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MicroRNA-216b is downregulated in hepatocellular carcinoma and inhibits HepG2 cell growth by targeting Forkhead box protein M1

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Abstract. – OBJECTIVE: Aberrantly expressed microRNAs (miRs) may play critical roles in the regulation of tumorigenicity of various cancers. The present study was designed to investigate the expression, function and the underlying mechanism of miR-216b in hepatocellular carcinoma (HCC).

MATERIALS AND METHODS: The expression of miR-216b and FOXM1 in 24 paired HCC tissues and adjacent normal tissues was determined by Real-time PCR. The proliferative activity of HepG2 cells was determined by MTT assay. We analyzed cell cycle progression by flow cytometry, apoptosis by cell death enzyme-linked immunosorbent assay (ELISA) and cleaved-caspase-3 by western blot. Luciferase reporter assay was employed to verify whether FOXM1 serves as a target of miR-216b in vitro.

RESULTS: The expression of miR-216b was significantly decreased in HCC tissues compared with that in adjacent normal tissues, whereas FOXM1 expression was increased. In addition, FOXM1 and miR-216b expression were inversely correlated in HCC tissues. Ectopic expression of miR-216b produced a suppressive effect on the growth of HepG2 cells and induced cell cycle arrest and apoptosis. We further demonstrated that miR-216b targets the 3' untranslated region (UTR) of FOXM1 directly to suppress the expression of FOXM1, and that suppression of FOXM1 produced the similar effects to miR-216b

CONCLUSIONS: These data suggest that down-regulation of miR-216b directly contributes to the up-regulation of FOXM1, which may confer the tumorigenicity of HCC cells. MiR-216b may serve as a potential therapeutic agent for HCC.

Key Words:
microRNAs, Cell cycle, Apoptosis, Hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is one of the common lethal malignancies in Asia-Pacific areas and it is the third leading cause of cancer-related death globally[1,2]. Since standard treatment such as surgical resection and chemotherapy failed to produce satisfactory efficacy, development of novel agents for targeted therapy may be one of the options to tackle this challenging issue. Thus, identification of new therapeutic targets or small biological molecules that have potentials to be utilized clinically is the key point in basic research of HCC.

MicroRNAs (miRs), a class of small noncoding single RNA strands that are discovered decades ago, have been shown to play critical roles in the tumor biology[3,4]. MicroRNAs are able to bind to the 3'UTR of mRNAs to degrade the targeted mRNAs or block the protein translation process consequently[5,6]. Altered expressions of several microRNAs, which act corporately with tumor suppressors or inducers, are often observed in HCC, and are functionally associated with dysregulated molecular pathways[7]. For example, miR-34c is involved in p53 regulated apoptosis and has multiple targets in HCC[8-10]; miR-17-92 clusters were found to be highly expressed to promote hepatocarcinogenesis via a number of target genes[11]. Located at chromosome 2p16, miR-216b has been identified as a tumor suppressor in various studies through several mechanisms. For example, down-regulation of miR-216b contributes to nasopharyngeal carcinogenesis by inducing KRAS pathway[12]; miR-216b targets CKII to induce cellular senes-

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cence in colorectal cancer cells[13]. Few studies investigated the role of miR-216b in liver cancer, it was only demonstrated by Liu et al[14] recently that miR-216b altered IGF pathway in HCC, and its activation depends on p53. This finding indicated the critical role of miR-216b in regulating hepatocarcinogenesis. However, more researches need to be done to prove its function. Besides, it is still unknown whether it regulates cell apoptosis or cell cycle transition via other novel mechanisms.

Herein by bioinformatics analysis, we discovered that miR-216b potentially binds to the 3'UTR of the mRNA of forkhead box protein M1 (FOXM1), a novel transcriptional factor that is critical in promoting cell cycle transition[15]. We showed that miR-216b expression is significantly down-regulated in HCC and an inverse correlation between miR-216b and FOXM1 expression was discovered. Experimental studies identified that miR-216b suppressed proliferation by inducing apoptosis and cell cycle arrest, which is mediated by the direct repression of the critical oncogene FOXM1. Our study provided the clues for the role of miR-216b-FOXM1 interaction in regulating the cancer cell biology of HCC for the first time, and revealed the therapeutic potential of miR-216b for the treatment of HCC.

Materials and Methods

Tumor Sample Collection

We analyzed 24 pairs of HCC and adjacent normal tissues for miR-216b and FOXM1 expression. The tissues were collected at Weifang Traditional Chinese Hospital between December 2014 and August 2015. All the patients were not subjected to any therapy before the surgery. The adjacent normal tissues were at least 2 cm away from the tumor margin. All samples were histologically examined and immediately kept in liquid nitrogen until use.

