miR-141 inhibits proliferation, migration and invasion in human hepatocellular carcinoma cells by directly downregulating TGFβR1

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Abstract. MicroRNAs (miRNAs) have been demonstrated to have crucial roles in modulating various human cancers. The present study examined the involvement of miR-141 in modulating the proliferation, migration, and invasion of hepatocellular carcinoma (HCC) cells. Notably, the results demonstrated that miR-141 was significantly downregulated in primary liver tumor tissues from patients compared with adjacent non-tumor tissues. Overexpression of miR-141 in HCC cells robustly impaired migration and invasion and suppressed proliferation in vitro, by arresting cells at the G2/M phase. Further analyses revealed that miR-141 overexpression downregulated transforming growth factor β receptor 1 (TGF\u00b3R1) by directly binding to its 3' untranslated region. In addition, the mRNA and protein levels of TGFBR1 were both significantly increased in HCC patient tissues compared with matched adjacent non-tumor tissues. Silencing of TGFBR1 produced similar effects in vitro to miR-141 overexpression. Furthermore, the downstream protein SMAD family member 2 was downregulated following overexpression of miR-141 or silencing of TGFβR1. These findings indicated that miR-141 inhibited HCC cell proliferation and invasion by directly downregulating TGF\u00b3R1 and its downstream signaling cascade, providing insights into a potential novel strategy for HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent and the third most lethal type of cancer, making it a highly aggressive malignancy. Over six million new cases and a similar number of deaths are reported worldwide each year (1,2). Although surgical resection and transplantation are effective for HCC treatment, patients who have undergone tumor resection have a high rate of recurrence primarily, due to intrahepatic spread and extrahepatic metastasis (3). Indeed, metastasis remains the primary obstacle to survival in patients with HCC. Understanding the essential molecular pathways for tumor metastasis is essential in order to improve therapeutic strategies. However, there is limited knowledge regarding the molecular and environmental mechanisms mediating the metastatic recurrence of HCC, and therapeutic choices for HCC treatment remain scarce. Thus, elucidating the mechanisms of HCC metastasis may provide a solid basis for the clinical prediction and prevention of HCC metastasis.

MicroRNAs (miRNAs) are small non-coding RNAs of ~22 nucleotides in length that negatively regulate the expression of various genes, primarily through interactions with the 3' untranslated regions (3'UTRs) of their mRNAs (4,5). miRNAs can post-transcriptionally modulate ~30% of human genes, indicating that they may serve as crucial regulators of human biological processes, including cancer development, cell proliferation, differentiation, and apoptosis (6,7). Several dozen miRNAs have been demonstrated to be aberrantly expressed in various human cancers. For example, miR-96, miR-128, and miR-193b are associated with pancreatic cancer (8-10); miR-200, miR-183, and miR-22 are associated with gastric cancer (11-13); and miR-185, miR-143, and miR-145 are associated with breast cancer (14,15). However, the role of miRNAs in HCC metastasis remains poorly understood. A more thorough understanding of deregulated miRNAs in HCC may elucidate the mechanisms of HCC development and may result in improvements of HCC diagnosis and treatment.

In a previous study from our group, a tumor tissue microarray was used to identify that miR-141 was significantly downregulated in renal cell carcinoma (RCC). That previous study also revealed that high expression of miR-141 dramatically inhibited the proliferation and metastasis of RCC cells both in vitro and in nude mouse models (16). Recently, miR-141 was reported to be correlated with metastasis in various types of cancer, including gastric cancer (17,18), non-small cell lung cancer (19), pancreatic cancer (20), and bladder cancer (21). Several studies have also demonstrated

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that miR-141 functions as a metastasis suppressor by targeting E2F transcription factor 3 (E2F3), zinc-finger E-box binding homeobox 2 (ZEB2), and T cell lymphoma invasion and metastasis 1 (Tiam1) in HCC (22-24). However, these studies were not in-depth analyses of the clinical value of miR-141 in HCC or its functional roles.

The present study demonstrated for the first time that miR-141 directly targeted transforming growth factor β receptor 1 (TGF β R1), which is an important factor in HCC growth, migration and invasion. These findings suggest that miR-141 may have clinical applications owing to its effects on proliferation and invasion.

