value represents the comparison between groups (* $p < 0.05$, ** $p < 0.01$).

Fig. 8. TUG1/miR-142-3p/ZEB1 axis contributed to the proliferation and EMT in vivo. A. Growth curve of tumor volumes and representative tumor photographs were generated and obtained after 28 days. B. Images of Ki-67 immunostaining were obtained from the transplanted tumors. C. The levels of miR-142-3p and ZEB1 in tumor tissues were detected by qRT-PCR. D. Western blotting was utilized to detect the expression level of E-cadherin and N-cadherin. Data are represented as the mean $\pm$ SD of three independent experiments. The p-value represents the comparison between groups (* $p < 0.05$, ** $p < 0.01$).

TUG1 regulates ZEB1 by competitively binding with miR-142-3p

In recent years, the patterns of ceRNA interactions have been widely reported. In a ceRNA model, lncRNA release or upregulate target mRNA of miRNAs by competitively binding with the same miRNAs Zhang *et al.* [27-31]. TUG1 participates in the progression and development of multiple tumors by acting as a ceRNA Zhang *et al.* [22, 24, 26, 32-36]. To determine whether TUG1 acts as a ceRNA in HCC, we applied bioinformatics analysis (<http://starbase.sysu.edu.cn/browseNcRNA.php>) to identify the binding site between TUG1 and miR-142-3p (Fig. 7A). Thereafter, we performed a series of experiments (RNA immunoprecipitation [RIP], RNA pull-down, and luciferase reporter assay) to verify the binding relationship between TUG1 and miR-142-3p. As illustrated in Fig. 7B, the fold enrichment of TUG1 in the RIP experiments was markedly higher in the beads harboring argonaute-2 (Ago2). IgG was used as the negative control, and small nuclear ribonucleoprotein U1 subunit 70 (SNRNP70) binding with U1 was used as the positive control. In the RNA pull-down assay, biotinylated miR-142-3p captured more TUG1 than did biotinylated miR-142-3p-mut (Fig. 7C). The luciferase reporter assay showed that overexpression of miR-142-3p markedly decreased the luciferase activity of wild-type TUG1 but not mutant TUG1 (Fig. 7D). These findings confirmed the interaction between TUG1 and miR-142-3p. Additionally, we assessed the effects of TUG1 on ZEB1 and EMT-related proteins. Knockdown of TUG1 inhibited the expression of ZEB1 and EMT progression in SMMC-7721 cells (Fig. 7E). Finally, the positive correlation between TUG1 and ZEB1 was verified by Spearman's correlation analysis (Fig. 7F). Taken together, these data showed that TUG1 facilitated ZEB1-mediated EMT acquisition in HCC cells by acting as a ceRNA through competitively sponging miR-142-3p.

TUG1/miR-142-3p/ZEB1 axis contributes to cell proliferation and the EMT acquisition in vivo

To further confirm that the TUG1/miR-142/ZEB1 axis affects the tumorigenesis of HCC *in vivo*, SMMC-7721 cells stably transfected with single hairpin-TUG1 (sh-TUG1) or control vector were inoculated into nude mice. Tumors were substantially smaller in the sh-TUG1 group than in the control group; tumor volume and weight were also significantly reduced by silenced TUG1 (Fig. 8A). Tumors formed from stably sh-TUG1-transfected SMMC-7721 cells exhibited weakened positivity for Ki-67 compared with control cells (Fig. 8B). qRT-PCR assays revealed that the expression level of miR-142-3p was increased in tumor tissues derived from TUG1-downregulated SMMC-7721 cells (Fig. 8C), whereas the expression level of ZEB1 was decreased in tumor tissues derived from TUG1-downregulated SMMC-7721 cells (Fig. 8C). Moreover, western blot analysis revealed that silenced TUG1 significantly reduced the level of N-cadherin but increased the level of E-cadherin (Fig. 8D). These *in vivo* data further demonstrated the oncogenic function of the TUG1/miR-142-3p/ZEB1 axis.

