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Downregulation of Human Farnesoid X Receptor by miR-421 Promotes Proliferation and Migration of Hepatocellular Carcinoma Cells

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Abstract

The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that is highly expressed in liver, kidney, adrenal gland, and intestine. It plays an important role in regulating the progression of several cancers including hepatocellular carcinoma (HCC). So it is necessary to study the regulation of FXR. In this study, we found that the expression of miR-421 was inversely correlated with FXR protein level in HCC cell lines. Treatment with miR-421 mimic repressed FXR translation. The reporter assay revealed that miR-421 targeted 3′ untranslated region of human FXR mRNA. Furthermore, downregulation of FXR by miR-421 promoted the proliferation, migration, and invasion of HCC cells. These results suggest that miR-421 may serve as a novel molecular target for manipulating FXR expression in hepatocyte and for the treatment of HCC. *Mol Cancer Res; 10(4); 516–22. ©2012 AACR.*

Introduction

The farnesoid X receptor (FXR) is a ligand-activated transcription factor and a member of the nuclear receptor superfamily that is mainly expressed in liver, intestine, kidney, and adrenal gland (1). Ligand-activated FXR binds to response elements of target genes either as a classical FXR-RXRα (retinoid X receptor alpha) heterodimer or as a monomer (2–4). FXR plays an important role in regulating the metabolisms of bile acid, cholesterol, triglyceride, and glucose (5). Recent studies show that FXR is also involved in the regulation of liver regeneration and protection against the liver carcinogenesis (6). Therefore, FXR has received increasing attention as a therapeutic target for the treatments of certain metabolic diseases and liver carcinoma.

As a multiple functional transcriptional factor, FXR regulates so many target genes, but its own regulation is not well known. It has been reported that several transcriptional coregulators, including PGC-1α, Brg-1, p300, CARM1, PRMT1, and ASCOM, can potentially modulate FXR expression (7). Moreover, glucose and insulin (8) as well as the cytokines tumor necrosis factor-α and interleukin-1 (9) have also been identified to regulate FXR level. Recently, posttranslational modifications, such as phosphorylation, ubiquitination, and sumoylation, have been reported to be involved in FXR regulation (7).

miRNAs are a family of small (about 19–22 nucleotides) noncoding RNAs that have been shown to be crucial posttranscriptional regulators of gene expression. Each miRNA can regulate multiple genes by targeting mRNAs in partial sequence homology, leading to mRNA degradation or translation repression. miRNAs play important roles in cellular processes of differentiation, proliferation, apoptosis, and metabolic homeostasis. Recently, a growing amount of evidences have proved that miRNAs are involved in the pathogenesis of human cancers and have been proposed as potential novel targets for the therapy of malignancies.

Using online database searches (miRBase, TargetScan, and miRanda), several miRNAs were predicted to potentially target 3′ untranslated region (UTR) of human FXR mRNA, and miR-421 was with a high score of probability to regulate FXR. Previous reports have shown that miR-421 is associated with pancreatic cancer, gastric cancer, and neuroblastoma (10–12). However, it is not clear whether miR-421 is involved in hepatocellular carcinoma (HCC). In this study, we show that miR-421 is inversely correlated with FXR protein level in HCC cells. Treatment with miR-421 leads to downregulation of FXR and promotes the proliferation, migration, and invasion of HCC cells. These results suggest that miR-421 may serve as a novel molecular target for manipulating FXR expression in hepatocyte and for the treatment of HCC.

Materials and Methods

Cell culture

Human hepatocellular carcinoma cell lines, HepG2 and Hep3B, were obtained from American Type Culture

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Collection. Huh7, PLC, and hepatic cell L02 were purchased from China Type Culture Collection. All cells were cultured in Dulbeco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 U/mL) at 37° in 5% CO_2 humid incubator.

