

## MicroRNA-142-5p Overexpression Inhibits Cell Growth and Induces Apoptosis by Regulating FOXO in Hepatocellular Carcinoma Cells

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Abnormal expression of microRNA (miR)-142-5p has been reported in hepatocellular carcinoma (HCC). However, little information is available regarding the functional role of miR-142-5p in HCC. We aimed to explore the effects of miR-142-5p aberrant expression on HCC cell growth and cell apoptosis, as well as the underlying mechanism. Human HCC cell lines HepG2 and SMMC-7721 cells were transfected with miR-142-5p mimic, inhibitor, or a corresponding negative control. Cell viability, cell cycle distribution, and cell apoptosis were then analyzed. In addition, protein expression of Forkhead box, class O (FOXO) 1 and 3, a Bcl-2-interacting mediator of cell death (Bim), procaspase 3, and activated caspase 3 was measured. After transfection with miR-142-5p inhibitor, FOXO1 and FOXO3 were overexpressed, and then the cell viability and cell apoptosis were determined again. The relative cell viability in both HepG2 and SMMC-7721 cells was significantly reduced by miR-142-5p overexpression ( $p < 0.05$ ). miR-142-5p overexpression displayed a significant blockage at the G<sub>1</sub>/S transition and significantly increased the percentages of G<sub>0</sub>/G<sub>1</sub> phase. Moreover, the results showed that miR-142-5p overexpression significantly induced cell apoptosis and statistically elevated the protein expression levels of FOXO1, FOXO3, Bim, procaspase 3, and activated caspase 3. However, the cells transfected with miR-142-5p inhibitor showed contrary results. Additionally, the effects of miR-142-5p inhibitor on cell viability and apoptosis were reversed by overexpression of FOXO. In conclusion, our results suggest that miR-142-5p overexpression shows an important protective role in HCC by inhibiting cell growth and inducing apoptosis. These effects might be by regulating FOXO expression in HCC cells.

**Key words: MicroRNA-142-5p; Hepatocellular carcinoma (HCC); Cell growth; Cell apoptosis; Forkhead box, class O (FOXO)**

### INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive tumor with a poor prognosis (1). It has been reported that HCC is now the sixth most common cancer worldwide and the third highest cause of cancer death (2). It is becoming an increasing health threat worldwide, particularly in developing countries. It has been estimated that over 700,000 cases are diagnosed every year (3). Moreover, in most patients, HCC is often diagnosed at an advanced stage because of the absence of specific early symptoms (4). Although tremendous advances have been made in recent years in the treatment of HCC, the 5-year survival rate of HCC still remains poor, ranging from 6.5% to 8.3% (5). It has been acknowledged that tumor cell growth and cell apoptosis are responsible for the

development of malignancies, including HCC. Therefore, there is an urgent need to obtain a better understanding of the underlying mechanisms that inhibit cell growth and induce cell apoptosis for HCC cells.

MicroRNAs (miRNAs) are small (19–23 nucleotides long), single-stranded, noncoding RNAs that modify post-transcriptional gene expression by interaction with target mRNAs (6). miRNAs have been reported to be involved in the development and progression of many cancers by regulating cell proliferation, apoptosis, differentiation, and migration (7,8). Recently, an increasing number of studies have shown the critical roles of miRNAs in HCC (9–11). Among miRNAs, miR-142-5p has been implicated in several human diseases, such as gastric cancer (12), gastric mucosa-associated lymphoid tissue (MALT) lymphoma

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(13), and lung cancer (14). In addition, miR-142-5p was recently found to be downregulated in hepatitis B virus (HBV)-related HCC (10,15,16). However, little information is available regarding the effects of miR-142-5p on HCC cell growth and apoptosis.

Therefore, in the present study, we explored the functional role of miR-142-5p in HCC cell growth and apoptosis, as well as the underlying mechanism. The expression pattern of miR-142-5p was altered, and then cell viability, cell cycle distribution, and cell apoptosis were determined. Furthermore, the expression of Forkhead box, class O (FOXO) 1 and 3, Bim, procaspase 3, and activated caspase 3 was measured to reveal the underlying mechanism of cell growth and apoptosis. Additionally, after transfection with the miR-142-5p inhibitor, the expression of FOXO1 and FOXO3 was overexpressed, and then these effects on cell viability and cell apoptosis were analyzed again. Our results might provide a new insight into a potential therapeutic target for HCC.