Discussion

HCC, a highly vascularized tumor, is one of the most prevalent fatal malignancies worldwide, and its increasing incidence has become a major health problem. HCC is often accompanied by frequent metastasis (intrahepatic or extrahepatic), and limited therapeutic approaches have resulted in a high mortality rate. Therefore, it is an urgent need to explore the underlying molecular mechanisms of HCC metastasis.

Integrative genomic studies have shown that ~98% of the human genome transcripts are ncRNAs. Based on length, these ncRNAs can be divided into two groups: short ncRNAs (<200 nucleotides) and long ncRNAs (>200 nucleotides). As a type of typical short ncRNAs, miRNAs have been shown to be dysregulated in many cancer types. Additionally, miRNAs can participate in tumorigenesis by regulating target genes via translational suppression or mRNA degradation Bash-Imam *et al.* [37-42]. For example, Tang *et al.* [43]. reported that dysregulated miR-218 is associated with the clinicopathological characteristics of cervical

cancer patients. Wu *et al.* showed that miR-655-3p functioned as a tumor suppressor in HCC by regulating a disintegrin and metalloproteinase domain-containing protein 10 and beta-catenin pathway Wu *et al.* [44].

In this study, we demonstrated that miR-142-3p was downregulated in HCC tissues and cell lines. Furthermore, our data showed that low levels of miR-142-3p were positively associated with vascular invasion ($P=0.021$), cirrhosis ($P=0.000$), tumor size ($P=0.000$), and Edmondson grade ($P=0.011$) and predicted poor prognosis in HCC patients. Because high metastasis ability is a main feature of HCC, we focused on the effects of miR-142-3p on metastasis and the EMT phenotype. The cellular experiments illustrated that miR-142-3p overexpression significantly repressed cell metastasis and reversed the EMT. EMT events have been widely found in the metastasis process of various cancers Ruan *et al.* [45-47]. ZEB1 is a critical regulator of the EMT Zhang *et al.* [48, 49]. In the current study, we found that miR-142-3p expression was inversely correlated with ZEB1 expression in HCC cells and tissues. The results of the luciferase reporter assays revealed that ZEB1 is a direct downstream target of miR-142-3p in HCC. Furthermore, rescue assays showed that overexpressed ZEB1 abolished the effects of miR-142-3p on the migration, invasion, and EMT process. Therefore, miR-142-3p exerts its function in HCC by targeting ZEB1.

Endogenous transcripts containing miRNA response elements (MREs) can interact with or co-regulate each other by acting as endogenous miRNA sponges or ceRNAs, thereby forming large-scale regulatory network across the transcriptome Salmena *et al.* [31, 50]. Numerous studies have documented that lncRNAs harbor potential MREs and function as competitive platforms for miRNAs. For instance, Ye *et al.* [51] demonstrated that the lncRNA GAS5 suppresses cell growth and the EMT in osteosarcoma by regulating the miR-221/ARHI pathway. Huang *et al.* [52] reported that the novel long intergenic noncoding RNA upregulated in CRC (UCC) promoted CRC progression by sponging miR-143. TUG1 plays a role in the regulation of many cancer types by functioning as a ceRNA Wang *et al.* [22, 32, 34, 35, 53]. Based on the results of our bioinformatics analysis, it was hypothesized that miR-142-3p could be negatively regulated by TUG1. Our findings revealed that the level of TUG1 in HCC tissues was inversely correlated with that of miR-142-3p. In addition, TUG1 negatively regulated the expression level of miR-142-3p. RIP, RNA pull-down, and luciferase reporter assays were further performed to confirm the interaction between miR-142-3p and TUG. Finally, silenced TUG1 significantly reduced the levels of ZEB1 and EMT-related proteins.

The results of this study showed that miR-142-3p acted as a tumor suppressor in HCC, and its downregulation was associated with poor prognosis in HCC patients. Cellular and mechanistic assays revealed that the TUG1-miR-142-3p-ZEB1/EMT axis contributes to the metastasis of HCC cells and might be a therapeutic target for this disease.

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Disclosure Statement

No conflict of interests exists.

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