Materials and methods

Patients and tissue samples. Clinical HCC tissues and adjacent non-tumor tissues (≥ 3 cm from the tumor), used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, were collected from 19 patients undergoing hepatectomy at Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). These specimens were obtained from patients with HCC from April to August, 2013. Patients aged 50-60 years and diagnosed with only primary hepatocellular carcinoma were selected as a late stage study. Informed consent was obtained from all patients prior to enrollment in the study. The Ethics Committee of Tongji Medical College approved the present study. Clinical pathological reports were collected. Tissue samples were immediately snap-frozen in liquid nitrogen at the time of surgery and stored at -80°C.

Cell culture and materials. The human hepatocellular carcinoma cell lines Huh7 and SK-HEP-1 and the human liver cell line LO2 (HL-7702) were purchased from the China Center for Type Culture Collection (Wuhan, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin sodium, and 0.1 mg/ml streptomycin sulfate in a humidified incubator at 37°C with an atmosphere containing 5% CO₂.

RNA extraction and RT-qPCR. Total RNA was extracted from the patient liver tissues or cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The RNA concentrations and A260/A280 ratios were measured with a plate reader (BioTek Instruments, Inc., Winooski, VT, USA). Subsequently, reverse transcription was performed using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). qPCR was performed using a SYBR Green qPCR Supermix UDG kit (Invitrogen; Thermo Fisher Scientific Inc.). The primers for miRNA detection were purchased from Ribobio Co., Ltd. (Guangzhou, China), and the primers for mRNA detection were purchased from Genewiz, Inc. (Jiangsu, China; Table I). U6 small nuclear RNA and GAPDH were used as internal controls for miRNA and mRNA, respectively. Primer sequences were as follows: TGFβR1, forward 5'-CACAGAGTGGGAACAAAAGGT-3' and reverse 5'-CCAATGGAACATCGTCGAGCA-3'; GAPDH, forward 5'-GGTGAAGGTCGGAGTCAACGG-3' and reverse 5'-GAGGTCAATGAAGGGGTCATTG-3'; miR-141, forward 5'-GGCGTCACAGGCATTCACAGTC-3' and reverse 5'-TCT TCCTCCTTCCTTCTCCG-3'; and U6, forward 5'-CTCGCTT CGGCAGCACATATACT-3' and reverse 5'-CGGCTGCAGA TGAGATAG-3'. qPCR was performed on a Roche Light 480 system (LightCycler 480; Roche Applied Science, Penzberg, Germany). All reactions were performed in triplicate. Relative fold changes in mRNA expression were calculated using the formula 2^{-ΔΔCT}.

Protein isolation and immunoblotting. Cells or tissues were washed twice with ice-cold PBS, scraped into RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% v/v Triton X-100, 1 mM ethylenediaminetetraacetic acid, 0.1% m/v SDS, 1% m/v glycodeoxycholate, and 150 mM NaCl) with freshly added protease inhibitor cocktail and phenylmethylsulfonyl fluoride for 30 min on ice, and then centrifuged at 12,000 x g at 4°C for 10 min. The supernatants were then collected. Protein (50 μ g) was separated on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membrane (0.45 μ m; EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk, and the primary antibody was added at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The following antibodies were used: rabbit polyclonal anti-TGFβR1 (sc-399; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit polyclonal anti-SMAD family member 2 (ab40855; 1:1,000; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-phosphorylated (p-) Smad2 (ab188334; 1:1,000; Abcam), mouse monoclonal anti-β-actin (sc-81178; 1:200; Santa Cruz Biotechnology, Inc.), anti-mouse secondary horseradish peroxidase (HRP)-conjugated antibodies, and anti-rabbit secondary HRP-conjugated antibodies (ab205718; 1:2,000; Abcam). Reactive bands were visualized with ECL reagent (Pierce; Thermo Fisher Scientific, Inc.) on a Bio-Rad Image Analysis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein expression was quantified using QuantityOne v4.6.2 software (Bio-Rad Laboratories, Inc.).