Western blot

miR-421 mimic (5′-AUCAACAGACAUUAAUUGGGCGC-3′), miR-421 mimic negative control (mimic NC, 5′-UUCUCCGAACGUGUCACGUTT-3′), miR-421 inhibitor (5′-GCGCCC AAUUAAUGUCUGUUGAU-3′, a single-stranded modified RNA that has a complimentary sequence to mature miRNA) and miR-421 inhibitor negative control (inhibitor NC, 5′-CAGUACUUUUGUGUAGUACAA-3′) were synthesized by Shanghai GenePharma. miR-421 inhibitor can antagonize the function of miR-421 mimic or the endogenous miR-421 in the cells. Cells were cultured in the presence or absence of miR-421 mimic/miR-421 inhibitor or corresponding mimic NC/inhibitor NC. Then the whole cell proteins were extracted and the protein concentrations were determined by Bradford protein assay reagent (Bio-Rad). Subsequently, the total proteins (50 μg per well) were separated with 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). After blocked 1 hour with 5% milk in Tris-buffered saline and Tween 20 (TBST; 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.5% Tween 20), the membranes were incubated with goat anti-human FXR antibody (Santa Cruz Biotechnology), or rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology) at 4°C overnight. After washed 3 times with TBST, the membranes were incubated with horseradish peroxidase (HRP)-labeled rabbit anti-goat immunoglobulin G (IgG; Invitrogen) or HRP-conjugated goat anti-rabbit IgG (Invitrogen) for 1 hour at room temperature. Finally, the membranes were washed 3 times with TBST and then detected with enhanced chemiluminescence reagents (Pierce).

Real-time reverse transcriptase PCR for mature miRNA

For the quantification of mature miR-421, polyadenylation and reverse transcription were carried out with the All-in-One miRNA quantitative reverse transcriptase PCR (qRT-PCR) Detection Kit (GeneCopoeia) according to the manufacturer's protocol. Then the qRT-PCR) was done using SYBR green-based assays with a forward primer for the mature miR-421 (5′-ATCAACAGACAT TAATTGGGCGC-3′) and the reverse universal qPCR primer supplemented in the kit. The level of mature miR-421 was normalized with U6 snRNA, and the forward and reverse primers for human U6 snRNA in qPCR were 5′-CGCTTCGGCAGCACATATACTAA-3′ and 5′-TATGGA ACGCTTCACGAATTTGC-3′, respectively.

qPCR detection for mRNA

Total RNA was extracted from cells with TRIzol reagent (Invitrogen), and the first-strand cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen). The expression of FXR mRNA was examined by qPCR using SYBR green-based assays with the forward primer (5′-GCG CGTCAGCAGGGAGGATC-3′) and the reverse primer (5′-CACACAGTTGCCCCCGTTTTT AC-3′). β-Actin was used as an internal control with the pimers 5′-ACCCCGTGCTGCTGACCG AG-3′ (forward) and 5′-TCCCGGCCAGCCAGGTCCA-3′ (reverse).

Plasmid construction

The DNA fragment corresponding to 2021-2056nt of human FXR mRNA containing the miR-421 recognition site (FXRMIRE421) was synthesized by Sangon Biotech and cloned into pMIR-REPORT vector (Promega) at *Spe*I and *Hind*III site downstream of the luciferase gene, and the resulting plasmid was named as pMIR/FXRMIRE. Similarly, the DNA fragment containing mutations in miR-421 recognition site (5′-TTAAATTGATTGTTACTTCAATTCTATCTGTT GAACTAGGGAAAATC-3′, the mutated bases were underlined) was synthesized and cloned into the pMIR-REPORT, and the resulting plasmid was named as pMIR/FXRMIRE-Mut. The promoter region (−572 to +10) of human *SHP* (small heterodimer partner, a typical target gene of FXR) was amplified by PCR with the oligonucleotides 5′-TCGGGGTAC**C**TCCTAGACTGGA CAGT-3′ (forward primer) and 5′-GGAAGATCTCTTCCAGCTCTCTGGCT-3′ (reverse primer) using HepG2 cell–derived genomic DNA as template. After digested with *Kpn*I and *Bgl*II, the amplified fragment was inserted into the luciferase reporter plasmid pGL3-Basic (Promega), and the resulting vector was named as phSHP-Luc.

Transient transfections and luciferase assays

Huh7 cells or HepG2 cells were grown to 70% to 80% confluence in 24-well plates. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with luciferase reporters (pMIR-REPORT, pMIR/FXRMIRE, pMIR/FXRMIRE-Mut, or phSHP-Luc) in the presence or absence of miR-421 mimic/miR-421 inhibitor or corresponding mimic NC/inhibitor NC. Transfection efficiency was monitored by cotransfection of pMIR-REPORT-β-gal (Promega). Cell extracts were prepared after transfection, and the luciferase and β-galactosidase assays were conducted according to manufacturer's instruction (Promega). Transfection experiments were carried out 3 times in triplicate. Data were represented as the ratios of luciferase activities/β-galactosidase activities.