Cell Culture

HCC cell line HepG2 was obtained from The Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). Cells were propagated in a 37°C humidified incubator, and the CO_2 concentration was 5%.

Transfection of miRs or siRNAs

The miR-216b mimics, miR-216-inhibitor and their negative control nucleotides were purchased from RiboBio Biotechnology Co. Ltd. (Guangzhou, China). The small interfering RNA (siRNA) for FOXM1 and its negative control were synthesized by Invitrogen (Shanghai, China). In order to transfect the cells with miRs or siRNAs, cells grown at 80% confluence were cultured in serum-free and antibiotics-free medium. Both the microRNAs and the siRNAs were transfected at the final concentration of 100 nmol/L using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. 6 hours post-transfection, the culture medium was then refreshed with a complete medium that contained 10% FBS.

Cell Proliferation Assay

Cells were plated into 96-well plates at the density of 5000 cells per well. The cell viability was detected using MTT assay at the time points of 24 h and 72 h. For MTT assay, the MTT reagent (Sigma, St. Louis, MO, USA) was dissolved in PBS at 5 mg/ml, and 20 µl of solution were added to each well 4h before the end of the incubation. Then the culture media were removed and the formazan crystals were dissolved in 200 µl DMSO. The absorbance at 490 nm was acquired on a micro-plate reader.

Real-time PCR

The total RNA from the tumor samples and the cells were isolated using TRIzol Reagent (Invitrogen) according to the standard protocol. The RNA samples were reverse transcribed using the Bulge Stem-loop miR-216b RT primer (RiboBio) or U6 specific RT primer (RiboBio). For detection of FOXM1 mRNA, the RNA samples were transcribed using the OligodT primer (Promega, Madison, WI, USA). The reverse transcription procedure was conducted using the GoScript Reverse Transcription System (Promega). The cDNAs were then amplified with SYBR Green Master Mix (Promega) using the specific miR-216b primers (RiboBio), U6 primers (RiboBio) and primers for FOXM1 and GAPDH on an ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, MA, USA). U6 and GAPDH were the internal controls for miR-216b and FOXM1, respectively. Primers for FOXM1 and GAPDH were as follows:

FOXM1: F: ATACGTGGATTGAGGACCACT, R: TCCAATGTCAAGTAGCGGTTG;
GAPDH: F: TGTGGGCATCAATGGATTTGG, R: ACACCATGTATTCCGGGTCAAT.

Western Blot

The cells were treated with Western and IP lysis buffer (Beyotime Bioinstitute), and the lysates were collected. The lysates were then sonicated, and added with 1/5 volume of 5X SDS loading sample buffer (Beyotime), followed by heating at 100°C for 5 min. The proteins were then separated on Sodium dodecyl sulfate polyacrylamide gels by electrophoresis (SDS-PAGE), followed by transferring to Polyvinylidene Fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Membranes were then blocked at room temperature for 2h in 5% Bovine Serum Albumin (BSA, Sigma). The blocked membranes were incubated with rabbit anti-human cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human p27 (Santa Cruz Biotechnology), rabbit anti-human cleaved-caspase-3 (Cell Signaling Technology Inc., Beverly, MA, USA), rabbit anti-human FOXM1 (Abcam, Cambridge, MA, USA), and mouse anti-human GAPDH (ZSGB, Beijing, China) at 4°C for 16 h, and subsequently washed with PBS 0.1% Tween 20 for 3 times. HRP-conjugated secondary antibodies (ZSGB) were then incubated at room temperature for 1 h. The proteins were visualized by a Pierce™ ECL detection kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Luciferase Reporter Assay

The 3'UTR of FOXM1 was inserted into the multiple cloning sites of the pmirGLO plasmid (Promega). HepG2 cells were plated in 24-well plates and co-transfected with 1 μg construct and 100 nM miR-216 mimics, miR-216-inhibitor or negative control. After incubation for 48 h, the luciferase activity was measured by a Dual-Luciferase system (Promega). Finally, the firefly luciferase activity was normalized to the renilla luciferase activity.

Cell Apoptosis Assay

Apoptosis level was measured by a cell death detection Enzyme-Linked Immunosorbent Assay (ELISA) kit (Roche Diagnostics, Indianapolis, IN, USA) as per the manufacturer's protocol. This ELISA assay specifically detects the mono- and oligo-nucleosomes that are released from apoptosis. The experiments were performed 5 times.