## MATERIALS AND METHODS

### *Cell Culture*

Human HCC cell line HepG2 and SMMC-7721 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml penicillin (Gibco BRL, Grand Island, NY, USA), and 100 mg/L streptomycin (Gibco BRL) under 37°C at 5% CO<sub>2</sub>.

### *Cell Transfection*

The miR-142-5p mimic, inhibitor, pcDNA3.1-FOXO1, pcDNA3.1-FOXO3, and corresponding negative controls were designed and generated by GenePharma (Shanghai, P.R. China). Briefly, the cells ( $2 \times 10^5$  cells/well) were plated on 96-well plates. The cells were then transiently transfected with miR-142-5p mimic, inhibitor, pcDNA3.1-FOXO1, pcDNA3.1-FOXO3, or control according to the manufacturer's manual. The transfection was performed by using Lipofectamine 2000 (Invitrogen, USA). After 48 h, the cells were harvested for further analyses.

### *Cell Viability*

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay according to a previously described standardized method (17). In brief, these cells were inoculated into 96-well plates and transfected with miR-142-5p mimic, inhibitor, and the corresponding negative control. After 48 h of transfection, 0.5 mg/ml MTT (Gibco BRL-Life Technologies, Carlsbad, CA, USA) was added to the cells and incubated for 4 h at 37°C. Dimethyl sulfoxide

(DMSO; 100  $\mu$ l; Sigma-Aldrich) was then added to dissolve the formazan crystals. Absorbance at 590 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

### *Apoptosis Assay*

The cell apoptosis was measured by flow cytometry analysis with an Annexin-V/FITC and propidium iodide (PI) apoptosis detection kit (BioVision, USA), according to the manufacturer's standards. Briefly, after 48 h of transfection, the cells were collected and suspended in annexin-binding buffer and exposed to Annexin-V-FITC and PI for 15 min in the dark at room temperature. The apoptotic percentage of cells was quantified by flow cytometry.

### *Cell Cycle Analysis by Flow Cytometry*

Cell cycle analysis was implemented by flow cytometry according to a previous protocol (18). Briefly, the cells were plated in 96-well plates and transfected with miR-142-5p mimic, inhibitor, and the corresponding negative control for 48 h at 37°C. The cells were then harvested, fixed with cold 70% ethanol at 4°C overnight, and washed with phosphate-buffered saline (PBS). Thereafter, the cells were resuspended in PI (50  $\mu$ g/ml) with RNase A (100  $\mu$ g/ml) followed by incubation for 30 min in the dark at room temperature. Flow cytometry was carried out to analyze the percentage of cells in G<sub>1</sub>, S, and G<sub>2</sub>/M phases using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA.)

### *Western Blotting Analysis*

Western blot was carried out to detect the protein levels according to a previous study (19). After transfection with the miR-142-5p mimic, inhibitor, and the corresponding negative control for 48 h, the cells were collected to extract the total protein. The protein concentration was determined using the BCA Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. After that, the protein samples were subjected to a 10%–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Thereafter, the cells were washed three times with PBS and blocked in 5% defatted milk in Tris-buffered saline with Tween (TBST) for 1 h. The membranes were then incubated with the following primary antibodies overnight at 4°C: anti-FOXO1 antibody (Abcam, Cambridge, UK), anti-FOXO3 antibody (Abcam), anti-Bcl-2 interacting mediator of cell death (Bim) antibody (Cell Signaling Technology Inc., Beverly, MA), anti-procaspase 3 antibody (Cell Signaling Technology), and anti-active caspase 3 antibody (Cell Signaling Technology). After being washed three times with TBST, the membranes were

incubated with horseradish peroxidase (HRP)-conjugated antibody for 2 h. GAPDH was used as a loading control. The protein bands were visualized with enhanced chemiluminescence (ECL) (Beyotime, P.R. China).