Analysis of cell viability, colony formation, and cell cycle distribution. Dimethyl thiazolyl diphenyl tetrazolium (MTT; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) assays were performed to determine cell viability in 96-well plates according to standard procedures. Cells were seeded at a density of 3,000 cells/well containing 100 μ l culture medium. At 24-h intervals, 20 μ l of 5 mg/ml MTT was added, and plates were incubated at 37°C for 4 h. The medium was then removed, and 150 μ l dimethyl sulfoxide was added per well to dissolve the precipitate. The optical density was tested at a wavelength of 490 nm. Wells with 100 μ l culture medium and no cells were used as blanks.

For colony formation assays, single-cell suspensions (2,000 cells/well) were plated in each well of 6-well plates. After 1 week of culture, the surviving colonies were fixed with 95% methanol for 10 min, stained with 0.1% crystal violet for 20 min, imaged, and counted. The experiments were repeated at least three times. For cell cycle assays, the cells were fixed in

Primers	Forward (5'-3')	Reverse (5'-3')	Product size (bp)
TGFβR1	CACAGAGTGGGAACAAAAAGGT	CCAATGGAACATCGTCGAGCA	143
GAPDH	GGTGAAGGTCGGAGTCAACGG	GAGGTCAATGAAGGGGTCATTG	112

Table I. Primers for qRT-PCR.

70% ethanol, stored at -20°C for at least 24 h, and then stained with propidium iodide prior to flow cytometry (ZE5; Bio-Rad Laboratories, Inc.).

In vitro migration and invasion assay. Twenty-four-well Transwell plates (8 µm pore size; Corning Inc., Corning, NY, USA) were used to measure the migratory and invasive abilities of the cells. Cells were serum starved for 24 h, trypsinized, and suspended in serum-free medium at 5×10^4 cells/200 µl. For Transwell migration assays, 5x10⁴ Huh7-NC or Huh7-141 cells were added into the upper of Transwell chambers containing uncoated membranes. For invasion assays, a similar number of cells was seeded into the upper chamber of the Transwell that were precoated with 200 mg/ml Matrigel (BD Biosciences). Then, 600 µl DMEM supplemented with 10-20% FBS was added to the lower of the Transwells. After incubation at 37°C for 20 h, the cells were removed and washed with PBS three times. Non-migratory or non-invasive cells on the top chambers were gently removed using cotton wool. The cells on the underside of the membranes were fixed with 95% methanol for 10 min, air-dried, stained with 0.1% crysal violet for 15 min, and counted under an inverted light microscope (magnification, x200).

miRNA and small interfering RNA (siRNA) transfection. For establishment of cell lines, cells were infected with lentivirus (GV369 vector; GeneChem Co., Ltd., Shanghai, China) after reaching 30-50% confluence, according to the manufacturer's recommendations. After 48 h of incubation, the infection efficiency was detected by evaluating green fluorescent protein (GFP) expression under a fluorescence microscope; infection of at least 90% of cells was considered effective. Successful infection was also confirmed using RT-qPCR. After identification, the cells were cultured and harvested for subsequent experiments.

siRNA against TGFβR1 and negative control oligonucleotides and siRNA against miR-141 and negative control oligonucleotides were synthesized by GenePhama Co., Ltd. (Shanghai, China). The oligonucleotides used in these studies were as follows: TGFβR1 siRNA, 5'-AGAAAUGAAGUGGC AUUAATT-3' (sense) and 5'-UUAAUGCCACUUCAUUUC UTT-3' (antisense). siRNA against miR-141 and the negative control oligonucleotides were ready-made by GenePhama Co., Ltd. Oligonucleotide transfection was performed using Lipofectamine 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h post-transfection, the cells were harvested for subsequent experiments.

Bioinformatic analysis. The prediction of miRNA targets was performed using these publicly available algorithms:

TargetScan (http://www.targetscan.org; Whitehead Institute, Cambridge, MA, USA), MiRanda (http://cbio.mskcc. org/mirnaviewer; Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and StarBase (http://starbase.sysu.edu.cn; Sun Yat-sen University, Guangzhou, China).

