miR-29b suppresses proliferation and induces apoptosis of hepatocellular carcinoma ascites H22 cells via regulating TGF-β1 and p53 signaling pathway

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Abstract. MicroRNA (miR)-29b is a key tumor regulator. It can inhibit tumor cell proliferation, induce apoptosis, suppress tumor invasion and migration, thus delaying tumor progression. Our previous studies revealed an increased level of miR-29b in hepatoma 22 (H22) cells in ascites tumor-bearing mice. The present study investigated the effect of miR-29b on proliferation and apoptosis of hepatocellular carcinoma ascites H22 cells and its association with the transforming growth factor-β1 (TGF-β1) signaling pathway and p53-mediated apoptotic pathway. Briefly, H22 cells were transfected with miR-29b-3p (hereinafter referred to as miR-29b) mimic or miR-29b inhibitor. MTS cell proliferation assay and flow cytometry were used to analyze cell viability and apoptosis. The expression change of the TGF-β1 signaling pathway and p53-mediated apoptotic pathway were detected by reverse transcription-quantitative PCR, western blotting and immuno-fluorescence. Furthermore, cells were treated with exogenous TGF-β1 and TGF-β1 small interfering RNA to evaluate the crosstalk between TGF-β1 and p53 under miR-29b regulation. The overexpression of miR-29b decreased cell viability, increased cell apoptosis, activated the TGF-β1 signaling pathway and p53-mediated apoptotic pathway. Conversely, these effects were reversed by the miR-29b inhibitor. Moreover, the effect of miR-29b mimic was further increased after treating cells with exogenous TGF-β1. The activation of the TGF-β1 signaling pathway and p53-mediated apoptotic pathway induced by miR-29b overexpression were reversed by TGF-β1 inhibition. In summary, these data indicated that miR-29b has an important role in proliferation and apoptosis of H22 cells by regulating the TGF-β1 signaling pathway, the p53-dependent apoptotic pathway, and the crosstalk between TGF-β1 and p53.

Introduction

Hepatic carcinoma is the sixth most common cancer (4.7% of all registered cancer cases) and the fourth leading cause of cancer-related mortality (8.2% of the all cancer-related deaths) worldwide according to findings from 2018 (1). Due to the absence of typical symptoms in the early stage, most patients are diagnosed at an advanced stage, thus having a poor prognosis (2). Uncured hepatic carcinoma in terminal stages is often combined with severe ascites (3). Understanding the molecular mechanisms of hepatic carcinoma is vital for identifying new therapeutic targets.

MicroRNAs (miRNAs or miRs) are short-chain (19-25 nucleotides in length) non-coding RNA that have an important role in a variety of human diseases, including cardiovascular disease, immune response and cancer (4,5). miR-29b is a key tumor regulator (6) that can inhibit tumor cell viability, prompt cell apoptosis, suppress tumor invasion and migration and delay tumor progression (7,8). miR-29b frequently acts as a tumor suppressor and is suppressed in multiple tumors such as non-small cell lung cancer, hepatocellular carcinoma and colorectal cancer (9-11). Our previous studies revealed an increased level of miR-29b in hepatoma 22 (H22) cells in ascites tumor-bearing mice treated with supercritical-carbon dioxide fluid or scutellarin administration. The treatment further decreased ascites and improved survival (12,13).

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Numerous malignant tumor mediators have been associated with miR-29b. The protein p53 is a key cell division checkpoint-monitoring DNA mutant (14). The function of p53 in cancer is usually impaired or mutated, which enables abnormal cell proliferation (15). Our previous studies reported that p53 may induce apoptosis in H22 cells (13,16). Additionally, both miR-29b and p53 had a consistent trend in H22 ascites, in accordance with cell apoptotic degree (12). Numerous studies have indicated a regulatory circuitry between miR-29b and p53 (10,17). miR-29b can reduce the levels of the SET domain bifurcated histone lysine methyltransferase 1 (SETDB1) and ultimately upregulate p53. Similarly, p53 can also increase the expression of miR-29b, thus forming an antitumor circle (10). However, to date, to the best of our knowledge, no studies have reported the regulation between p53 and miR-29b of malignant ascites.

Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine that regulates cell growth, differentiation, migration and apoptosis (18). It is another malignant tumor mediator associated with miR-29b (19). TGF-β1 signaling is a double-edged sword in the regulation of cancer (20). In the initial stages of cancer, TGF-β1 often has an antitumor effect, inhibiting cell proliferation, cell cycle arrest in G1 and inducing apoptosis (21). However, in advanced tumors, deletion or mutation of TGF-β1 or Smads has been revealed (22,23). When mutated, TGF-β1 loses its antitumor function and enhances tumor cell epithelial-mesenchymal transition (EMT), leading to invasion and metastasis (24,25). Interestingly, TGF-β1 can improve p53 expression in vascular senescence and apoptosis of hepatic stellate cells (26,27). Moreover, a previous study has demonstrated a crosstalk between TGF-β1 and p53 (28).

The aim of this study was to investigate the effect of miR-29b on proliferation and apoptosis of hepatocellular carcinoma ascites H22 cells and its association with the TGF-β1 signaling pathway and p53-mediated apoptotic pathway. The crosstalk between TGF-β1 and p53 in hepatocellular carcinoma ascites H22 cells was also explored. The findings of the present study provided further understanding of molecular mechanisms of hepatic carcinoma.

Materials and methods

Materials. miR-29b-3p (hereinafter referred to as miR-29b) mimic [sense strand (5' to 3'): UAGCACCAUUUGAAAUCAGUGUU and antisense strand (3' to 5'): AUCGUGGUAAACUUUAGUCACAA; product no. miR10000127-1-5], mimic negative control (product no. miR1N0000002-1-5), miR-29b inhibitor (5' to 3': AUCGUGGUAAACUUUAGUCACAA; product no. miR20000127-1-5), inhibitor negative control (product no. miR2N0000002-1-5), TGF-β1 small interfering RNA (siRNA) (siTGF-β1, 5' to 3': CCAGAAATATAGCAACAAT), and control siRNA (product no. siP0000003-1-5) were purchased from Guangzhou RiboBio Co., Ltd. Recombinant human TGF-β1 protein was obtained from PeproTech, Inc. Recombinant human TGF-β1 was dissolved in citric acid provided by Multi Sciences (Lianke) Biotech, Co., Ltd. TGF-β1 negative control (TGF-β1 NC) was 1 mM citric acid which was the solvent of the recombinant human TGF-β1. TGF-β1 has cross-reactivity towards mouse according to the manufacturer's instructions. Lipofectamine™ RNAiMAX Transfection Reagent was provided by Thermo Fisher Scientific, Inc.

MTS CellTiter 96 AQueous one solution cell proliferation assay solution was acquired from Promega Corporation. Annexin V-FITC/PI double staining apoptosis detection kit was supplied by BestBio Biotechnology Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, Opti-MEM™ I reduced serum medium (Opti-MEM), fetal bovine serum (FBS), penicillin/streptomycin solution and TRIzol reagent were obtained from Thermo Fisher Scientific, Inc. Reverse transcription primers were designed by Sangon Biotech Co., Ltd. Specific antibodies for TGF-β1 (cat. no. AF1027), p53 (cat. no. AF0865), phosphorylated (p)-Smad3 (cat. no. AF3362), Smad7 (cat. no. AF5147), B-cell lymphoma-2 (Bcl-2) (cat. no. AF6139), Bcl-2-Associated X protein (Bax) (cat. no. AF0120), and β-actin (cat. no. AF7018) were obtained from Affinity Biosciences Pty Ltd. RIPA lysis buffer, phenylmethylsulfonyl fluoride (PMSF), cocktail protease inhibitor, and phosphatase inhibitors A and B were purchased from Servicebio Technology Co., Ltd. Horseradish peroxidase (HRP) and goat anti-rabbit immunoglobulin G (H+L) (cat. no. E030120-01) were obtained from EarthOx Life Sciences. Fluorescein isothiocyanate (FITC; product no. GB22301), Cyanine3 (CY3; product no. GB21303) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Servicebio Technology Co., Ltd. Chloroform, isopropanol and anhydrous ethanol were at least of the analytic grade.