#### Statistical Analysis

Each experiment was performed in triplicate. The data are expressed as the mean  $\pm$  standard deviation (SD). Statistical analyses were carried out with GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). Significance between groups was analyzed by *t*-tests or one-way analysis of variance (ANOVA) with post hoc test. Statistical significance was defined as a value of  $p < 0.05$ .

## RESULTS

### Overexpression of miR-142-5p Reduces HCC Cell Viability

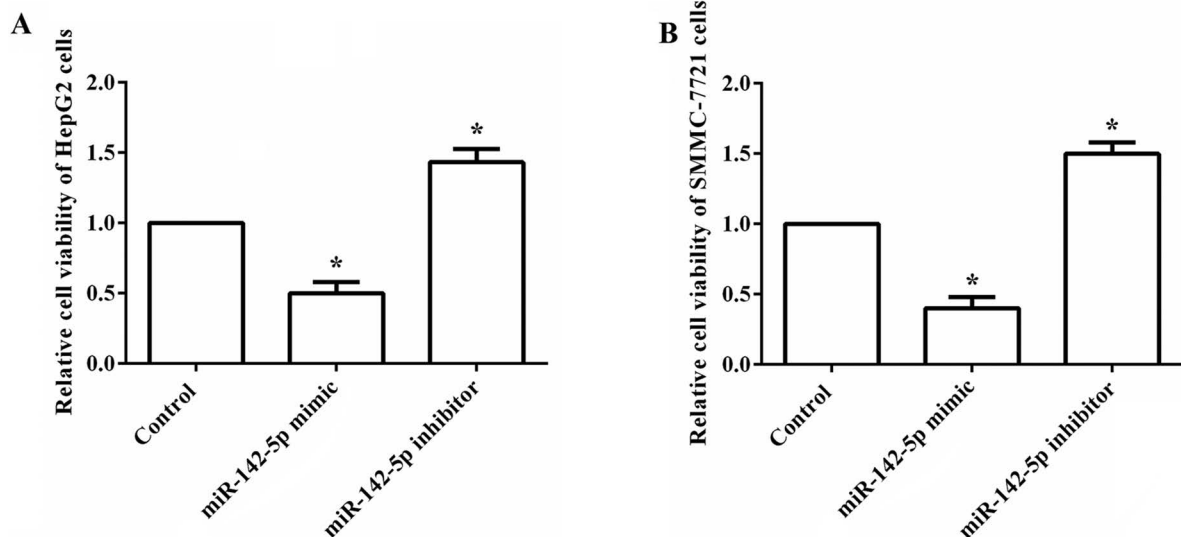
To explore the functional role of miR-142-5p in HCC, we overexpressed and suppressed the expression of miR-142-5p in human HCC cell lines HepG2 and SMMC-7721 cells. The effect of aberrant expression of miR-142-5p on cell viability was then analyzed. As shown in Figure 1A and B, the relative cell viability in both HepG2 and SMMC-7721 cells was significantly decreased by overexpression of miR-142-5p compared to the control group, but was statistically increased by suppression of miR-142-5p (all  $p < 0.05$ ). The results suggested that overexpression of miR-142-5p could reduce HCC cell growth.

### Overexpression of miR-142-5p Induces Cell Cycle Arrest at the $G_0/G_1$ Phase in HCC Cells

We further investigated the effects of miR-142-5p abnormal expression on cell cycle. Flow cytometry assay was performed. As shown in Figure 2A and B, both the HepG2 and SMMC-7721 cells transfected with the miR-142-5p mimic displayed a significant blockage of  $G_1/S$  transition compared to the control group, and there was a distinct increase in the percentages of the  $G_0/G_1$  phase. However, the cells transfected with the miR-142-5p inhibitor showed contrary results. The miR-142-5p inhibitor remarkably decreased the percentages of the  $G_0/G_1$  phase but showed an increase in the  $G_1/S$  transition. The results indicated that overexpression of miR-142-5p induces cell cycle arrest at the  $G_0/G_1$  phase in HCC cells.