Vector construction and dual luciferase reporter assays. The wild-type (wt) 3' untranslated region (UTR) of the human TGFβR1 gene, which was predicted to interact with miR-141, and a mutant (mut) variant, were constructed and cloned into the GV272 vector (GeneChem, Inc., Daejeon, Korea). For luciferase reporter activity assays, cells were seeded in 24-well plates and allowed to reach 70% confluence prior to transfection. Next, wt TGF\u00b3R1 3'UTR reporter vector, mut TGF\u00b3R1 3'UTR reporter vector, or the negative control vector were cotransfected with the Renilla firefly luciferase-expressing vector into the cells using Lipofectamine 2000 reagent. Cell lysates were collected 24 h later, and luciferase activity was determined with a Dual-Luciferase Reporter System (Promega Corporation, Madison, WI, USA). Relative Renilla luciferase activities were used as an internal control for transfection efficiency.

Statistical analysis. All values are presented as means \pm standard deviations. MTT assays, RT-qPCR, dual luciferase reporter assays, and cell cycle analysis were performed in triplicate. Statistical analyses were performed with GraphPad Prism (version 5.01 for Windows; GraphPad, Inc., La Jolla, CA, USA). Differences between two groups were assessed by Student's t-tests. Differences among multiple groups were assessed with one-way analysis of variance, and Tukey's post hoc test. The correlation between levels of miR-141 and TGF β R1 mRNA in HCC tissues was analyzed with Pearson's correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-141 is frequently downregulated in human HCC tissues and cell lines. Based on published reports (25-29) and our previous studies (16,30) of miR-200 family members in several types of cancer, the present study attempted to clarify the role of the miR-200 family member miR-141 in human HCC progression. To investigate the clinical implications of miR-141 in HCC progression, first the expression levels of miR-141 were determined in 19 pairs of primary HCC tissues and their matched adjacent non-tumor tissues, using miRNA-based RT-qPCR. The results demonstrated that miR-141 was significantly downregulated in 89% (17 of 19; P=0.012) of HCC tissues compared with their matched non-tumor tissues (Fig. 1A), suggesting that decreased expression of miR-141



Figure 1. miR-141 is frequently downregulated in human HCC tissues and cell lines. (A) miR-141 expression was determined by RT-qPCR in HCC tissues and adjacent non-tumor liver tissues (n=19). Expression was plotted as relative levels of miR-141 (log_{10}) to the U6 internal control. (B) RT-qPCR analysis of miR-141 levels in normal LO2 hepatocytes and HCC cell lines SK-HEP-1 and Huh7. (C) Infection of Huh7 cells with miR-141-expressing or negative control vector lentivirus. (D) miR-141 levels were further confirmed by RT-qPCR. Data are presented as means ± standard deviation from three independent experiments. *P<0.05 and ***P<0.001 with comparisons indicated by lines. HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GFP, green fluorescent protein; N, normal; T, tumor; NC, negative control.

occurred frequently in HCC and that miR-141 may function as a suppressor of HCC development.

To investigate the role of miR-141 in HCC *in vitro*, the expression levels of miR-141 were detected in three human liver cell lines. The results revealed that miR-141 expression was substantially lower in two liver cancer cell lines, SK-HEP-1 and Huh7, compared with the normal liver LO2 cells (Fig. 1B). Next, stable cell lines were established via infection of the Huh7 cells with either a lentivirus harboring an miR-141-overexpressing vector (cells termed Huh7-141) or a control empty vector (cells termed Huh7-NC). The efficiency of infection was >90%, as evidenced by the expression of GFP encoded in the lentiviral vector (Fig. 1C). The efficiency of miR-141 overexpression was further confirmed by RT-qPCR (Fig. 1D).