Cell Cycle Assay

The above-transfected cells were collected and fixed with 70% ethanol; cells were then washed with PBS followed by t 0.01 mg/mL RNase treatment. Cells were then stained with propidium iodide (PI, Beyotime Bioinstitute) (50 μg/mL) in a dark room at 37°C for 1 h. The samples were sorted and analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

All data were expressed as means ± SD, the differential expression of miR-216b and FOXM1 in clinical specimens was examined by paired *t*-test, and unpaired *t*-test was performed for cellular experiments. The correlation between FOXM1 and miR-216b expression was determined by Pearson correlation analysis. $p < 0.05$ was deemed statistically significant.

Results

The Expression of miR-216b is Down-Regulated in HCC Tissues and Correlates with Increased FOXM1 expression

To study the role of miR-216b in HCC, we collected 24 pairs of HCC tissues and the adjacent normal tissues to examine the expression of miR-216b. As shown in Figure 1A, 22 out of 24 HCC tissues showed a down-regulated miR-216b expression. The mean expression of miR-216b was also significantly decreased (Figure 2B). Conversely, FOXM1, which is a potent cell cycle regulator, was significantly up-regulated in HCC tissues (Figure 2C). We further observed an inverse correlation between FOXM1 mRNA and miR-216b expression in HCC tissues (Figure 2D), suggesting the possible involvement of miR-216b and FOXM1 in the regulation of the tumor biology of HCC.

MiR-216b Inhibits Cell Growth by Inducing Cell Cycle Arrest and Apoptosis

We, then, overexpressed miR-216b in the HCC cell line HepG2 to analyze its function. The efficacy of miR-216 mimics was confirmed by Real-time PCR (Figure 2A). A strong growth inhibition effect was observed at the time points of 24 h and 72 h when miR-216b was overexpressed (Figure 2B). Analysis of the cell cycle progression by flowcytometry revealed that the cell cycle was blocked in G1 phase in miR-216b overexpressing cells, as exemplified by the increased