Cell culture. NCTC1469, Hepa1-6 and H22 cells were all provided by iCell Bioscience, Inc. NCTC1469 and Hepa1-6 were cultured in DMEM medium, while H22 was cultured in RPMI-1640 medium containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

RNA and protein extraction. NCTC1469, Hepa1-6, and H22 cells were seeded in 6-well plates at a concentration of $2x10^5$ cells/ml at 2 ml per well and cultured for 48 h. TRIzol reagent and chloroform (15,000 x g, at 4°C for 15 min), isopropanol (12,000 x g, at 4°C for 5 min) and anhydrous ethanol (7,500 x g, at 4°C for 5 min) were utilized to extract and purify total RNA. Cell disruption reagent containing RIPA, PMSF, cocktail and phosphatase inhibitors A and B at ratios of 100:1:2:1:1 was used to extract the total protein (14,000 x g, at 4°C for 10 min).

Transfection and interference in H22 cells. H22 cells were diluted with RPMI-1640 and seeded at a concentration of $2x10^5$ cells/ml in 96-well or 6-well plates at a volume of 100 µl or 1 ml per well, respectively. Cells were first starved for 12 h. Lipofectamine™ RNAiMAX Transfection Reagent was used for transfection according to the manufacturer's instructions; specific steps were as follows: Opti-MEM containing transfection reagent (at a ratio of 50:3) or gene sequence complex (at a ratio of 50:0.5) (10 nM) was prepared. Transfection concentrations of miR-29b mimic, mimic negative control, miR-29b inhibitor, inhibitor negative control, siTGF-β1 and control siRNA were 50, 50, 100, 100, 80 and 80 nM, respectively. Opti-MEM-transfection reagent complex was mixed with Opti-MEM-gene sequence complex and incubated for 5 min at room temperature. The complex was then added to H22 cells (96- or 6-well plate) at 10 or 100 µl/well for 48 h. After 6 h of transfection at 37°C, 10 ng/ml recombinant human TGF-β1 was added to the wells, and cells were cultured

Table I. Primer sequences used for reverse transcription-quantitative qPCR.

Gene name		Primer (5'-3')
U6	RT-primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAAT
	Forward	AGAGAAGATTAGCATGGCCCCTG
	Reverse	AGTGCAGGGTCCGAGGTATT
miR-29b	RT-primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACACT
	Forward	CGCGTAGCACCATTTGAAATC
	Reverse	AGTGCAGGGTCCGAGGTATT
GAPDH	Forward	AATGGTGAAGGTCGGTGTGAACG
	Reverse	TCGCTCCTGGAAGATGGTGATGG
TGF-β1	Forward	GCAGTGGCTGAACCAAGGAGAC
	Reverse	GCCGTGAGCTGTGCAGGTG
Smad3	Forward	CACAGCATGGACGCAGGTTCTC
	Reverse	CACTGAGGCACTCCGCAAAGAC
Smad7	Forward	GCAAGATCGGCTGTGGCATCC
	Reverse	GCTGATGAACTGGCGGGTGTAG
p53	Forward	CCTGGGAGAGACCGCCGTAC
	Reverse	CTAGGCTGGAGGCTGGAGTGAG
Bax	Forward	GCGAGTGTCTCCGGCGAATTG
	Reverse	TGGTGAGCGAGGCGGTGAG
Bcl-2	Forward	CCGTCGTGACTTCGCAGAGATG
	Reverse	GGTGTGCAGATGCCGGTTCAG

RT, reverse transcription; miR, microRNA.

for 48 h in the TGF-β1 interference groups. Cells were harvested for further experimentation.

RNA and protein extraction from H22 cell interference with miR-29b or TGF-β1. H22 cells were transfected with or without miR-29b mimic, mimic negative control, miR-29b inhibitor, or inhibitor negative control. H22 cells were also transfected with miR-29b mimic or mimic negative control, as well as siTGF-β1 or control siRNA. Total RNA and protein were extracted as aforementioned.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA extracted from NCTC1469, Hepa1-6 and H22 cells or from H22 cells with transfection or interference was reversely transcribed to cDNA using the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (product serial no. MR101-01) or HiScript II Q RT SuperMix (product no. R223-01) (both from Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. The cDNA was amplified and quantified using the miRNA Universal SYBR qPCR Master Mix (product no. MQ101-01) or SYBR quantitative polymerase chain reaction (qPCR) Master Mix kit (product no. Q711-02) (Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. The thermocycling conditions were as follows: cDNA was subjected to a temperature of 95˚C for 30 sec (or 5 min for miRNA); 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec; and then 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. Primer sequences are presented in Table I. U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal reference. The gene expression was quantified using the following formulas: $\Delta\Delta Cq = (Cq_{\text{sample (or Hepa1-6 and H22)}} - Cq_{\text{internal reference}}) - (Cq_{\text{control (or NCTC1469)}} - Cq_{\text{internal reference}})$, fold change = $2^{-\Delta\Delta Cq}$ (29).

Cellular viability assay. H22 cells were seeded in 96-well plates and transfected as aforementioned. Then, 20 µl MTS reagent was added to each well for 4 h. The optical density (OD) value at 490 nm was determined using a microplate reader (Thermo Fisher Scientific, Inc.). The cell viability was calculated as follows: [(OD control-OD blank)-(OD transfection-OD blank)]/(OD control-OD blank) x100%. The experiment was conducted in triplicate.

Cell apoptosis. H22 cells were seeded at a concentration of $2x10^5$ cells/ml in 6-well plates and transfected as aforementioned. Annexin V-FITC/PI double staining apoptosis detection kit (cat. no. BB-4101; BestBio Biotechnology Co., Ltd.) was then applied according to the manufacturer's instructions. Specific steps were as follows: 400 µl 1X Annexin V was used to bind the suspension cells. Then, 5 µl Annexin V-FITC was added to stain the cells with incubation for 15 min at 2-8˚C in a dark place. Finally, 10 µl PI was added to stain the cells with incubation for 15 min at 2-8˚C in a dark place. BD LSRFortessa (BD Biosciences) was used to detect the apoptotic rate. FlowJo (version 10.0; BD Biosciences) was used for analysis.