Cell proliferation assay

Cell proliferation was determined by the CCK8 (Beyotime Inst Biotech) according to manufacture's instructions. Briefly, 2×10^3 cells per well were seeded in 96-well plates and cultured for 24 hours. Then, the cells were transfected with miR-421 mimic/miR-421 inhibitor or corresponding mimic NC/inhibitor NC. After 24, 48, or 72 hours, 10 μL WST-8 dye was added to each well. Subsequently, the cells were incubated at 37°C for 3 hours,

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and the absorbance was determined at 450 nm using a microplate reader.

Cell migration and invasion assays

Cell migration and invasion were determined with Transwell migration and extracellular matrix-coated invasion chambers (Millipore) according to the manufacture's instruction. Briefly, the HCC cells were harvested and resuspended in serum-free medium after pretreatment for 24 hours with miR-421 mimic/miR-421 inhibitor or corresponding mimic NC/inhibitor NC. Then 1×10^5 cells were plated into the top well of a Transwell migration chamber (for migration assay) or an extracellular matrix-coated invasion chamber (for invasion assay). The bottom well of the chamber contained 500 μL Dulbecco's Modified Eagle's Medium supplemented with 5% FBS (for migration assay) or 10% FBS (for invasion assay). After 24 hours incubation, the nonmigrating/noninvading cells were removed with a cotton swab, and the migrating/invading cells on the underside of the membrane were stained with cell stain solution (Millipore) for 10 minutes. After washed 3 times with water, the stain of each membrane was removed with 100 μL extraction buffer (Millipore) and quantitated with a colorimetric microplate reader at 570 nm.

Statistical analysis

Each experiment was carried out as least 3 times, and data are shown as the mean ± SD. The differences were evaluated with the Student *t* test for 2-group comparisons and one-way ANOVA for 3-group comparisons. All statistical analyses were conducted with SPSS 13.0 software. $P < 0.05$ was considered statistically significant.

Results

FXR protein level is inversely correlated with miR-421 in HCC cell lines

The FXR protein and mature miR-421 levels were separately examined by Western blot (Fig. 1A and B) and real-time RT-PCR (Fig. 1C) in 4 human HCC cell lines and hepatic cell L02. The results showed that there was a significant inverse correlation between miR-421 and FXR protein levels (Pearson correlation, $r = 0.922$, $P < 0.05$). Moreover, compared with L02 hepatic cells, HepG2 and Hep3B cells had a lower level of FXR protein and a higher expression of miR-421. These results suggest that the FXR protein may be decreased along with the increased miR-421 in HCC cells.

miR-421 downregulates FXR protein expression in HCC cell lines

To investigate whether miR-421 was involved in the downregulation of FXR, the HCC cell lines HepG2, Hep3B, and Huh7 cells were separately transfected with miR-421 mimic. As shown in Fig. 2A, treatment with miR-421 mimic induced a repression of FXR protein. Conversely, transfection of miR-421 inhibitor resulted in a significant increase of FXR protein in the above 3 HCC cells (Fig. 2B).