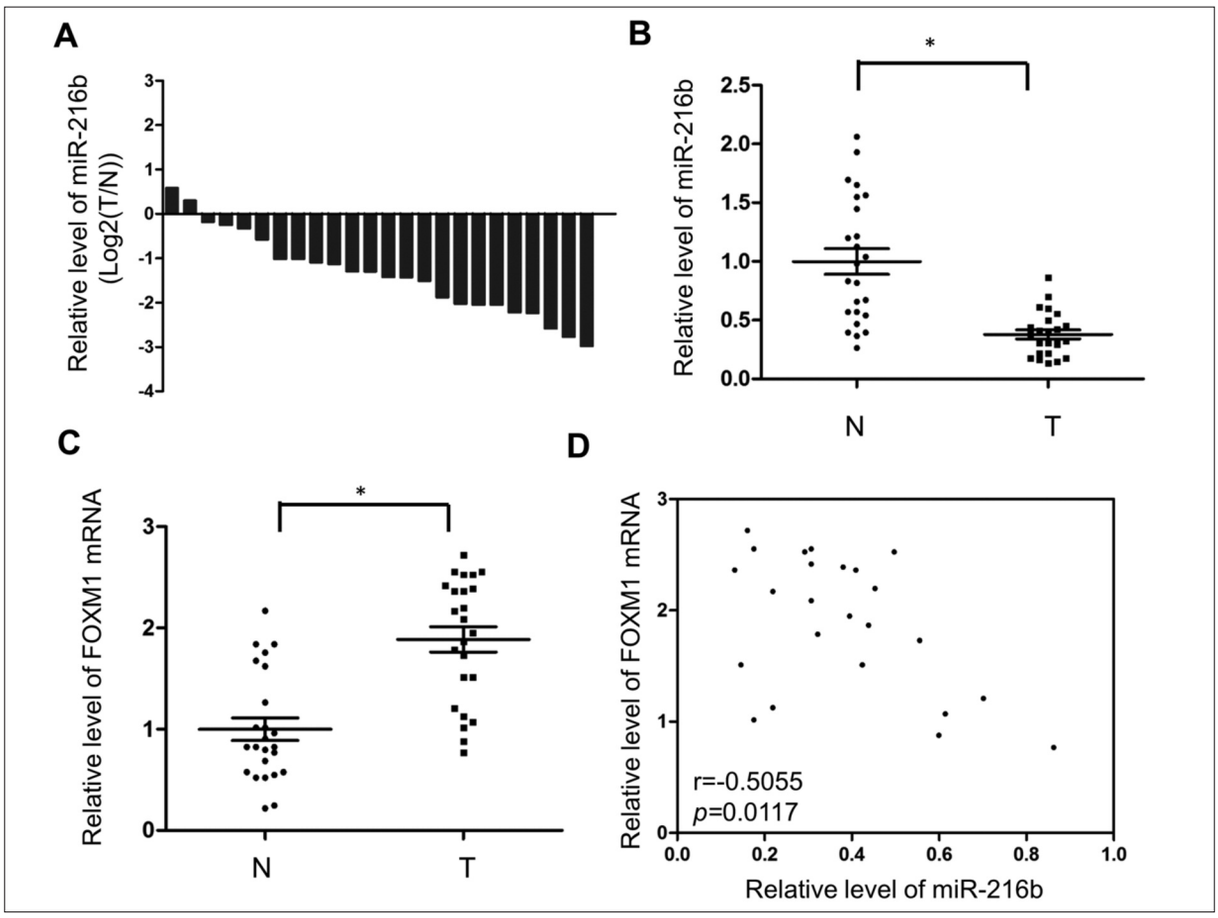

Figure 1. The expression of miR-216b is down-regulated in HCC tissues and correlates with increased FOXM1 expression. ***A,*** MiR-216b expression in HCC tissues is down-regulated in 22 out of 24 patients. The relative expression of miR-216b was log transformed, and each bar represents an individual. ***B,*** The relative expression of miR-216b is down-regulated, and ***(C)*** the relative expression of FOXM1 is up-regulated in HCC tissues. U6 was used to normalize miR-216b, and GAPDH was used to normalize FOXM1. *$p < 0.05$. ***C,*** FOXM1 and miR-216b expression was inversely correlated. N, adjacent normal tissue; T, HCC tumor tissue.

cell number in the G1 phase and decreased cell number in the S phase (Figure 2C). In addition, miR-216b resulted in a decreased expression of the positive cell cycle regulator cyclin D1 and an increased expression of negative regulator p27 (Figure 2D and E). Moreover, cell death ELISA and Western blot of cleaved-caspase-3 together showed the induction of apoptosis by miR-216b (Figure 2F, G and H). These data demonstrated that miR-216b exerts an antigrowth function by inducing apoptosis and cell cycle arrest.

FOXM1 Serves as a Target for miR-216b

The above experiments confirmed the inverse correlation between FOXM1 and miR-216b expression. Then, we asked whether miR-216b functions through FOXM1. Intriguingly, bioinformatics databases miRanda (www.microrna.org) and TargetScan (www.targetscan.org) both predicted that FOXM1 serves as a possible target for miR-216b (Figure 3A), we thus experimentally tested their relationship using luciferase reporter assay. As shown in Figure 3B, miR-216b significantly inhibited the expression of the luciferase gene, whereas transfection of its inhibitor increased the luciferase activity. Importantly, miR-216b transfection resulted in a significant decrease in the protein level of FOXM1, whereas miR-216 inhibitor increased FOXM1 expression (Figure 3C). These data established that FOXM1 is one of the direct targets of miR-216b.

Knockdown of FOXM1 Exhibited Similar Effects to miR-216b Overexpression

If miR-216b inhibits HCC cell growth through FOXM1, then inhibiting FOXM1 should gener-