Western blot analysis. The protein content of the samples was determined by bicinchoninic acid (BCA) method. Proteins were
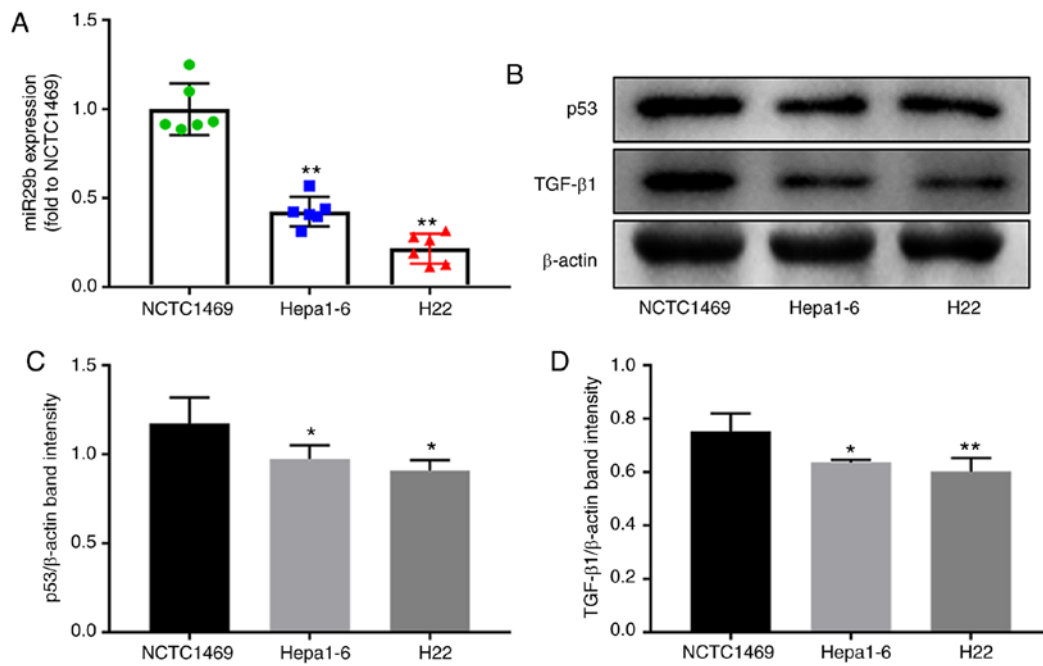
Figure 1. Expression levels of miR-29b, TGF-β1 and p53 in NCTC1469, Hepa1-6 and H22 cells. (A) miR-29b expression. Values represent the means ± SD (n=6). (B) Western blot analysis of TGF-β1 and p53. (C and D) The intensity of TGF-β1 and p53 relative to β-actin. Values represent the means ± SD (n=3). *P<0.05 and **P<0.01 vs. NCTC1469. miR, microRNA; TGF, transforming growth factor; H22, hepatoma 22; SD, standard deviation.

loaded at 50 μg per lane. Proteins collected were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature in 5% skimmed milk diluted in Tris-buffered solution-Tween-20 (0.1%, TBST). Subsequently, samples were incubated with specific primary antibodies (1:2,000 for TGF-β1 and p53; 1:1,000 for p-Smad3, Smad7, Bcl-2, Bax and β-actin) overnight at 4˚C and then with the appropriate secondary antibody (1:3,000 in TBST) for 1 h at room temperature. The protein bands were observed using an enhanced chemiluminescence advanced kit according to the instructions of the manufacturer (product no. RPN2232; GE Healthcare; Cytiva). Quantity one analysis software (version 4.6.2; Bio-Rad Laboratories, Inc.) was used to analyze band intensity.

Immunofluorescence. H22 cells were seeded in 6-well plates and transfected as aforementioned. After being washed 3 times with phosphate-buffered saline (PBS), cells were collected, and 4% paraformaldehyde was added to fix the cells at room temperature for 24 h. H22 cell pellets were obtained using centrifugation at 800 x g for 5 min at room temperature. Samples were then embedded in paraffin and cut into 4-μm sections. The sections were incubated in 5% bovine serum albumin solution (BSA) at room temperature for 30 min, then incubated with specific primary antibodies (1:1,000 for TGF-β1) overnight at 4˚C, and finally incubated with secondary antibody (1:500) conjugated with horseradish peroxidase (HRP) at room temperature for 1 h. Next, sections were stained with FITC-conjugated antibody (1:300) at room temperature for 10 min. Sections were incubated with another primary (1:2,500 for p53) overnight at 4˚C and secondary antibody (HRP-conjugated goat anti-rabbit IgG (H+L); cat. no. GB23303; Servicebio Technology Co., Ltd.; 1:500) for

1 h at room temperature and stained with CY3 antibody (1:300) at room temperature for 10 min. Finally, DAPI (2 μg/ml) was used to stain the cell nucleus at room temperature for 10 min. A fluorescence microscope (Nikon Corporation) was used to observe the intensity of fluorescence at a magnification of x200.

Statistical analysis. Data analysis was performed by SPSS software (version 23.0; IBM Corp.). The significance of different groups was assessed by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post hoc test following ANOVA. P<0.05 was considered to indicate a statistically significant difference. Graphs were drawn by GraphPad Prism software version 7 (GraphPad Software, Inc.).

Results

Expression of miR-29b, TGF-β1 and p53 in NCTC1469, Hepa1-6 and H22 cells. RT-qPCR and western blot analysis were used to evaluate the different expression levels of miR-29b, TGF-β1 and p53 in NCTC1469, Hepa1-6 and H22 cells. As demonstrated in Fig. 1, the expression of miR-29b in Hepa1-6 and H22 was lower compared with NCTC1469 cells (P<0.01). Moreover, lower protein expression levels of TGF-β1 and p53 were revealed in Hepa1-6 and H22 compared with NCTC1469 cells (P<0.05).