### Overexpression of miR-142-5p Induces Cell Apoptosis in HCC Cells

We next assessed the effect of miR-142-5p aberrant expression on the induction of apoptosis in HCC cells. As indicated in Figure 3A and B, the results demonstrated that overexpression of miR-142-5p significantly increased the percentages of apoptotic HepG2 cells compared to the control group, and suppression of miR-142-5p statistically decreased the percentages of apoptotic cells (both  $p < 0.05$ ). The effects of miR-142-5p on cell apoptosis in SMMC-7721 cells were similar with those in HepG2 cells (Fig. 3C and D). The results demonstrated that



**Figure 1.** Overexpression of miR-142-5p reduces HCC cell viability. Human HCC cell lines HepG2 and SMMC-7721 cells were transfected with miR-142-5p mimic, inhibitor, and corresponding negative control, and then cell viability was analyzed. (A) The relative cell viability in HepG2 cells was significantly decreased by miR-142-5p overexpression, but was statistically increased by miR-142-5p suppression. (B) The relative cell viability in SMMC-7721 cells was markedly decreased by miR-142-5p overexpression, but remarkably was increased by miR-142-5p suppression. miR, microRNA. \* $p < 0.05$  compared to the control group.

overexpression of miR-142-5p could induce cell apoptosis in HCC cells.

#### *Overexpression of miR-142-5p Controls Cell Growth and Apoptosis by Regulating FOXO in HepG2 Cells*

To further understand the underlying mechanisms of the effects of miR-142-5p on cell growth and apoptosis, HepG2 cells were transfected with the miR-142-5p mimic or inhibitor, and then the expression of FOXO1, FOXO3, Bim, procaspase 3, and activated caspase 3 was determined by Western blotting. As shown in Figure 4, we found that the expressions of FOXO1, FOXO3, Bim, procaspase 3, and activated caspase 3 were all significantly elevated by miR-142-5p overexpression compared to the control group, but were statistically decreased by miR-142-5p suppression. The results indicated that the effects of miR-142-5p on cell growth and apoptosis might be by regulating the expression of FOXO.

#### *Overexpression of FOXO Reverses the Effects of miR-142-5p Inhibitor on Cell Viability and Cell Apoptosis*

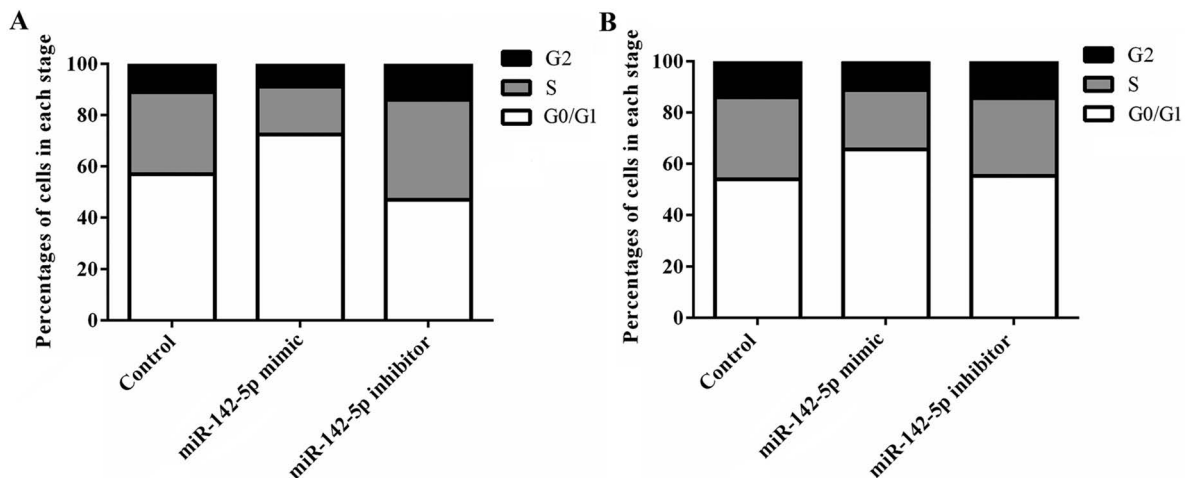
On the basis of the above results, we speculated that overexpression of FOXO might affect the effects of the miR-142-5p inhibitor on cell viability and cell apoptosis. To confirm the results, after transfection with the miR-142-5p inhibitor, the expression of both FOXO1 and FOXO3 was overexpressed by pcDNA3.1. The cell viability and cell apoptosis were analyzed again. As shown in Figure 5A and B, the results showed that cell viability was significantly increased by the miR-142-5p inhibitor in both HepG2 and SMMC-7721 cells (both  $p < 0.05$ ), but these effects were reversed by overexpression of FOXO1

and FOXO3. Similarly, we found that the percentages of apoptotic cells were statistically decreased by the miR-142-5p inhibitor (both  $p < 0.05$ ), but overexpression of FOXO altered these effects (Fig. 5C and D).