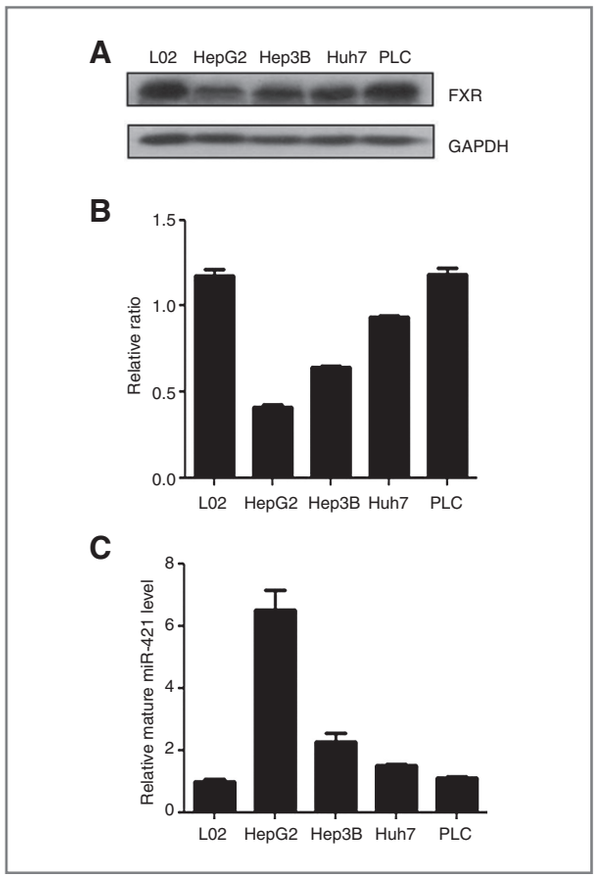

Figure 1. miR-421 is inversely correlated with FXR protein level in HCC cell lines. A, Western blot analysis of FXR protein in L02, HepG2, Hep3B, Huh7, and PLC. B, the relative protein ratio of FXR/GAPDH for (A). C, real-time RT-PCR analysis of mature miR-421 in HepG2, Hep3B, Huh7, PLC, and L02 cells. The values were the mature miR-421 normalized with U6 snRNA relative to that in L02 cells.

However, the FXR mRNA was not affected by the treatment of miR-421 mimic (Fig. 2C).

SHP is a typical target gene of FXR, and ligand-activated FXR activates the transcription of *SHP* by binding to *SHP* promoter region. To inquire whether the downregulation of FXR protein by miR-421 is functional, the reporter assay was conducted with phSHP-Luc construct. As shown in Fig. 2D, treatment with miR-421 mimic significantly decreased both the basal and the specific FXR ligand GW4064-induced transcriptional activities of *SHP* promoter in HepG2 cells. In contrast, the transfection of miR-421 inhibitor significantly restored both the GW4064-induced and basal transcriptional activity in HepG2 cells (Fig. 2E). These results indicated that miR-421–induced downregulation of FXR protein led to decrease of FXR function in HCC cells.

miR-421 targets 3′-UTR of FXR mRNA

The above studies clearly showed that miR-421 suppressed FXR translation. We then examined whether miR-421 modulated FXR protein level by targeting 3′-UTR

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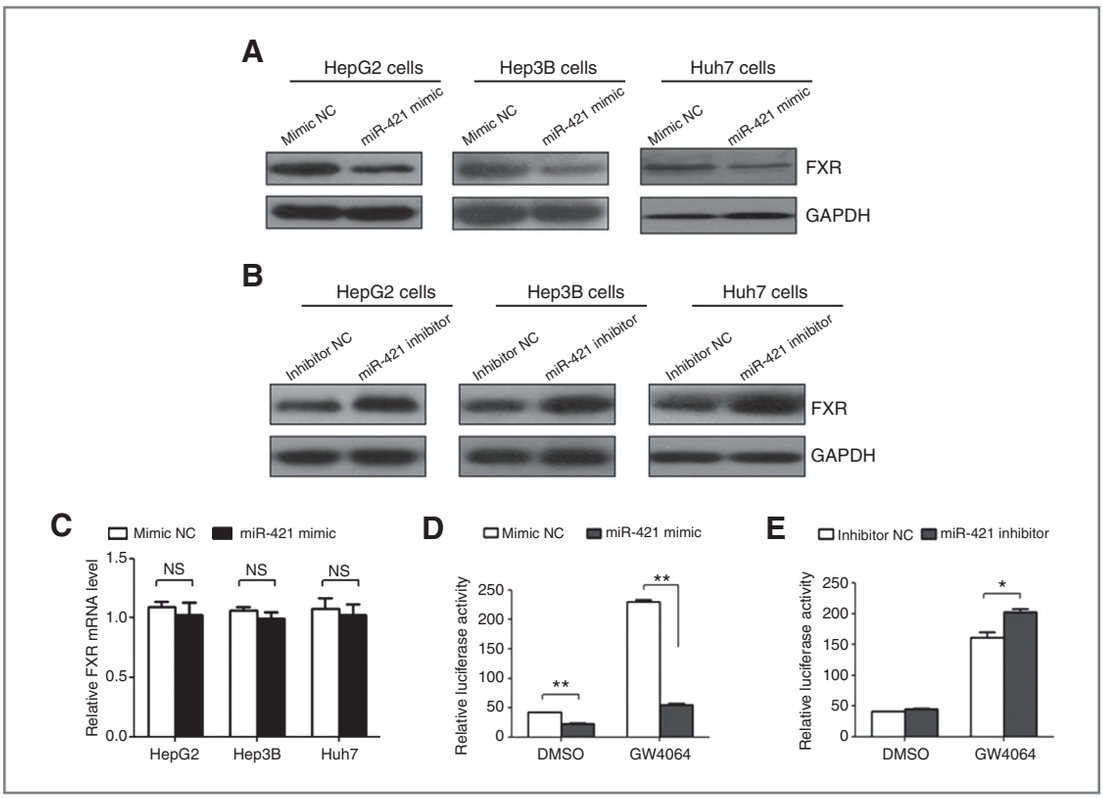