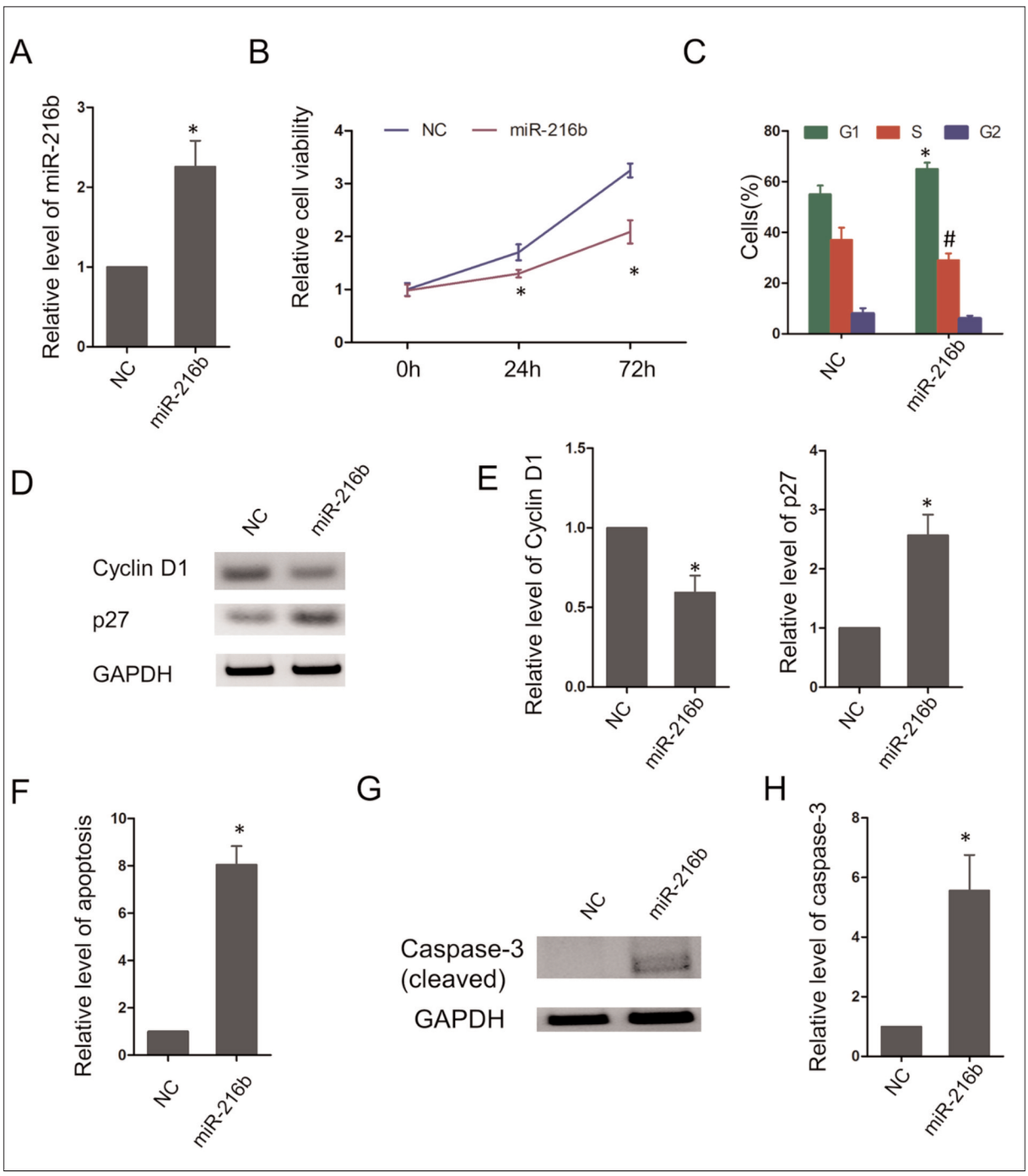

Figure 2. MiR-216b inhibits cell growth by inducing cell cycle arrest and apoptosis. ***A,*** Transfection of miR-216b mimics increases its expression. ***B,*** MiR-216b inhibits the growth of HepG2 cells at indicated time. Cells were transfected with NC or miR-216b, cell viability was determined by MTT assay. $*p < 0.05$ *vs*. NC at each time points. ***C,*** MiR-216b induces cell cycle arrest. $*p < 0.05$ *vs*. G1 of NC, $^{\#}p < 0.05$ *vs*. S of NC. ***D,*** MiR-216b regulates the expression of cell cycle regulators. ***E,*** The statistical analysis of the Western blot data in ***(D)***, $*p < 0.05$ *vs*. NC. ***F,*** MiR-216b induces apoptosis as determined by cell death ELISA. $*p < 0.05$ *vs*. NC. ***G,*** and ***H,*** MiR-216b induces the expression of apoptosis marker cleaved-caspase-3. $*p < 0.05$ *vs*. NC cells were transfected with NC or miR-216b for 72h, cell cycle was measured by flow cytometry, apoptosis was measured by cell death ELISA and cyclin D1, p27 and cleaved-caspase-3 proteins were detected by Western blot, GAPDH was used as internal control. NC, negative control.