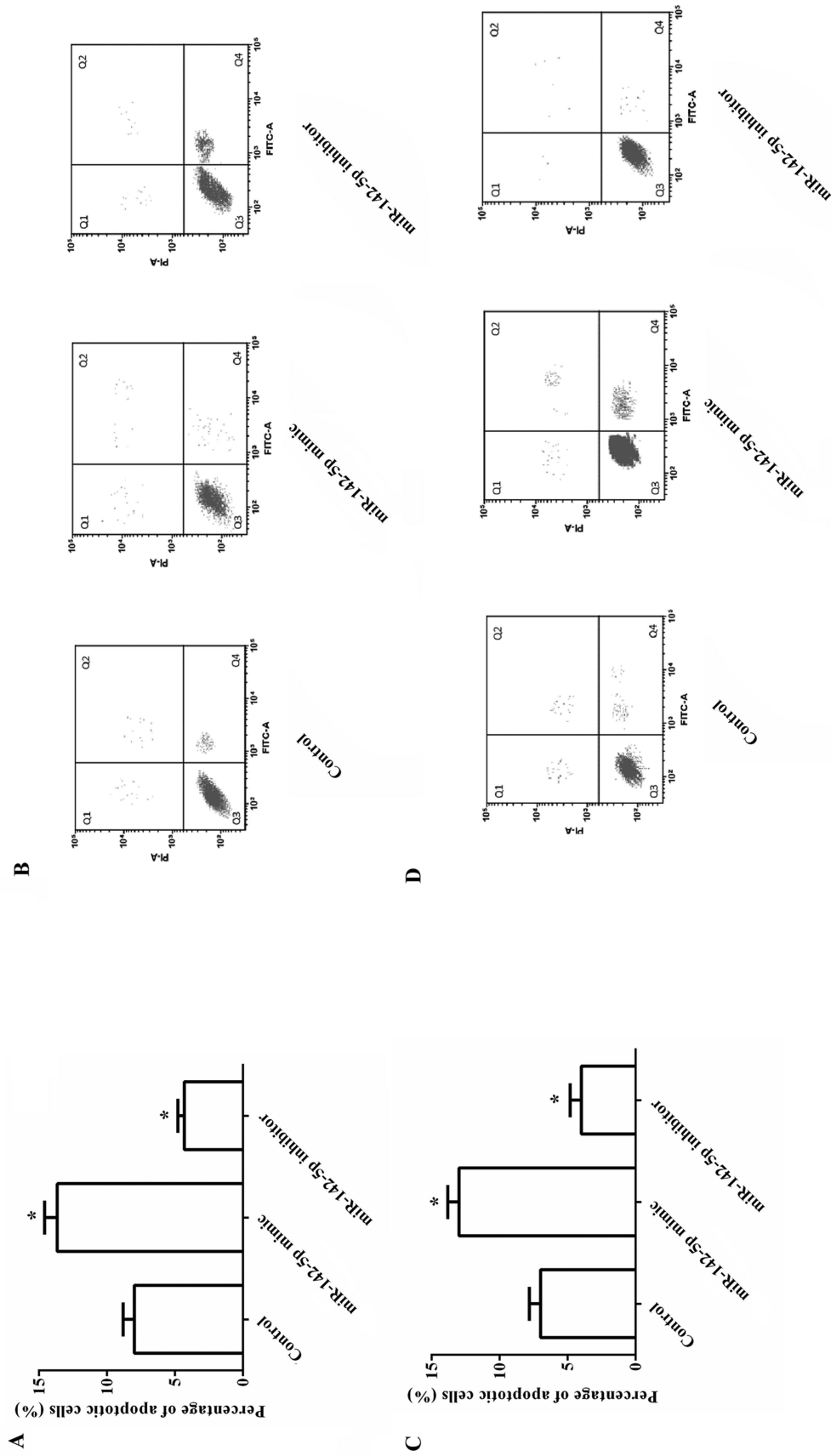
## DISCUSSION

In the present study, the functional role of miR-142-5p in HCC was investigated. Here we provided the evidence that miR-142-5p overexpression played a significant protective role in HCC. We found that, in both HepG2 and SMMC-7721 cells, miR-142-5p overexpression significantly reduced the relative cell viability, statistically induced cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase, and remarkably promoted cell apoptosis. However, miR-142-5p suppression reversed these results. The underlying mechanism regarding the cell growth and apoptosis was the regulation of FOXO expression in HCC cells. miR-142-5p might be considered a potential therapeutic target for the treatment of HCC.

miR-142 has been reported to be a T-cell-specific and hematopoiesis-specific miRNA (20,21). miR-142 is composed of two different mature transcripts (miR-142-3p and miR-142-5p). The miR-142-5p gene is located on chromosome 17, which has been found to be overexpressed in hematopoietic organs (21). Recently, it has been reported that miR-142-5p is also highly expressed in other pathological conditions, such as tumors (12,13), inflammation (22), immunologically related disorders (23,24), and renal fibrosis combined with inflammation (25,26). Also, the downregulation of miR-142-5p is responsible for acute stressor (27), early normal macrophage differentiation (28), and adaptive hypertrophy (29). Moreover,

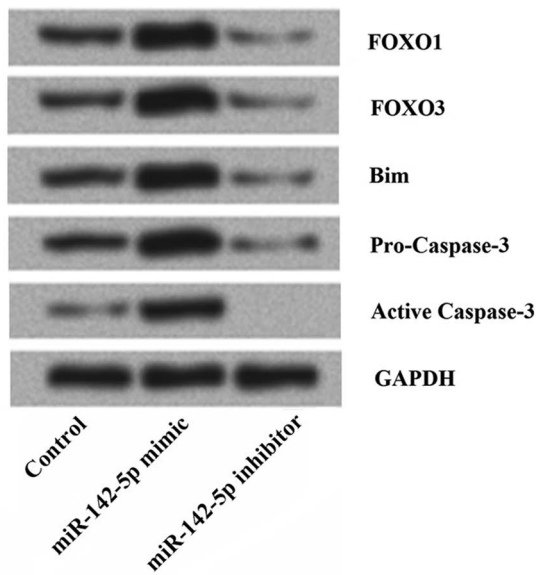


**Figure 2.** Overexpression of miR-142-5p induces cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase in HCC cells. The effects of miR-142-5p abnormal expression on cell cycle distribution were determined by flow cytometry. (A) A significant increase in the percentages of the G<sub>0</sub>/G<sub>1</sub> phase was found in the HepG2 cells that were transfected with the miR-142-5p mimic. (B) A significant increase in the percentages of the G<sub>0</sub>/G<sub>1</sub> phase was found in the HepG2 cells that were transfected with the miR-142-5p mimic. miR, microRNA.