Figure 2. miR-421 mimic downregulates FXR protein in HCC cells. A, Western blot analysis of FXR protein after transfection with 50 nmol/L miR-421 mimic or mimic negative control (mimic NC) for 48 hours in HepG2, Hep3B, and Huh7 cells. B, Western blot analysis of FXR protein after transfection with 50 nmol/L miR-421 inhibitor or inhibitor negative control (inhibitor NC) for 48 hours in HepG2, Hep3B, and Huh7 cells. C, real-time PCR analysis of FXR mRNA after transfection with 50 nmol/L miR-421 mimic or mimic NC for 24 hours in HepG2, Hep3B, and Huh7 cells. D and E, HepG2 cells were cotransfected with the reporter plasmid phSHP-Luc and miR-421 mimic (D) or miR-421 inhibitor (E). After 48 hours, the cells were treated with 10 μmol/L GW4064 (a specific FXR ligand) or vehicle DMSO for 24 hours. Then the Luciferase assay was conducted. The data were the firefly luciferase activities normalized with the β-galactosidase activities. Each column represents the mean ± SD of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$, versus negative control.

of FXR mRNA. As shown in Fig. 3A, the online database searches (miRBase, TargetScan, miRanda) predicted a potential recognition element of miR-421 in 3′-UTR of human FXR mRNA (FXRMIRE421, 2023 to 2045). Furthermore, cotransfection with miR-421 mimic in Huh7 cells (with a lower endogenous miR-421) resulted in a decrease of the luciferase activity of pMIR/FXRMIRE plasmid containing the FXRMIRE421 (Fig. 3B). Whereas, cotreatment with miR-421 inhibitor in HepG2 cells (with a higher endogenous miR-421) led to significant restoration of the luciferase activities of pMIR/FXRMIRE (Fig. 3C). However, the luciferase activity of pMIR/FXRMIRE-Mut plasmid containing mutations in miR-421 recognition site was not affected by the cotransfection of miR-421 mimic or miR-421 inhibitor. These results indicated that miR-421 directly targeted 3′-UTR of FXR mRNA and resulted in the translation inhibition of FXR.

Downregulation of FXR by miR-421 induces the proliferation of HCC cells

As shown in Fig. 4A, the CCK-8 assay showed that treatment with miR-421 mimic for 48 or 72 hours dramatically increased the proliferation of Huh7 cells. In contrast, inhibition of endogenous miR-421 with miR-421 inhibitor significantly suppressed the growth of HepG2 cells (Fig. 4B). Furthermore, a rescue experiment showed that the ectopic expression of FXR (without 3′-UTR) significantly attenuated the miR-421–induced proliferation in HepG2 cells (Fig. 4C), which indicated that downregulation of FXR was involved in the miR-421–inducued proliferation in HCC cells.

Downregulation of FXR by miR-421 promotes the migration and invasion of HCC cells

As shown in Fig. 5, the Transwell migration and invasion assays showed that treatment with miR-421 mimic led to obviously increased migration (Fig. 5A) and invasion (Fig. 5B) of Huh7 cells. In contrast, transfection with miR-421 inhibitor resulted in significantly reduced migration (Fig. 5C) and invasion (Fig. 5D) of HepG2 cells. Moreover, the ectopic expression of FXR (without 3′-UTR) significantly attenuated the miR-421–promoted migration (Fig. 5E) and invasion (Fig. 5F) of HepG2 cells, which indicated that downregulation of FXR by miR-421 was associated with the increased migration and invasion of HCC cells.