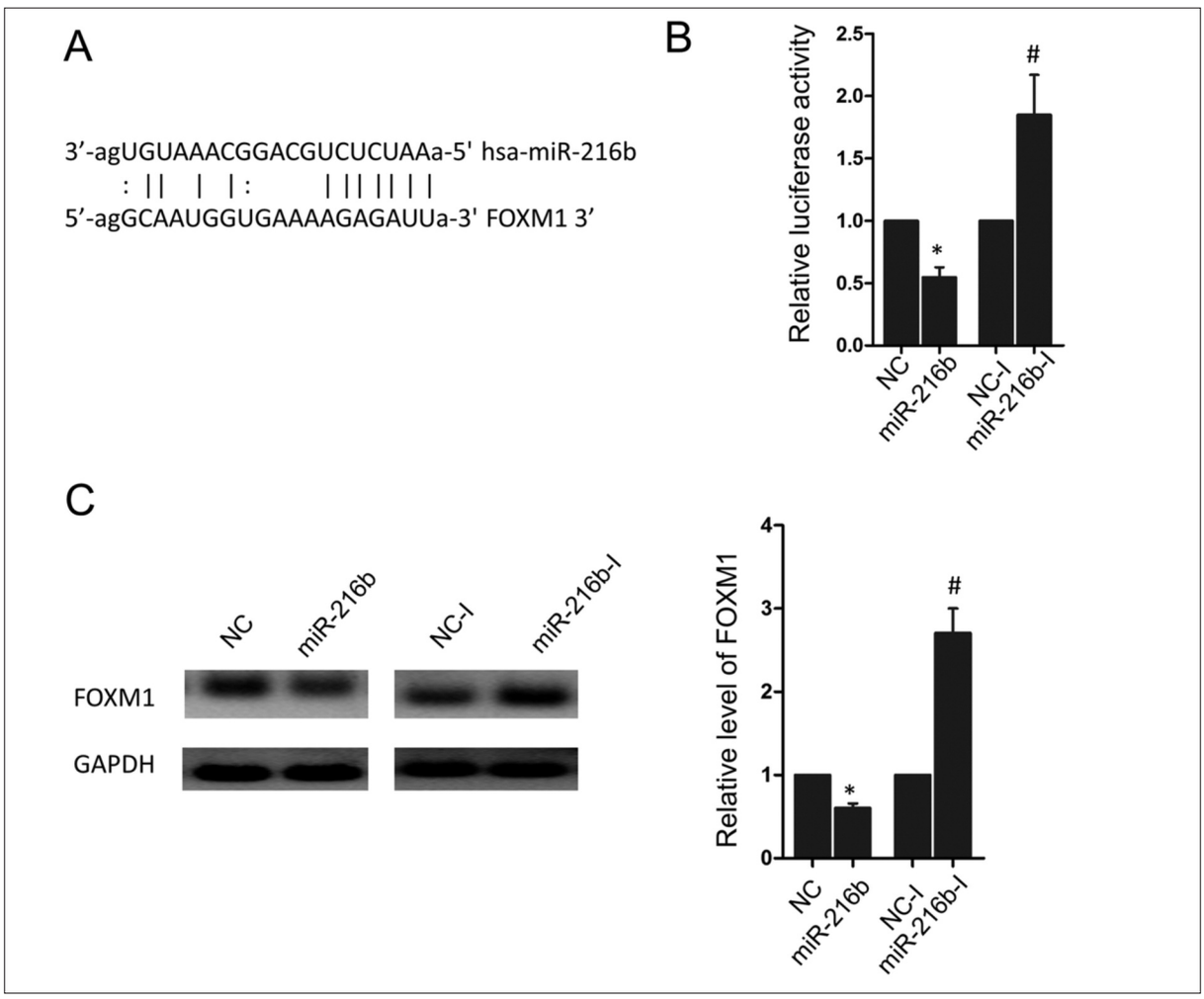

Figure 3. FOXM1 serves as a target for miR-216b. ***A,*** The schematic of the predicted base pairing between miR-216b and FOXM1 3'UTR. ***B,*** Luciferase activity was inhibited by miR-216b and enhanced by miR-216b-I. MiR-216b, miR-216b-I, NC or NC-I were co-transfected into HepG2 cells with a FOXM1-3'UTR luciferase reporter; luciferase activity was measured 48h after transfection. ***C,*** MiR-216b affects FOXM1 expression. MiR-216b, miR-216b-I, NC or NC-I were transfected into HepG2 cells, FOXM1 protein was detected 48h after transfection. $^{*}p < 0.05$ vs. NC, $^{\#}p < 0.05$ *vs*. NC-I. NC, negative control; miR-216b-I, miR-216 inhibitor; NC-I, negative control for the miR-216 inhibitor.

ate similar effects. To test this hypothesis, FOXM1 was efficiently silenced by its specific siRNA (Figure 4A). Cell viability assay confirmed the inhibited growth activity in cells that had FOXM1 knocked down (Figure 4B). Detailed analyses by flow cytometry, Western blot and cell death ELISA showed that silencing endogenous FOXM1 resulted in cell cycle arrest at the G1 phase (Figure 4C) and apoptosis (Figure 4D, E and F), which was consistent with the effects produced by miR-216b mimics. These results confirmed that FOXM1 is functionally relevant to miR-216b, and thus might mediate the anti-growth effect of miR-216b on HepG2 cells.