**Figure 3.** Overexpression of miR-142-5p induces cell apoptosis in HCC cells. The effects of the miR-142-5p aberrant expression on cell apoptosis were investigated. (A, B) The percentages of apoptotic cells in HepG2 cells were significantly increased by miR-142-5p overexpression, but statistically were decreased by miR-142-5p suppression. (C, D) The percentages of apoptotic cells in SMMC-7721 cells were markedly increased by miR-142-5p overexpression, but were remarkably decreased by miR-142-5p suppression. miR, microRNA. \* $p < 0.05$  compared to the control group.





**Figure 4.** Overexpression of miR-142-5p controls cell growth and induces apoptosis by regulating FOXO in HepG2 cells. The effects of miR-142-5p aberrant expression on the expression of FOXO1, FOXO3, Bim, procaspase 3, and activated caspase 3 were determined by Western blotting. The results showed that the expressions of FOXO1, FOXO3, Bim, procaspase 3, and activated caspase 3 were all significantly elevated by miR-142-5p overexpression, but were statistically decreased by miR-142-5p suppression. miR, microRNA; FOXO, Forkhead box, class O; Bim, Bcl-2-interacting mediator of cell death.

several studies have implied that miR-142-5p is downregulated in HCC and might be responsible for the pathogenesis of HCC (10,15,16). However, the exact mechanism by which miR-142-5p exerts its effector function remains elusive. In the present study, we focused on the functional role of miR-142-5p in HCC cell growth and cell apoptosis, as well as the underlying mechanism.