miR29b expression with transfection of miR29b mimic and inhibitor in H22 cells. RT-qPCR was applied to detect the miR-29b expression in H22 cells transfected with miR-29b mimic or inhibitor. The results revealed that with the transfection of miR-29b mimic, the expression of miR-29b significantly increased (P<0.01) (Fig. 2A). Concurrently, the expression of miR-29b was significantly downregulated with miR29b inhibitor transfection (P<0.05) (Fig. 2A).
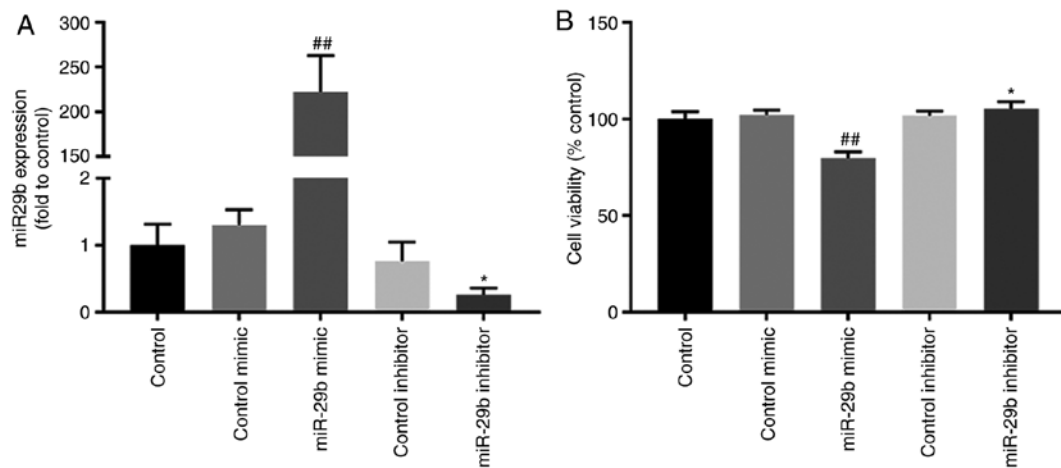
Figure 2. miR-29b expression and cell proliferation after miR29b mimic and inhibitor interference in H22 cells. (A) miR29b expression. (B) Cell viability. Values represent the means ± SD (n=6). ##P<0.01 vs. control mimic; *P<0.05 vs. control inhibitor. miR, microRNA; H22, hepatoma 22; SD, standard deviation.
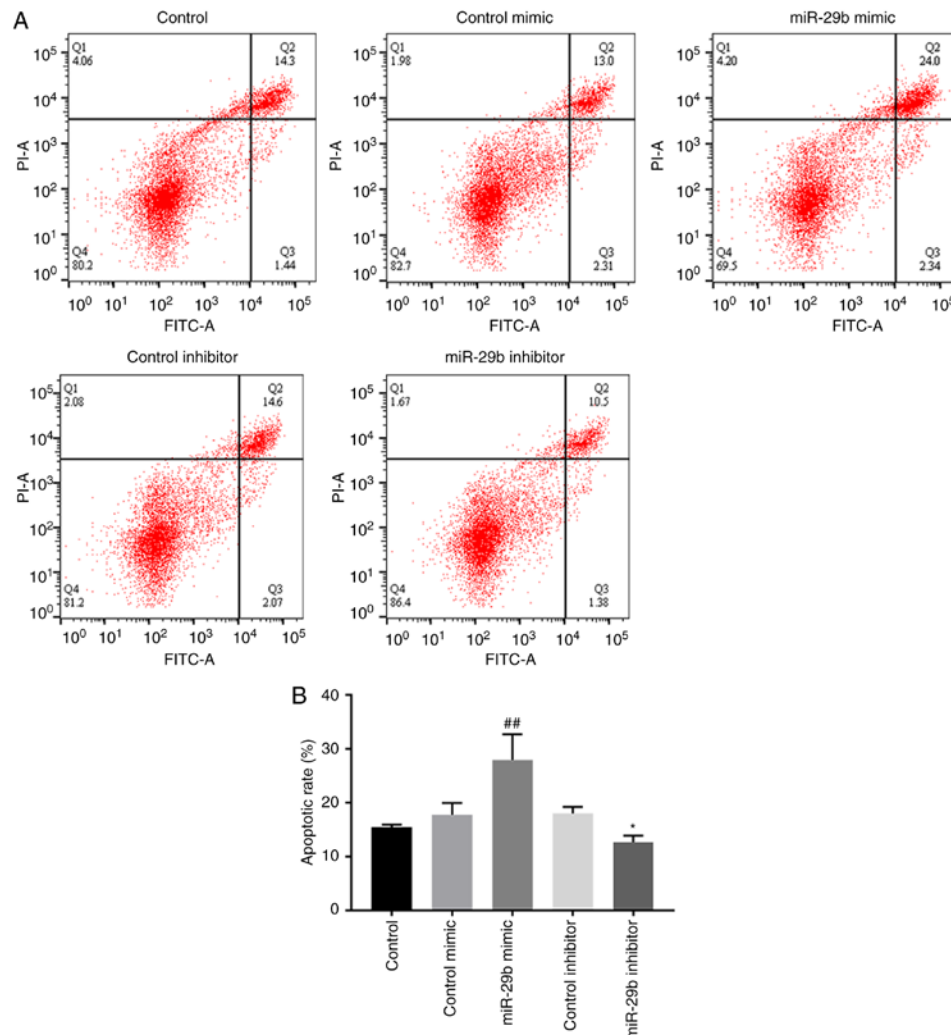

Figure 3. Effect of miR-29b on cell apoptosis in H22 cells. (A) Apoptosis in H22 cells. (B) Apoptotic rate. Values represent the means ± SD (n=3). ##P<0.01 vs. control mimic; *P<0.05 vs. control inhibitor. miR, microRNA; H22, hepatoma 22; SD, standard deviation.

Effect of miR-29b on cell proliferation in H22 cells. To investigate the effect of miR-29b on hepatocellular carcinoma ascites cell proliferation, H22 cells were transfected with miR-29b mimic or miR-29b inhibitor. Transfection with miR-29b

mimic in H22 cells inhibited cell proliferation, compared with the control group (P<0.01) (Fig. 2B). Conversely, miR-29b inhibitor increased cell proliferation compared with the control group (P<0.05) (Fig. 2B).
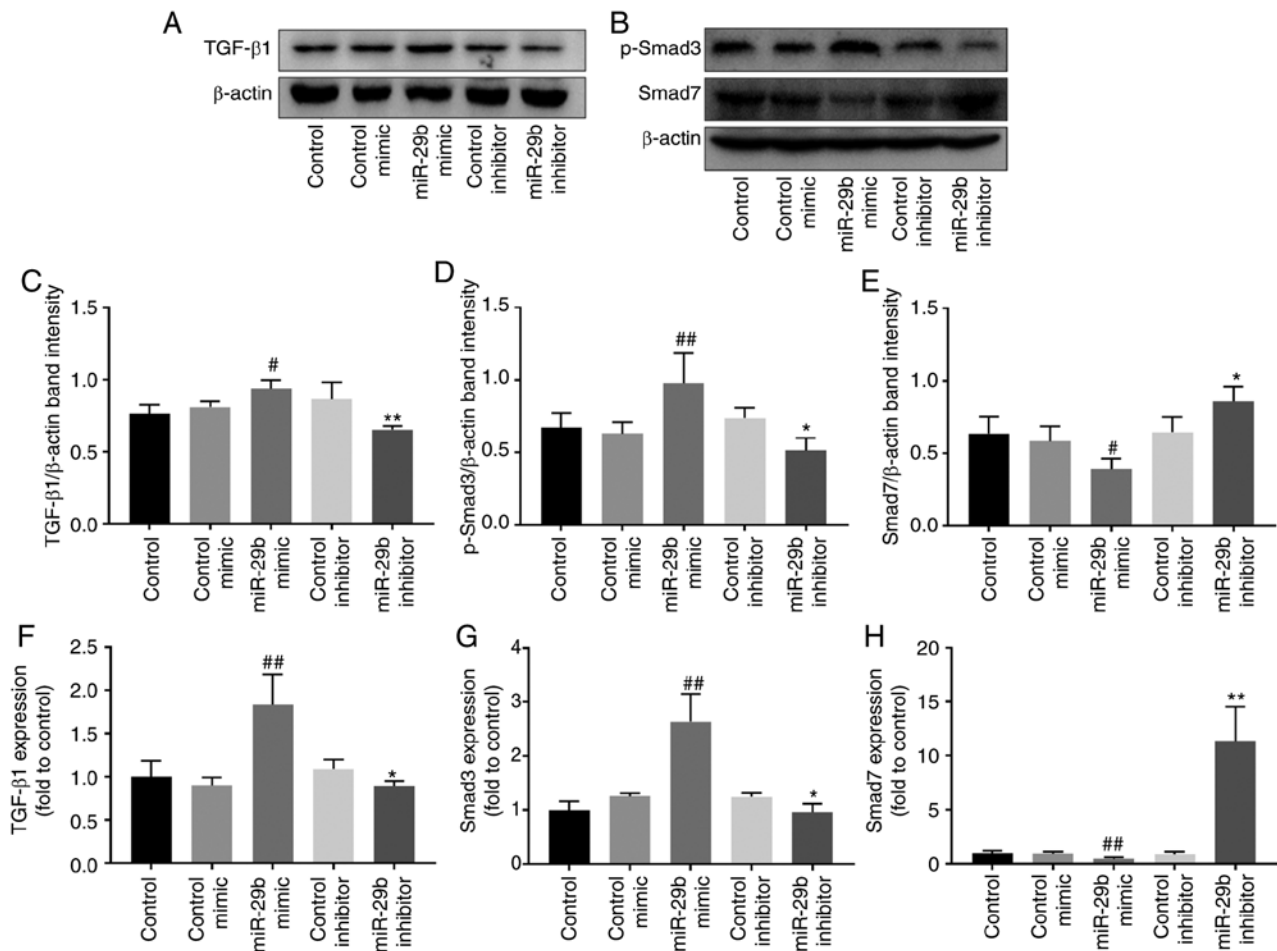
Figure 4. Effect of miR-29b on the TGF-β1 signaling pathway in H22 cells. (A and B) Western blot analysis of TGF-β1, p-Smad3 and Smad7. (C-E) Protein intensities of TGF-β1, p-Smad3 and Smad7. Values represent the means ± SD (n=3). (F-H) mRNA levels of TGF-β1, Smad3 and Smad7. Values represent the means ± SD (n=5). #$P<0.05$ and ##$P<0.01$ vs. control mimic; *$P<0.05$ and **$P<0.01$ vs. control inhibitor. miR, microRNA; TGF, transforming growth factor; SD, standard deviation; p-, phosphorylated.