Discussion

In the present study, we describe the tumor suppressive function of miR-216b and its underlying molecular mechanism. A significant down-regulation of miR-216b in HCC tumor tissues was detected, which is associated with up-regulation of FOXM1 expression. Functional tests verified that FOXM1 was targeted by miR-216b. Importantly, replenishment of miR-216b in HCC cells significantly suppressed proliferation by inducing apoptosis and cell cycle arrest, which is consistent with the FOXM1 knockdown. These findings evidenced the anti-tumor role of miR-

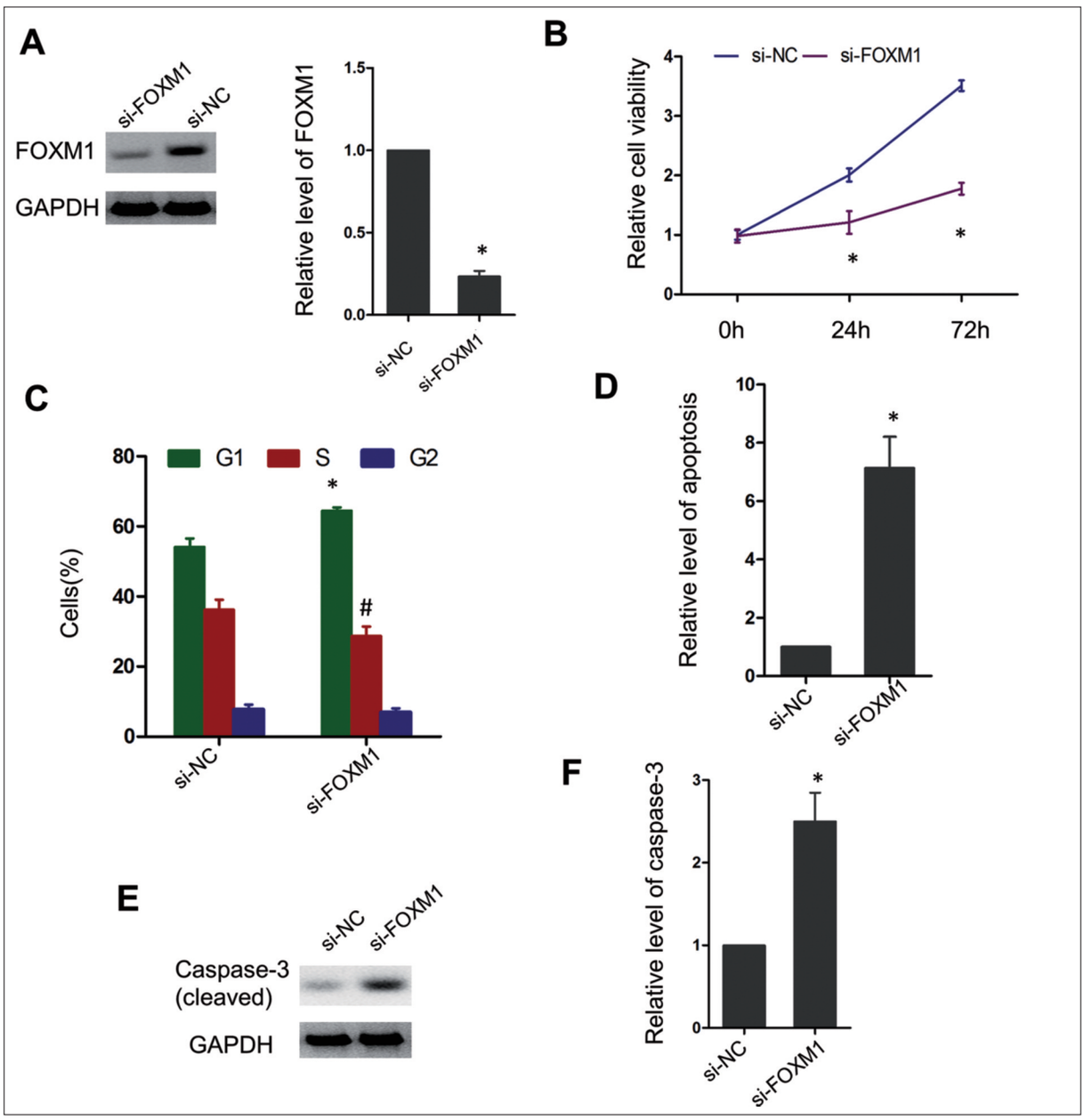

Figure 4. Knockdown of FOXM1 exhibited similar effects to miR-216b overexpression. ***A,*** Transfection of siRNA for FOXM1 efficiently reduced endogenous FOXM1 expression. ***B,*** Knockdown of FOXM1 inhibits the growth of HepG2 cells at indicated time. Cells were transfected with si-NC or si-FOXM1, cell viability was determined by MTT assay. $^{*}p < 0.05$ *vs*. si-NC at each time points. ***C,*** Knockdown of FOXM1 induces cell cycle arrest. $^{*}p < 0.05$ *vs*. G1 of si-NC, $^{\#}p < 0.05$ *vs*. S of si-NC. ***D,*** Knockdown of FOXM1 induces apoptosis as determined by cell death ELISA. $^{*}p < 0.05$ *vs*. si-NC. ***E,*** and ***F,*** Knockdown of FOXM1 induces the expression of apoptosis marker cleaved-caspase-3. $^{*}p < 0.05$ vs. si-NC. Cells were transfected with si-NC or si-FOXM1 for 72h, cell cycle was measured by flow cytometry, apoptosis was measured by cell death ELISA and cleaved-caspase-3 protein was detected by Western blot, GAPDH was used as internal control. si-FOXM1, small interfering RNA for FOXM1; si-NC, negative control small interfering RNA.

216b and highlighted its potential application for the treatment of HCC.

Emerging evidences have supported that microRNAs are a class of the important posttranscriptional regulators in the development of cancers[16,17], and a large number of microRNAs are implicated in the tumor biology of HCC by affecting various processes such as proliferation,

metastasis, apoptosis and autophagy. For example, Tang et al[18] described that miR-429 regulates Wnt signaling and promotes metastasis, Yin et al[19] reported that miR-193b modulates cisplatin-induced apoptosis. Ge et al[20] disclosed that miR-100 targets mTOR and IGF-IR to promote autophagy. Huang et al[21] showed that miR-128-3p inhibited proliferation of HCC. In this study, we demonstrated that miR-216b exerted multiple functions including suppressing proliferation and cell cycle transition and inducing apoptosis. The functions of miR-216b have been widely studied in other malignancies in nasopharynx, stomach, breast and colorectum[12,13,22,23]. Importantly, Liu et al[14], recently, demonstrated that down-regulation of miR-216b in HCC contributes to cell proliferation by affecting IGF signaling. Our data corroborated and reinforced their findings. Particularly, we firstly identified the role and mechanism of miR-216b in the regulation of cell cycle and apoptosis in HCC, which considerably deepened our understanding on the action this microRNA.