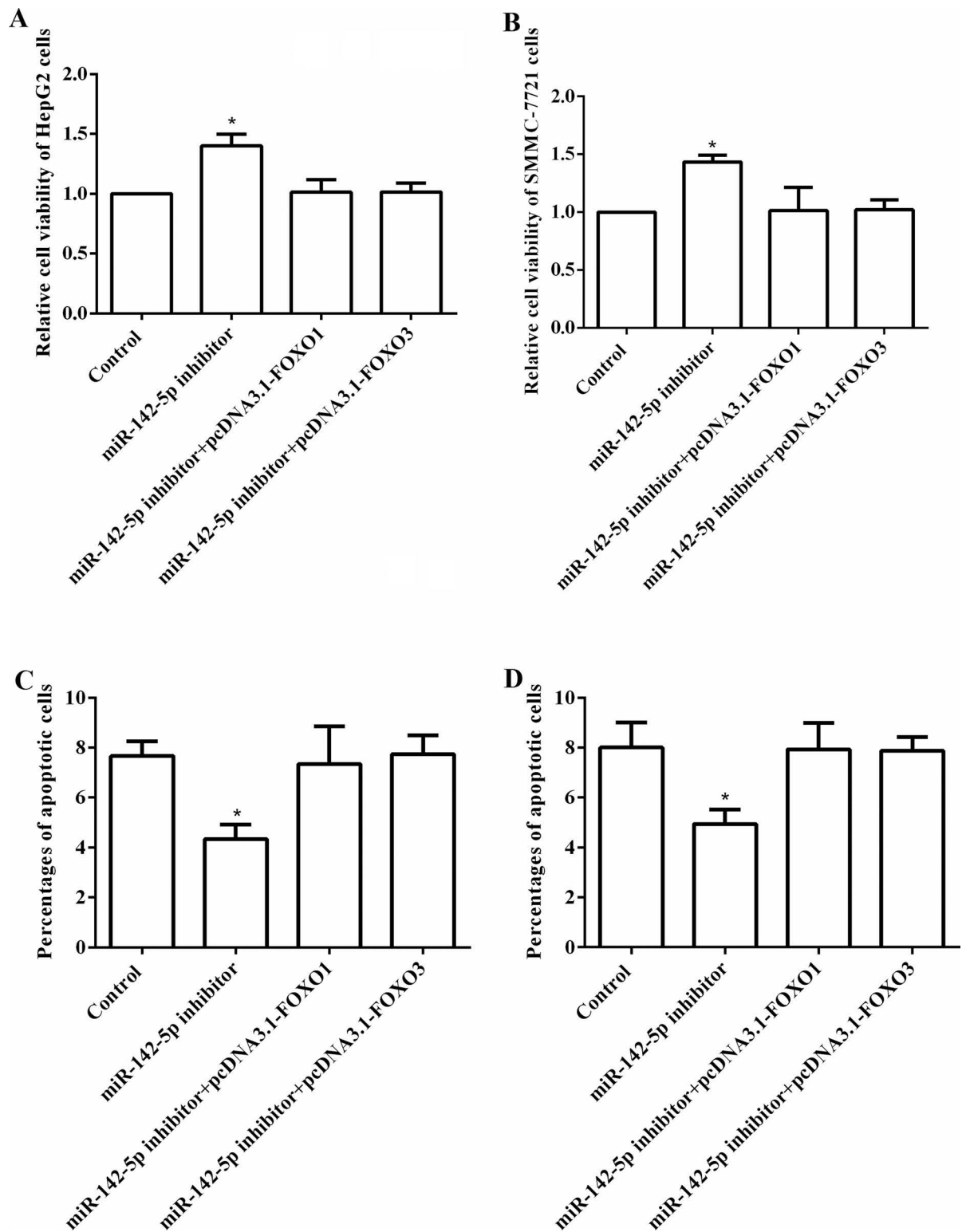
It has been demonstrated that cell growth and apoptosis are of great importance to the development and progression of cancer. Inhibition of cell growth and apoptosis appears to protect against malignancies. Therefore, a growing number of studies have paid great attention to the important topic, and a variety of miRNAs have been identified as potential therapeutic targets. To clearly reveal the possible function of miR-142-5p in HCC, we first altered the expression of miR-142-5p by transfection with the miR-142-5p mimic or inhibitor in two different HCC cell lines, HepG2 cells and SMMC-7721 cells. Cell viability, cell cycle distribution, and cell apoptosis were then analyzed. The data presented here demonstrated that overexpression of miR-142-5p significantly decreased the cell viability but increased cell apoptosis in both HepG2 cells and SMMC-7721 cells, while inhibition of miR-142-5p showed contrary results. As inhibition of