miR-141 negatively regulates HCC proliferation, migration, and invasion in vitro. Tumor cell proliferation, migration, and invasion are crucial processes of HCC progression. The significant downregulation of miR-141 in HCC tissues and cell lines, compared with normal tissues and cells, indicated that miR-141 may have a biologically significant role in HCC tumorigenesis. First, MTT assays, colony formation assays, and flow cytometry were used to investigate whether miR-141 affects the growth of HCC cells *in vitro.* MTT assays demonstrated that overexpression of miR-141 significantly reduced the numbers of viable Huh7 cells over time (Fig. 2A). The viability of Huh7-141 cells decreased by 52.37% by day 5 compared with Huh7-NC cells (Fig. 2A). These data implied that miR-141 suppressed HCC cell growth. Colony formation assays were employed to confirm these results. As illustrated in Fig. 2B, far fewer colonies were formed following miR-141 overexpression in HCC cells, compared with the control cells. In addition, cell cycle analysis revealed that overexpression of miR-141 increased cell cycle arrest at the S phase by 4.85%, with 5.86% fewer cells entering G2/M phase (Fig. 2C; P<0.05). Taken together, these observations suggested that miR-141 suppressed the proliferation of Huh7 cells by inducing cell cycle arrest.

To determine the effects of miR-141 on the migration and invasion abilities of HCC cells, Transwell assays were performed in vitro. In accordance with our hypothesis, ectopic miR-141 expression significantly suppressed HCC cell migration and invasion compared with control cells (Fig. 2D). These data revealed an obvious negative correlation between miR-141 expression and HCC migration and invasion.

miR-141 directly targets the 3'UTR of $TGF\beta RI$. To explore the molecular mechanisms through which miR-141 suppressed HCC migration, a search was performed to identify candidate gene targets of miR-141 that are involved



Figure 2. Overexpression of miR-141 suppresses hepatocellular carcinoma cell proliferation, migration and invasion *in vitro*. (A) At 24-h intervals, MTT assays were performed to measure the viability of control and miR-141-overexpressing Huh7 cells (Huh7-NC and Huh7-141, respectively). (B) Colony formation was evaluated after 1 week of culture. (C) Analysis of the cell cycle was performed via flow cytometry. (D) Migration and invasion were examined by Transwell assays for 20 h. *P<0.05 and **P<0.01 with comparisons indicated by lines. NC, negative control; OD, optical density.

in HCC pathogenesis. With a combination of miRNA target prediction programs, such as TargetScan and MiRanda, and through review of the literature, the 3'UTR of TGF β R1 was identified as complementary to the miR-141 sequence; thus, miR-141 may exert its biological functions by targeting TGF β R1 mRNA (Fig. 3A). To test this hypothesis, a luciferase-reporter plasmid (Luc-TGF β R1-wt) was constructed in which the nucleotides of the wild-type (wt) TGF β R1 3'UTR (complementary to miR-141) were inserted into a GV238 vector downstream of the luciferase reporter gene. A mutant reporter (Luc-TGF β R1-mut; in which the first six nucleotides in the miR-141 seed region were changed; Fig. 3A), as well as a negative control reporter (Luc-Ctrl) were also constructed. Luc-TGF β R1-wt, Luc-TGF β R1-mut, or Luc-Ctrl was transfected into Huh7 cells overexpressing miR-141. Luciferase assays revealed that the luciferase activity decreased significantly only in miR-141-overexpressing Huh7 cells that were transfected with Luc-TGF β R1-wt (Fig. 3B). However, no effect was observed following transfection with Luc-TGF β R1-mut (Fig. 3B).

To further investigate whether miR-141 reduced endogenous TGF β R1, the expression of TGF β R1 was detected in various cell lines by western blotting. The results demonstrated that the protein expression levels of TGF β R1 were higher in Huh7 cells compared with the other cell lines (Fig. 3C), and appeared to be inversely correlated with the expression levels



Figure 3. miR-141 downregulates TGF β R1 by directly targeting its 3'UTR in HCC cells. (A) The putative miR-141-binding site in the TGF β R1 3'UTR was predicted using TargetScan and MiRanda. The sequence that was changed to produce the mutant 3'UTR is shown in boxes. (B) Luciferase activity assays were performed after Huh7-NC and Huh7-141 cells were transfected with wt or mutant TGF β R1 3'UTR -containing reporter plasmids. (C) Protein expression of TGF β R1 in LO2 cells and HCC cell lines. (D) Protein expression of TGF β R1 in Huh7-141 cells compared with control cells. β -actin was used as internal control. *P<0.05 with comparisons indicated by lines. HCC, hepatocellular carcinoma; TGF β R, transforming growth factor β receptor 1; UTR, untranslated region; NC, negative control; wt, wild-type; mut, mutant.

of miR-141 (Fig. 1B). Additionally, overexpression of miR-141 in Huh7 cells significantly reduced the protein expression levels of TGF β R1 protein (Fig. 3D). These data demonstrated that miR-141 specifically bound to the 3'UTR of wt TGF β R1 (but not the mutant), and reduced the expression of TGF β R1. Therefore, the present results indicated that TGF β R1 was a specific target of miR-141.