Effect of miR-29b on cell apoptosis in H22 cells. Flow cytometry was used to verify whether miR-29b had an effect on hepatocellular carcinoma ascites cell apoptosis. As revealed in Fig. 3, miR-29b mimic could significantly increase cell apoptosis of H22 cells compared with the control mimic group ($P<0.01$), while the opposite effect was observed when using miR-29b inhibitor ($P<0.05$).

Effect of miR-29b on the TGF-β1 signaling pathway in H22 cells. Western blotting and RT-qPCR were used to further explore the regulation of miR-29b on the TGF-β1 signaling pathway. As revealed in Fig. 4, when compared with the control mimic group, miR-29b overexpression increased the protein and mRNA levels of TGF-β1 ($P<0.05$ and $P<0.01$, respectively). Concurrently, p-Smad3 protein and Smad3 mRNA levels were increased ($P<0.01$), while Smad7 protein and mRNA levels were decreased ($P<0.05$ and $P<0.01$, respectively). The opposite effects were observed when using miR-29b inhibitor ($P<0.05$ and $P<0.01$).

Effect of miR-29b on p53-mediated apoptotic pathway in H22 cells. Western blotting and RT-qPCR were used to verify the effect of miR-29b on the p53-mediated apoptotic pathway. miR-29b mimic increased the expression p53 protein and mRNA

levels ($P<0.01$), the protein ratio of Bax/Bcl-2 and mRNA level of Bax, compared with the control group ($P<0.05$). Furthermore, it decreased the mRNA level of Bcl-2. Conversely, miR-29b inhibitor induced the opposite effects ($P<0.05$ and $P<0.01$) (Fig. 5).

Effect of TGF-β1 on miR-29b-regulated cell proliferation in H22 cells. Exogenous TGF-β1 and siTGF-β1 were used to investigate whether the effect of miR-29b on H22 cell proliferation inhibition and apoptotic promotion was associated with the TGF-β1 signaling pathway. The results revealed that under miR-29b mimic and exogenous TGF-β1 stimulation, the proliferation of H22 cells was significantly inhibited compared with miR-29b mimic and TGF-β1 NC stimulation group ($P<0.01$). However, in cells transfected with miR-29b mimic and siTGF-β1, the proliferation of H22 cells was significantly increased compared with cells transfected with miR-29b mimic and control siRNA ($P<0.01$) (Fig. 6).

Effects of miR-29b overexpression and TGF-β1 suppression on the TGF-β1 signaling pathway. Transfection of siTGF-β1 in H22 cells reduced the mRNA level of siTGF-β1 by 43.75% compared with the group without any transfection ($P<0.01$). Furthermore, siTGF-β1 transfection could reverse TGF-β1 mRNA increase induced by miR-29b overexpression
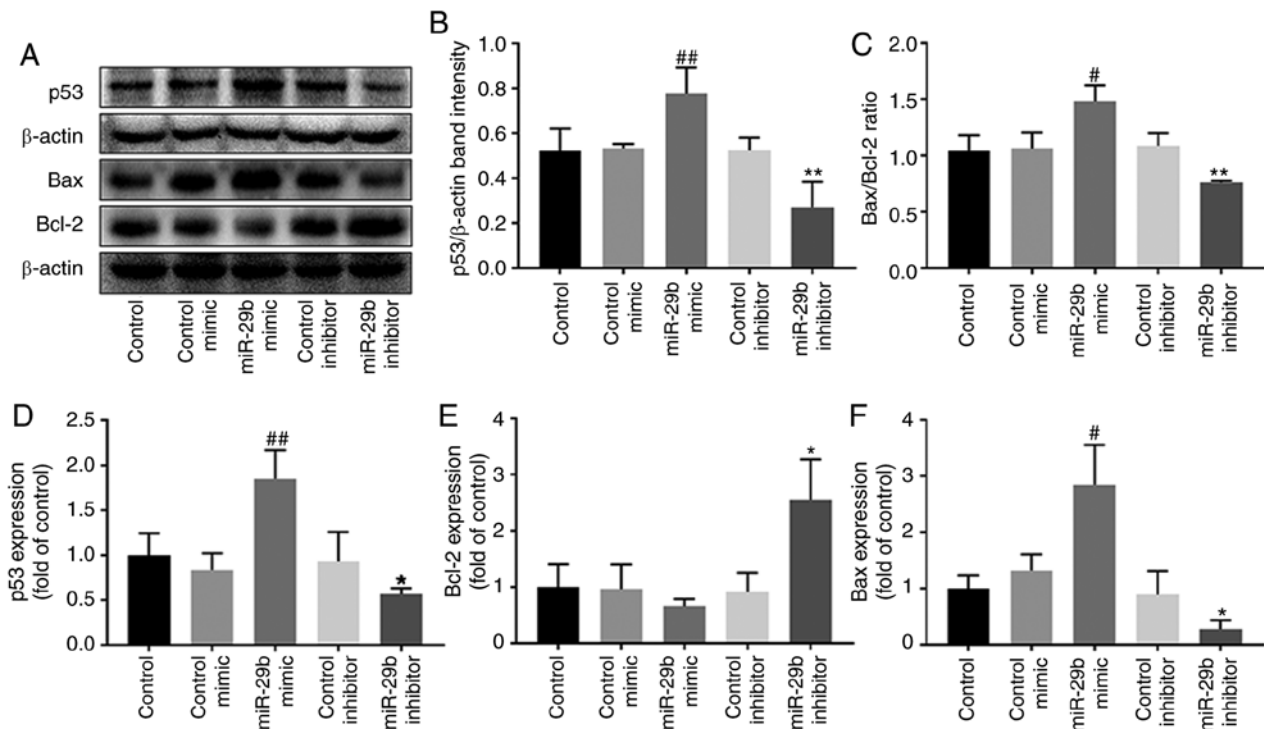
Figure 5. Effect of miR-29b on the p53-mediated apoptotic pathway in H22 cells. (A) Western blot analysis of p53, Bax and Bcl-2. (B) Protein intensity of p53. (C) Protein intensity ratio of Bax/Bcl-2. Values represent the means ± SD (n=3). (D-F) mRNA level of p53, Bax and Bcl-2. Values represent the means ± SD (n=5). [#]P<0.05 and [##]P<0.01 vs. control mimic; [*]P<0.05 and [**]P<0.01 vs. control inhibitor. miR, microRNA; H22, hepatoma 22; SD, standard deviation.
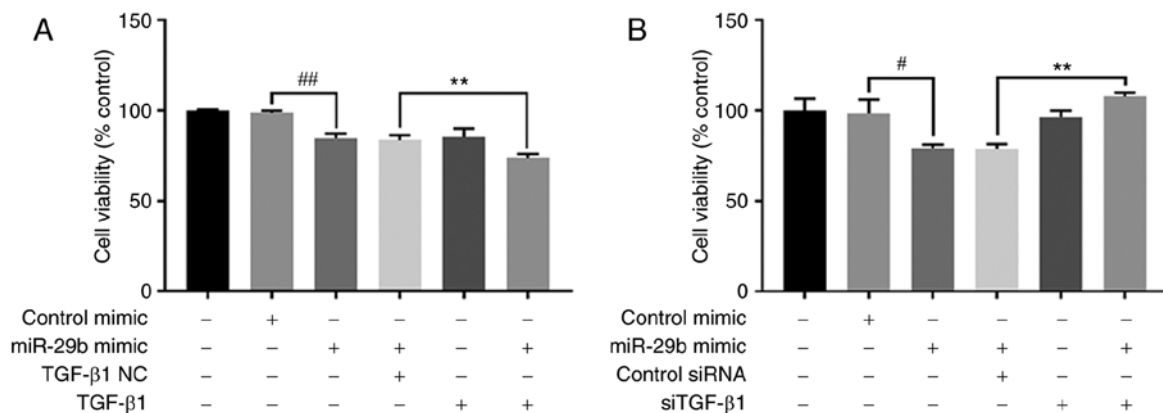