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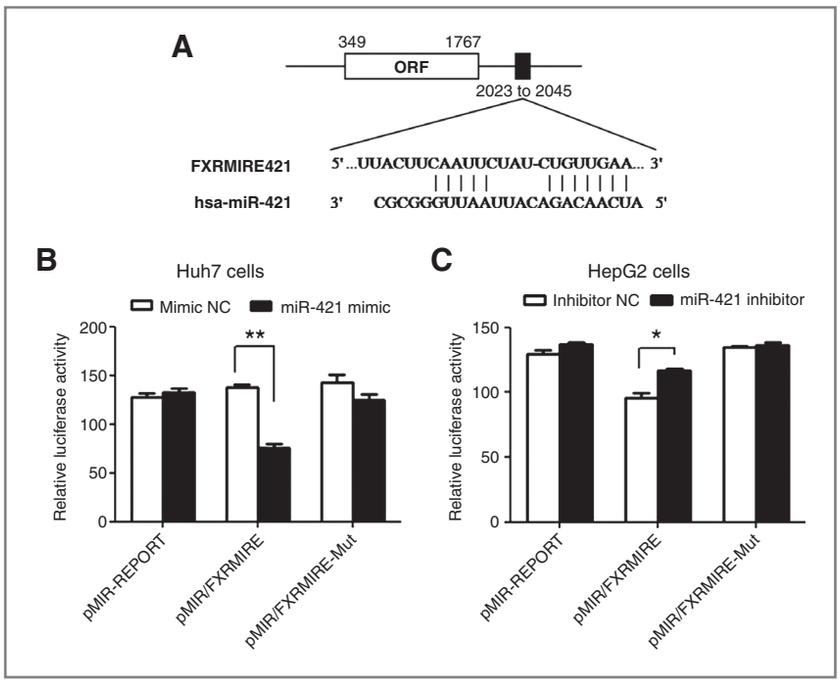

Figure 3. miR-421 represses FXR translation by targeting 3′-UTR of FXR mRNA in HCC cells. A, putative binding sites of miR-421 in the 3′-UTR of FXR mRNA. The potential miR-421 recognition element is located on +2023 to +2045 in the 3′-UTR of human FXR mRNA, in which the numbering refers to the 5′-end of mRNA as +1. B and C, the wild-type FXR 3′-UTR-containing reporter pMIR/FXRMIRE or the mutant FXR 3′-UTR-containg reporter pMIR/FXRMIRE-Mut were transiently cotransfected with 50 nmol/L miR-421 mimic or mimic NC into Huh7 cells (B), or with 50 nmol/L miR-421 inhibitor or inhibitor NC into HepG2 cells (C) for 24 hours. Then the Luciferase assay was conducted. The data were the firefly luciferase activities normalized with the β-galactosidase activities. Results represent 3 independent experiments carried out in triplicates. *, $P < 0.05$; **, $P < 0.01$, versus negative control.

Discussion

We have clearly showed in this study that the expression of miR-421 was inversely correlated with FXR protein level in HCC cell lines. miR-421 downregulated FXR through directly targeting the 3′-UTR of FXR mRNA. Treatment with miR-421 promoted proliferation, migration, and invasion of HCC cells, which were attenuated by the ectopic expression of FXR (without 3′-UTR).

Evidence suggests that miRNAs are often deregulated in human malignancies and can function as either oncogenes or tumor suppressors (13–15). Recent reports have shown that miR-421 may be an onco-miR in several human cancers including pancreatic cancer, gastric cancer, and neuroblastoma (10–12). However, it is not well known about the action mechanism of miR-421. It is predicted that an average miRNA can have more than 100 targets (16), but so far just 5 targets of miR-421 have been identified including DPC4/Smad4, ATM, CBX7, RBMXL1, and CNTN-1 (17–19). In this study, we showed that FXR is a novel target of miR-421 in HCC cells, and downregulation of FXR may be a new oncogenic mechanism of miR-421.

miRNAs can target mRNAs at the 3′-UTR/5′-UTR, even at the open reading frame (ORF) by partial sequence