Upregulation of $TGF\beta R1$ was inversely correlated with miR-141 expression in vivo. Because miR-141 was downregulated in HCC patient samples and because miR-141 was demonstrated to specifically target TGFBR1, we next investigated whether the expression of TGF β R1 was negatively associated with miR-141 levels in patient tissues. The mRNA expression levels of TGFβR1 were determined by RT-qPCR in the 20 pairs of HCC tissues and matched adjacent non-tumor tissues collected in the present study. The results demonstrated that TGFβR1 mRNA expression was significantly higher in HCC tissues compared with adjacent non-tumor tissues (Fig. 4A; P=0.027). In addition, western blotting revealed that the protein levels of TGF β R1 in HCC were significantly higher compared with adjacent non-tumor tissues (Fig. 4B). In Fig. 1A, miR-141 expression was demonstrated to be downregulated in HCC tissues. Indeed, the expression of miR-141 was significantly inversely correlated with TGF\u00b3R1 expression (Fig. 4C; P=0.048, R2=0.21). Taken together, these findings suggested that TGF β R1 was a functional target of miR-141 in HCC.

Knockdown of TGF β R1 has similar effects to miR-141 overexpression in vitro. To further investigate the specificity of miR-141 and TGF β R1, TGF β R1 expression was silenced using siRNA, and the effects were compared to the effects of miR-141 overexpression. Similar to the effects of miR-141 overexpression (Fig. 5A), western blotting results demonstrated that TGF β R1 siRNA transfection clearly reduced the expression of TGF β R1 and decreased the phosphorylation of its downstream protein Smad2 (Fig. 5B). MTT assays revealed that TGF β R1 knockdown significantly inhibited Huh7 cell growth (Fig. 5C), while cell cycle analysis revealed that TGF β R1 knockdown reduced cell cycle arrest at the G2/M phase by 5.35%, and increased the ratio of cells entering the S phase by 7.66% (Fig. 5D; P<0.05). Finally, silencing of TGF β R1 significantly impaired migration and invasion in Huh7 cells (Fig. 5E). These results further confirmed that TGF β R1 was a key factor involved in miR-141-induced suppression of HCC cell proliferation, migration, and invasion, by blocking TGF β signaling.

Inhibition of miR-141 promotes HCC cell proliferation, migration and invasion in vitro. A rescue experiment was performed, and the results revealed that inhibition of miR-141 restored HCC proliferation, migration, and invasion, as well as the expression of TGF_βR1 and p-Smad2 (Fig. 6). First, RT-qPCR results confirmed that specific siRNA against miR-141 (si-miR-141) significantly inhibited the expression of miR-141 in the stable Huh-141 cells (Fig. 6A). Silencing miR-141 in Huh7-141 cells increased the expression of TGFBR1 and the p-Smad2/Smad2 ratio (Fig. 6B). Additionally, MTT assays revealed that inhibition of miR-141 enhanced HCC cell growth. The viability of Huh7-141 cells transfected with si-miR-141 increased by 49.09% on day 5 compared with the cells transfected with si-miR-NC (Fig. 6C). Cell cycle analysis indicated that silencing of miR-141 increased the number of cells in G2/M phase by 3.55% (Fig. 6D). Finally, Transwell assays revealed that silencing of miR-141 restored HCC cell migration and invasion capacity (Fig. 6E). These data suggested that inhibition of miR-141 promoted HCC cell proliferation, migration, and invasion.