Figure 6. Effect of TGF-β1 on miR-29b-regulated cell proliferation in H22 cells. (A) Cells transfected with miR-29b mimic and treated with exogenous TGF-β1. (B) Cells transfected with miR-29b mimic and siTGF-β1. The control group referred to the group without any treatment. Values represent the means ± SD (n=5). [#]P<0.05 and [##]P<0.01; and [**]P<0.01. TGF, transforming growth factor; miR, microRNA; H22, hepatoma 22; NC, negative control; siTGF-β1, TGF-β1 small interfering RNA; SD, standard deviation.

(P<0.01). Smad3 also increased with the miR-29b overexpression (P<0.01); however, siTGF-β1 significantly reduced this effect (P<0.05). Smad7 mRNA was inhibited by miR-29b overexpression (P<0.01); whereas siTGF-β1 reversed this inhibition (P<0.05) (Fig. 7).

Effects of miR-29b overexpression and TGF-β1 suppression on TGF-β1 and p53 expression. Immunofluorescence was utilized to analyze the interaction between TGF-β1 and p53. The results revealed that inhibition of TGF-β1 could reduce the fluorescence intensity of TGF-β1 and p53. Concurrently, overexpression of miR-29b along with inhibition of TGF-β1 (siTGF-β1) significantly reduced the fluorescence intensity of

TGF-β1 and p53 compared with cells overexpressing miR-29b and treated with control siRNA (Fig. 8).

Effects of miR-29b overexpression and TGF-β1 suppression on the p53-mediated apoptotic pathway. RT-qPCR was used to investigate whether TGF-β1 could impact the expression of p53 and thus jointly regulate the cell growth process downstream of miR-29b in H22 cells. The mRNA levels of p53 and Bax demonstrated a significant downward trend (P<0.01 and P<0.05, respectively), while the levels of Bcl-2 mRNA increased in cells overexpressing miR-29b and treated with TGF-β1 siRNA compared with cells overexpressing miR-29b and treated with control siRNA (P<0.05) (Fig. 9).
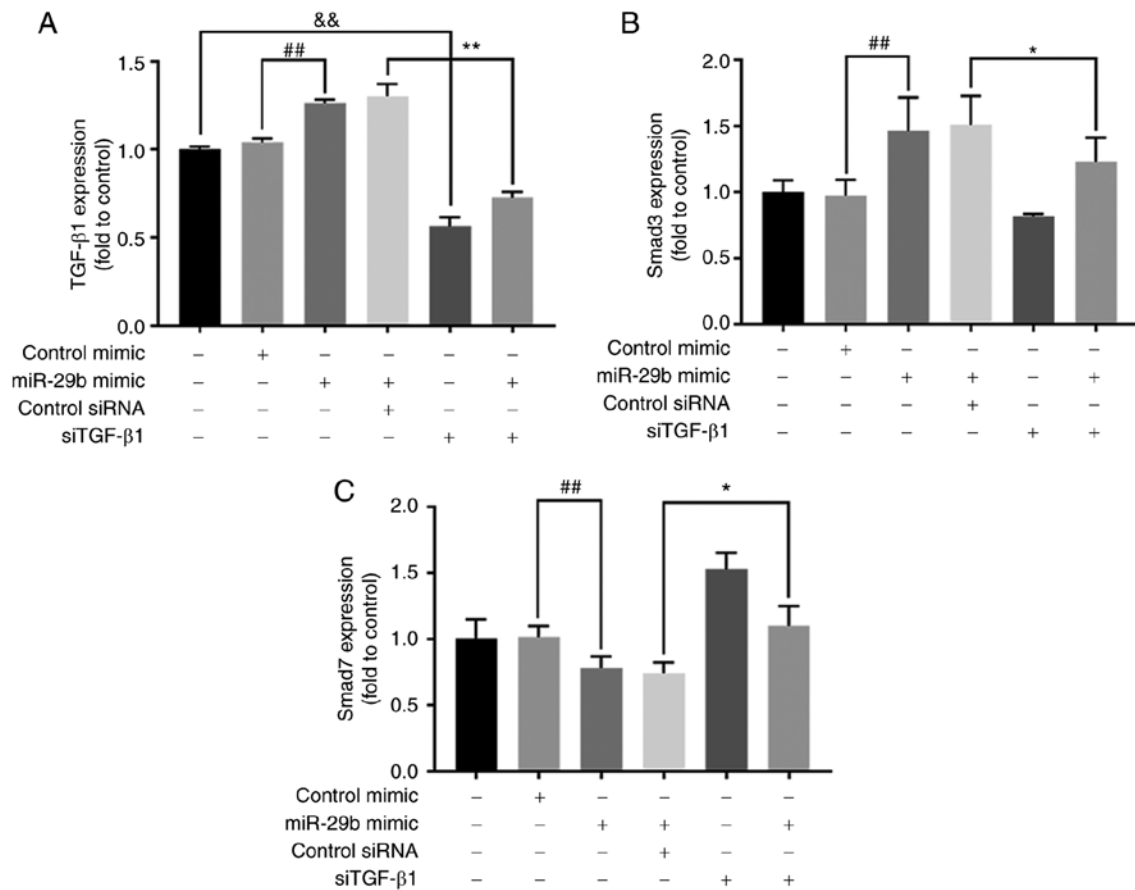
Figure 7. Effects of miR-29b overexpression and TGF-β1 suppression on the TGF-β1 signaling pathway. (A) TGF-β1 mRNA expression. (B) Smad3 mRNA expression. (C) Smad7 mRNA expression. The control group referred to the group without any treatment. Values represent the means ± SD (n=4). [##]$P<0.01$; [*]$P<0.05$ and [**]$P<0.01$; [&&]$P<0.01$. miR, microRNA; TGF, transforming growth factor; siTGF-β1, TGF-β1 small interfering RNA; SD, standard deviation.