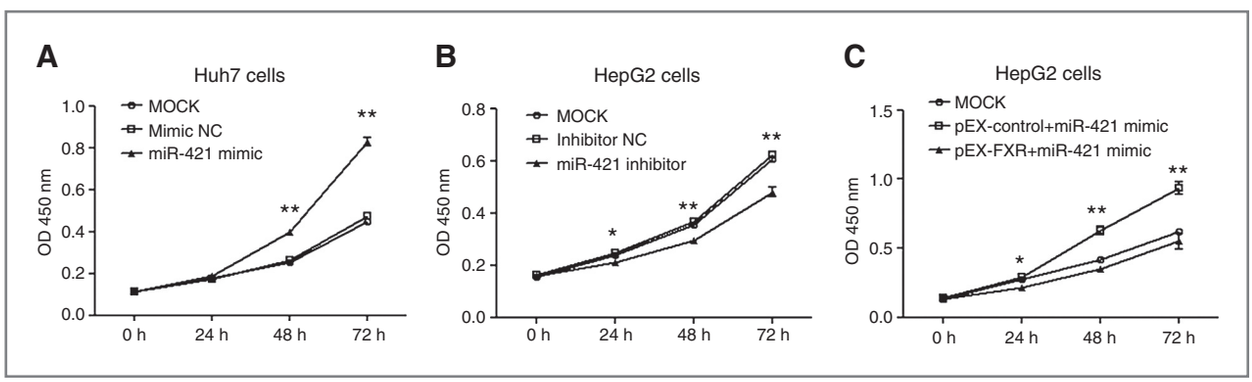

Figure 4. Downregulation of FXR by miR-421 promotes the proliferation of HCC cell. A, Huh7 cells were transfected with 50 nmol/L miR-421 mimic, mimic NC, or blank control culture medium (Mock) for 24, 48, and 72 hours, then the CCK-8 assay was conducted to detect the proliferation of Huh7 cells. B, HepG2 cells were transfected with miR-421 inhibitor, inhibitor NC, or Mock for 24, 48, and 72 hours, and then the CCK-8 assay was conducted. C, HepG2 cells were cotransfected with 50 nmol/L miR-421 mimic and FXR-expressing plasmid pEX-FXR (without 3′-UTR in FXR mRNA) for 24, 48,and 72 hours, then the cell proliferation was examined by CCK-8 assay. Data are means of 3 separated experiments ± SD. *, $P < 0.05$; **, $P < 0.01$, versus negative control.

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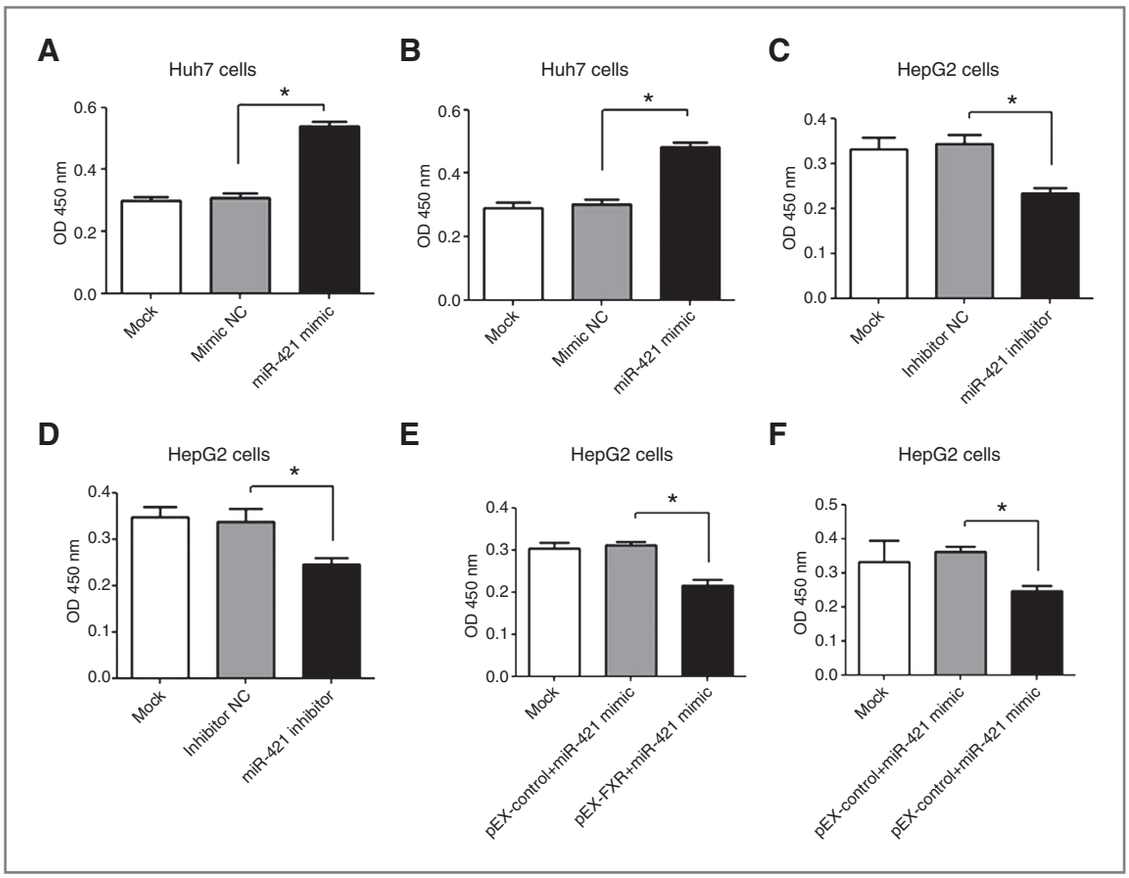