Discussion

Metastasis is a complex, multistep process through which primary tumor cells invade adjacent tissues and proliferate from microscopic growth to macroscopic secondary



Figure 4. TGF β R1 is upregulated in HCC tissues. (A) The expression levels of TGF β R1 were determined by RT-qPCR and (B) western blotting in tumor tissue samples and adjacent non-tumor liver tissue samples (n=19). GAPDH and β -actin were used as internal controls for RT-qPCR and western blotting, respectively. (C) Pearson's correlation analysis between the relative levels of miR-141 and TGF β R1 in HCC tissue samples. *P<0.05 with comparisons indicated by lines. TGF β R, transforming growth factor β receptor 1; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; N, normal; T, tumor.



Figure 5. Knockdown of TGF β R1 has similar effects to miR-141 overexpression. (A) TGF β R1 and downstream Smad2 and p-Smad2 protein expression levels were determined by western blotting following miR-141 overexpression, and (B) following TGF β R1 silencing by siRNA. The grayscale value ratio of p-Smad2 to total Smad2 is displayed in the graph inserts. (C) At 24-h intervals, MTT assays were performed to measure the viability of the control and TGF β R1-knockdown Huh7 cells (Huh7-si-NC and Huh7-si-TGF β R1, respectively). (D) Analysis of the cell cycle was performed via flow cytometry. (E) Transwell migration and invasion assays of the control and TGF β R1-knockdown Huh7 cells. *P<0.05 and ***P<0.001 with comparisons indicated by lines. TGF β R, transforming growth factor β receptor 1; Smad2, SMAD family member 2; p-, phosphorylated; si, small interfering; NC, negative control; OD, optical density.



Figure 6. Inhibition of miR-141 restored the effects induced by overexpression of miR-141. (A) RT-qPCR confirmed the successful silencing of miR-141 via transfection with a specific siRNA in the stable Huh7-141 cells. (B) Expression of TGF β R1, Smad2 and p-Smad2 were examined by western blotting following inhibition of miR-141 expression. The grayscale value ratio of p-Smad2 to total Smad2 is displayed in the graph insert. (C) MTT assays were utilized to measure the growth of Huh7-141 cells transfected with specific siRNAs against miR-141 (si-miR-141) or the negative control (si-miR-NC). (D) Flow cytometry was used to determine the cell cycle distribution. (E) Transwell assays were used to measure migration and invasion. ***P<0.001 with comparisons indicated by lines. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, small interfering; TGF β R, transforming growth factor β receptor 1; Smad2, SMAD family member 2; p-, phosphorylated; NC, negative control; OD, optical density.

tumors (31). Many studies have described numerous genes and gene products that can drive the metastatic process. In addition to alterations in protein-encoding genes, a class of non-coding small RNAs also contributes to cancer metastasis. For example, miRNAs can lead to silencing of their cognate target genes by cleaving mRNA molecules or by inhibiting their translation (32). Emerging evidence has suggested that miRNAs regulate diverse cellular pathways. Although miRNAs can function as oncogenes or tumor suppressors, the roles of miRNAs in mediating cancer metastasis remain unclear.

Hepatocarcinogenesis is a highly complicated process due to changes in signaling induced by mutation of tumor suppressors, chromosomal amplifications and deletions, and epigenetic alterations (33). Signaling cascades related to HCC proliferation and metastasis are activated as a result. Consequently, miRNAs may to some extent regulate the expression of key oncogenes (34). The altered expression of miRNA profiling is common in HCC; thus, research on these small RNAs has become a hotspot in attempts to control the crucial steps of cancer progression (35,36).

A previous study from our group demonstrated that miR-141 suppresses proliferation and metastasis in RCC *in vitro* and *in vivo* (16). The miR-200 family, including miR-141, miR-200c, miR-200b, miR-200a and miR-429, has been reported to be downregulated in HCC (22-24) and other tumor types (16,37,38). Although the importance of miR-141 in HCC development has previously been highlighted, studies

focusing on its functions remain limited. Therefore, based on our previous discovery that miR-141 downregulation is associated with RCC metastasis (16), the present study selected to examine the functions of miR-141 in HCC progression.