MicroRNAs functions to repress gene expression by binding to the 3'UTR region of their targeted mRNAs, owing to the ability of miR-216b in regulating cell cycle and apoptosis, we speculated that miR-216b may function through essential genes that are directly involved in these processes. We searched the TargetScan Human database (www.targetscan.org) and the miRanda database (www.microrna.org), both databases predicted that the sequence from position 240 to 261 of FOXM1 3'UTR matches imperfectly with miR-216b. Both the Western blot and luciferase activity assay verified our hypothesis. FOXM1 is a forkhead transcriptional factor that has been recently recognized to possess oncogenic potential[15,24,25]. Due to its essential roles in modulating the transcription activity of various factors involved in G1-S transition and G2-M transition, FOXM1 is tightly associated with proliferation[24]. Also, FOXM1 is involved in apoptosis by promoting the transcription of X-linked Inhibitor of Apoptosis (XIAP) and survivin[26], which are key negative regulators of apoptosis. Early studies have demonstrated that FOXM1 is essential for the development of HCC, and increased FOXM1 expression is closely associated with poor prognosis[27-29]. Here, we found that when FOXM1 was knocked down, HCC cells underwent apoptosis and cell cycle arrest, which is consistent with the previous studies[30,31], the same effect was detected when miR-216b was overexpressed. Of note, we found that cells in G1 phase were increased and cells in S phase were decreased after FOXM1 knockdown or miR-216b transfection, suggesting the existence of G1-S cell cycle arrest. Moreover, the altered cyclin D1 and p27kip expression are consistent with the defects in cell-cycle transition after miR-216b transfection. Several microRNAs exhibited regulatory ability on FOXM1 expression, such as miR-204 in esophageal cancer, miR-671-5p in breast cancer, miR-149 in colorectal cancer and miR-34a in HCC[32-35]. To the best of our knowledge, our findings identified the interaction between miR-216b and FOXM1 for the first time. Our study, therefore, provided novel insights into the master role of microRNAs related mechanisms in cell cycle and apoptosis regulation.

Conclusions

Our data demonstrate that miR-216b is significantly down-regulated and functions as a tumor suppressor in HCC. Overexpression of miR-216b suppressed FOXM1 expression, which consequently causes apoptosis and cell cycle arrest. This study provides a novel molecular basis of miR-216b in regulating the tumor biology of HCC, and identified miR-216b as a potential drugable biological molecule for the clinical intervention of HCC, further *in vivo* investigations may be warranted to verify its efficacy.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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