Figure 5. Downregulation of FXR by miR-421 promotes the migration and invasion of HCC cells. A and B, after transfected with miR-421 mimic, mimic NC, or Mock for 24 hours, the Huh7 cells were seeded in Transwell filters (A) or ECM gel-coated Transwell culture chambers (B) and incubated for 24 hours, then the Transwell migration assay (A) or invasion assay (B) were conducted. C and D, HepG2 cells were transfected with miR-421 inhibitor, inhibitor NC, or Mock for 24 hours, then the cells were seeded in Transwell filter, and after 24 hours incubation, migration assay (C) or invasion assay (D) were conducted. E and F, HepG2 cells were cotransfected with 50 nmol/L miR-421 mimic and FXR-expressing plasmid pEX-FXR (without 3′UTR in FXR mRNA) for 24 hours, then the cells were seeded in Transwell filter, and after 24 hours incubation, migration assay (E), or invasion assay (F) were conducted. Data are means of 3 separated experiments ± SD. *, $P < 0.05$ versus negative control.

homology, leading to translation repression/mRNA degradation (20–22). The current miRNA databases, such as miRBase, TargetScan, miRanda, and so on, are usually used for predicting the miRNAs for a target gene or predicting the targets of a miRNA based on the 3′-UTR sequences of mRNAs. In this study, we just analyzed and identified that miR-421 suppresses the translation of FXR protein through directly targeting the 3′-UTR of FXR mRNA. But it does not affect the level of FXR mRNA. More studies are warranted about whether miR-421 can regulate FXR by targeting 5′-UTR or ORF of FXR mRNA.

Besides involved in the regulation of bile acid, lipid, and glucose metabolisms, recent studies have shown that FXR can protect against tumorigenesis and inhibit cell growth in several cancers including HCC (23–25). Usually, FXR regulates cell proliferation and/or apoptosis through the function of its target genes. For example, SHP, a typical target gene of FXR, has been shown to suppress cell proliferation and promote apoptosis (23). It is reported that the expression of SHP was downregulated in human HCC, and mice with SHP deficiency developed spontaneous liver tumors (24, 26). The accumulating data suggest that FXR may be a pharmaceutical target for the treatment of the associated cancers including HCC.

Recently, the new evidences indicate that miRNAs are involved in the regulation of tumor-associated nuclear receptors such as estrogen receptor α (27, 28), progesterone receptor (29), glucocorticoid receptor (30), liver X receptor (31), and mineralocorticoid receptor (32). In this study, we showed that miR-421 downregulated the antitumor nuclear receptor FXR and promoted the proliferation, migration, and invasion of HCC cells, which suggest that miR-421 may serve as a novel molecular target for manipulating FXR expression in hepatocyte and for the treatment of HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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