Discussion

The protein p53 is considered one of the most popular tumor suppressor factors (14). As a cell cycle checkpoint, p53 can recognize cells with aberrant mutation, arresting the cell cycle, inhibiting cell growth and inducing cell apoptosis (30). Several p53 mutations or deficiencies have been revealed in malignant cells, enabling cancer cells to escape and proliferate (15). TGF-β1 is a pleiotropic cytokine that has been regarded as a double-edged sword in the progression of cancer (20). In the early stage of tumor development, TGF-β1 inhibits aberrant cell proliferation, arrests the cell cycle in G1, and induces apoptosis (21). However, in an advanced-stage tumor, TGF-β1 prompts cancer evolution by enhancing EMT, invasion, and metastasis (24,25).

The effect of TGF-β1 on H22 cells remains to be elucidated. In the present study, the protein expression levels of TGF-β1 and p53 were detected in normal hepatocytes and hepatoma cells. Compared with mouse normal hepatocytes NCTC1469 cells, hepatoma cells Hepa1-6 and H22 cells demonstrated low expression levels of TGF-β1 and p53 proteins. These results indicated that low levels of TGF-β1 and p53 may prompt hepatoma development. Meanwhile, the RT-qPCR results revealed low expression of miR-29b in Hepa1-6 and H22 cells compared with NCTC1469 cells, indicating that miR-29b may act as a hepatoma suppressor.

miRNAs have been considered as diagnostic and prognostic targets for cancer (31). A tumor-suppressing effect of miR-29b has been reported in non-small cell lung cancer, hepatocellular carcinoma and colorectal cancer (9-11). The present study demonstrated that overexpression of miR-29b could decrease cell viability and increase cell apoptosis in H22 cells. Conversely, inhibition of miR-29b caused the opposite effects, indicating that miR-29b is a suppressor in H22 cells. Furthermore, these results were consistent with our previous studies that found an increased level of miR-29b in H22 cells in ascites tumor-bearing mice treated with supercritical-carbon dioxide fluid or scutellarin administration (12,13).

Proliferation and apoptotic disorder have been widely recognized as important mechanisms in the course of a malignant tumor (32). Bcl-2 is the most common anti-apoptotic protein, which can form a heterodimer with the pro-apoptotic protein Bax, block the transmission of apoptosis signals, and promote the survival and growth of the cells. Bax can form dimers on its own and transmit apoptosis signals to the caspase family, eventually inducing cell apoptosis (33). Both Bcl-2 and Bax are regulators of the mitochondrial pathway (33). It is noteworthy that p53 can mediate Bcl-2 and Bax gene transcription to interact with the mitochondrial pathway and regulate apoptosis together (34,35). Moreover, it has been reported that miR-29b can induce apoptosis by directly targeting the prototypical anti-apoptotic molecules Bcl-2 in hepatocellular carcinoma cells or improving the level of Bax (11,36). Our data indicated that miR-29b overexpression could facilitate transcription and translation of p53, transcription of Bax and
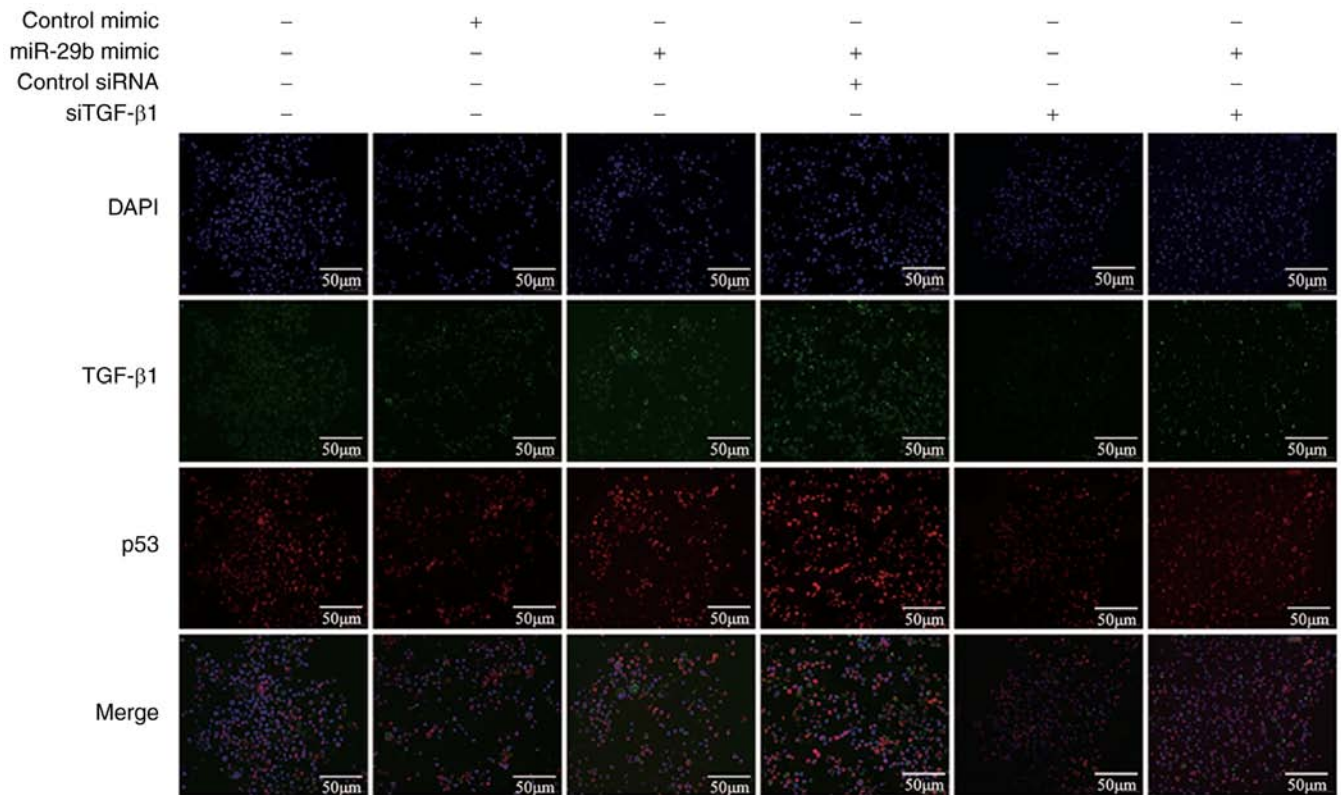
Figure 8. Effects of miR-29b overexpression and TGF-β1 suppression on TGF-β1 and p53 expression. miR, microRNA; TGF, transforming growth factor; siTGF-β1, TGF-β1 small interfering RNA.
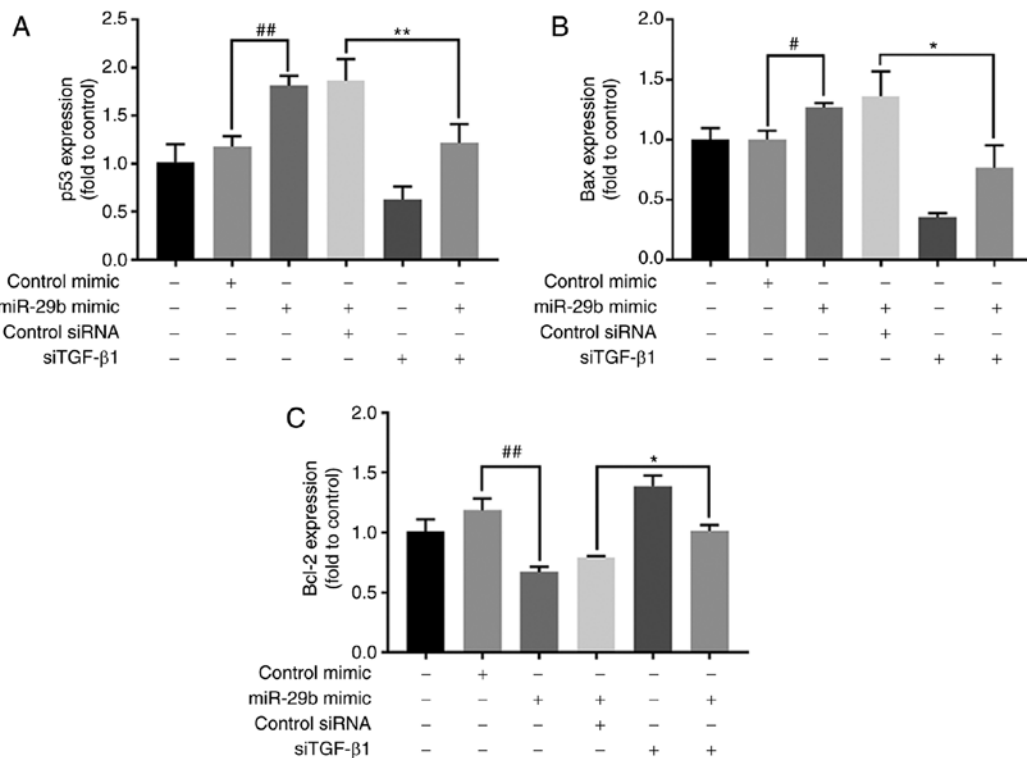