cell growth and promotion of cell apoptosis are associated with cell cycle arrest, we explored whether overexpression of miR-142-5p could induce cell cycle arrest. The results show that overexpression of miR-142-5p statistically induced cell cycle arrest at the  $G_0/G_1$  phase. However, our results were different from those of Kee et al. (30). In the research of Kee and coworkers, they found that miR-142-5p was upregulated in vascular smooth muscle cells (VSMCs) and promoted VSMC proliferation by downregulation of B-cell translocation gene 3 (BTG3). The main reason for the difference might be the different expressions of miR-142-5p in VSMCs and HCC as well as different regulatory mechanisms.

We further explored the possible mechanism of miR-142-5p abnormal expression on cell growth and cell apoptosis. FOXO transcription factors have been shown to modulate diverse cellular functions such as cell proliferation, metabolic processes, apoptosis, DNA repair, cell survival, and stress resistance (31–34). Additionally, activation of FOXO could induce specific gene expression, leading to cell cycle arrest. This function of FOXO implies a significant role in tumor inhibition (31). Recent research has shown the important molecular modulation of FOXO in liver cancer and also proposed the potential therapeutic target for developing new anti-tumor agents (35). Among the FOXO family, FOXO1 and FOXO3 are known to be responsible for cell proliferation, cell cycle arrest, and apoptosis (31,36,37). Previous studies proved that FOXO transcription factors directly induce Bim expression and promote apoptosis (38–40). It has also been reported that FOXO is involved in Fas-mediated apoptosis by activating the caspase family (41). Several miRNAs have been identified to show their carcinogenesis or carcinostasis by targeting FOXO (42). As indicated in our results, overexpression of miR-142-5p significantly increased all levels of FOXO1, FOXO3, Bim, procaspase 3, and activated caspase 3, suggesting that miR-142-5p controls cell growth and apoptosis by regulating the FOXO. To confirm the results, we further altered the expression of FOXO after transfection with the miR-142-5p inhibitor and then analyzed cell viability and cell apoptosis. As expected, the effects of the miR-142-5p inhibitor on cell viability and apoptosis were reversed by overexpression of FOXO1 and FOXO3. Our results were in line with the previous study, which additionally provided the evidence that miR-142-5p controls cell growth and apoptosis by regulating the FOXO.

## CONCLUSIONS

Our results suggest that miR-142-5p may serve as a critical protective factor in HCC. miR-142-5p overexpression inhibits cell growth and induces apoptosis. These effects might be by regulating FOXO expression.



**Figure 5.** Overexpression of FOXO reverses the effects of the miR-142-5p inhibitor on cell viability and cell apoptosis. After transfection with the miR-142-5p inhibitor, the expression of FOXO1 and FOXO3 was overexpressed, and then the cell viability and cell apoptosis were analyzed. (A, B) The cell viability in both HepG2 and SMMC-7721 cells was significantly increased by the miR-142-5p inhibitor, but these effects were reversed by overexpression of FOXO. (C, D) The percentages of apoptotic cells in both HepG2 and SMMC-7721 cells were markedly decreased by the miR-142-5p inhibitor, but these effects were reversed by overexpression of FOXO. miR, microRNA; FOXO, Forkhead box, class O. \* $p < 0.05$  compared to the control group.

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