In accordance with previous reports (22-24), the present results demonstrated the downregulation of miR-141 in HCC tumor tissues compared with matched adjacent non-tumor tissues. The present *in vitro* results suggested that enhanced miR-141 expression markedly suppressed HCC cell migration and invasion, blocked cell growth, and arrested cells at the G2/M phase. Both previous reports and our current findings implied that miR-141 was involved in HCC progression and may serve as a negative regulator of HCC metastasis. The present study, however, has several limitations. First, only one cell line was used *in vitro* for functional experiments. Second, a relatively small number of tissues was used to confirm the protein expressions in patients. Further studies will be required in the future to confirm these results in additional cell lines and larger patient cohorts.

Of note, the present study identified TGF\u00b3R1, an important receptor in the TGF^β signaling pathway, as a novel direct target of miR-141. TGF^β has emerged as a potent driver of multiple aspects of cancer progression, particularly induction of the epithelial-mesenchymal transition. Several reports have indicated that TGFB acts as a tumor suppressor by inhibiting epithelial cell proliferation. However, alterations in TGF^β signaling components could inactivate the tumor-suppressive abilities of TGF β and thus enable cancer cells to survive, escape the primary tumor, and form metastases, via the TGFB signaling pathway (39). TGF β signals through type I and type II receptors to form a heterotetrameric receptor complex. TGFBR1, one of the type II receptors of the TGF β ligand, is a transmembrane receptor serine-threonine kinase. Using computational prediction, TGF β R1 was identified as a potential target of miR-141. The present in vitro findings confirmed that the negative effects induced by miR-141 were significantly associated with the expression of TGF\u00b3R1 mRNA and protein. Loss-of-function assays also demonstrated that silencing TGFBR1 by siRNA dramatically suppressed the migration and invasion of Huh7 cells, in a manner similar to the miR-141 overexpression. A recent study reported that miR-140-5p suppressed HCC cell growth and metastasis by targeting TGF β R1 (40), indicating that TGFβR1 was likely an important modulator of HCC progression and that it could be simultaneously regulated by multiple miRNAs, such as miR-141 and miR-140-5p. Taken together, these results suggested that the downregulation of TGFβR1 may be a mechanism through which miR-141 modulates the growth and metastasis of HCC cells.

Three other important downstream proteins, including matrix metalloproteinases (MMPs), Jagged1, and focal adhesion kinase (FAK), have been reported to be activated by the TGF β signaling pathway. MMPs, particularly MMP2 and MMP9, are prominent enzymes in the degradation of the basement membrane and extracellular matrix (41) and can be enhanced by the TGF β signaling pathway (42,43). Additionally, MMP9 has been identified as a major contributor and a crucial factor in tumor-induced angiogenesis, due to its effects on induction of quiescent vasculature formation (44). Preliminary experiments from our group demonstrated that, although neither MMP2 nor MMP9 were predicted as

potential targets of miR-141, a significant change in their expression was associated with miR-141 (data not shown). Further studies will be needed to elucidate the underlying mechanisms through which miR-141 may regulate MMPs. Jagged1 and FAK are also activated by the TGF β /Smad pathway (45-47). Additionally, FAK has been demonstrated to be a target gene of miR-151, which promotes HCC cell migration and invasion (46). Taken together, these findings suggested that miR-141 may prevent HCC cell metastasis and invasion by attenuating the TGF β /Smad/MMP/Jagged1/FAK signaling cascades.

Collectively, the current study indicated that miR-141 partially inhibited HCC proliferation and invasion by directly targeting TGF β R1 and blocking the TGF β signaling pathway. However, the regulatory network of miRNAs involved in HCC is complex; the present study on the miR-141/TGF β R1 pathway provides only preliminary information on the overall signaling networks affecting HCC progression. Notably, one single miRNA may target multiple genes and thus control multiple signaling pathways. Nonetheless, the present study of miR-141 and TGF β R1 improved the current understanding of the mechanisms underlying HCC development and suggested that miR-141 may represent a novel therapeutic target for the treatment of HCC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YZ, ZX, JZ, HY contributed to the conception and design of the study. YZ, ZX, JZ contributed to the acquisition, analysis, and interpretation of the data. YZ drafted the manuscript. HY revised and approved the final version of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the work ensuring integrity and accuracy.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tongji Medical College and informed consent was obtained from all patients prior to enrollment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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