Figure 9. Effects of miR-29b overexpression and TGF-β1 suppression on the p53-mediated apoptotic pathway. mRNA expression of (A) p53, (B) Bax and (C) Bcl-2. The control group referred to the group without any treatment. Values represent the means ± SD (n=4). #P<0.05 and ##P<0.01; *P<0.05 and **P<0.01. miR, microRNA; TGF, transforming growth factor; siTGF-β1, TGF-β1 small interfering RNA; SD, standard deviation.

improve protein ratio of Bax/Bcl-2, while inhibiting the transcription of Bcl-2. An opposite effect was observed when using

miR-29b inhibitor. The results indicated that miR-29b has an important role in the activation of the p53-mediated apoptotic

pathway. Additionally, prompting heterodimer formation between Bax and Bcl-2 may contribute to the activation of miR-29b to the p53-mediated apoptotic pathway.

TGF-β1 is a pleiotropic cytokine regulating cell growth, differentiation, migration and apoptosis (18). The classic TGF-β1 signaling pathway is transduced by the Smad family (37). Smad3 transmits signals by binding to the TGF-β1 receptor from the cytoplasm to the nucleus; Smad7 negatively regulates the TGF-β1 signaling pathway and is considered as a candidate oncogene (38). In the present study, miR-29b overexpression activated the TGF-β1 signal, facilitating the transcription of Smad3 and phosphorylation of Smad3, and inhibiting transcription and translation of Smad7. Thus, it was hypothesized that the effect of miR-29b on cell proliferation and apoptosis in H22 cells may be regulated by the activation of the TGF-β1 signaling pathway and the p53-mediated apoptotic pathway. Furthermore, it was revealed that the inhibition of miR-29b expression could inactivate p53 and TGF-β1 pathways to a certain extent. However, this inactivation effect was modest compared with miR-29b overexpression. Thus, miR-29b over-expression was only focused on in further analysis.

Crosstalk between p53 and the TGF-β1 signaling pathway has been observed in multiple diseases, including cancer (26). TGF-β1 can activate p53, induce the combination between p53 and Smad and further regulate cell progression with TGF-β1 (39). Moreover, TGF-β1 can improve p53 expression in vascular senescence and apoptosis of hepatic stellate cells (24,25). To further determine whether miR-29b regulates H22 cells via the TGF-β1 signaling pathway and the crosstalk between p53 and TGF-β1 in H22 cells, miR-29b was overexpressed and cells were treated with exogenous TGF-β1. The results demonstrated that the cell viability was significantly reduced compared with cells transfected with miR-29b mimic but not treated with exogenous TGF-β1, whereas the proliferation inhibited by miR-29b overexpression was reversed with TGF-β1 inhibition and the expression of a key element of the TGF-β1 signaling pathway. These data indicated that TGF-β1 possessed an antitumor effect. Additionally, the inhibitory effect of miR-29b on H22 cell proliferation may be accomplished by TGF-β1 signaling pathway activation.

Next, the expression of a key element of the p53 regulated apoptotic pathway was further detected. Immunofluorescent results revealed that the p53 protein expression was in agreement with TGF-β1, i.e., both were reduced by siTGF-β1 transfection. Moreover, the improvement of p53 expression by miR-29b overexpression was reversed once the TGF-β1 was inhibited. Furthermore, the mRNA levels of p53 and Bax were decreased, and Bcl-2 was increased by TGF-β1 inhibition even when miR-29b was overexpressed. These data indicated that siTGF-β1 could reverse the activation of TGF-β1 signal pathway which was induced by miR-29b overexpression, as well as the p53-dependent apoptotic pathway. These data also indicated that a crosstalk existed between TGF-β1 and p53 under the regulation of miR-29b. Moreover, it was possible that miR-29b may inhibit H22 cell proliferation by regulating the TGF-β1 signaling pathway, the p53-dependent apoptotic pathway, and the crosstalk between TGF-β1 and p53.

However, there are some limitations in the present study that need to be further assessed. Firstly, all the data in the present study were obtained from H22 cells, miR-29b regulation of TGF-β1 and p53 signaling pathways should be investigated with additional cell lines in a future study. Secondly, as important endogenous regulatory molecules, miRNAs inhibit the translation of genes or directly accelerate mRNA degradation at the post-transcriptional level (40). Nevertheless, in the present study, when miR-29b was overexpressed, the level of TGF-β1 was significantly increased. There is no direct report on how miR29b upregulates TGF-β1 in cancer. Hence, the mechanism of miR29b upregulation of the expression level of TGF-β1 requires thorough investigation in a future study. Thirdly, the invasion assays were carried out to determine the effect of miR29b on H22 cell invasion. However, there was no significant difference between the control group and the miR-29b intervention group (data not shown). The aforementioned finding may be related to the cancer suppressive effect of TGF-β1 in the present study. As a result, further functional experiments also need to be carried out.

In conclusion, as a short-chain non-coding RNA, miR-29b has an important role in H22 cells. It can reduce proliferation and induce apoptosis of H22 cells by regulating the TGF-β1 signaling pathway, the p53-dependent apoptotic pathway and the crosstalk between TGF-β1 and p53. The present study enhanced the functional knowledge of miR-29b and the understanding of hepatic carcinoma molecular mechanisms. It may provide novel insight into possible targets for hepatic carcinoma therapy.

Acknowledgements

Funding

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL, DJ and JNC made substantial contributions to the conception and design of the study. YLL, BYC and JN acquired and interpreted the data of the study. WHY, JNZ, STG analyzed the data of the study. YL, DJ, ZRS drafted the study and revised it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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