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# FXR blocks the growth of liver cancer cells through inhibiting mTOR-s6K pathway

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ABSTRACT

The nuclear receptor Farnesoid X Receptor (FXR) is likely a tumor suppressor in liver tissue but its molecular mechanism of suppression is not well understood. In this study, the gene expression profile of human liver cancer cells was investigated by microarray. Bioinformatics analysis of these data revealed that FXR might regulate the mTOR/S6K signaling pathway. This was confirmed by altering the expression level of FXR in liver cancer cells. Overexpression of FXR prevented the growth of cells and induced cell cycle arrest, which was enhanced by the mTOR/S6K inhibitor rapamycin. FXR upregulation also intensified the inhibition of cell growth by rapamycin. Downregulation of FXR produced the opposite effect. Finally, we found that ectopic expression of FXR in SK-Hep-1 xenografts inhibits tumor growth and reduces expression of the phosphorylated protein S6K. Taken together, our data provide the first evidence that FXR suppresses proliferation of human liver cancer cells via the inhibition of the mTOR/S6K signaling pathway. FXR expression can be used as a biomarker of personalized mTOR inhibitor treatment assessment for liver cancer patients.



## 1. Introduction

The nuclear receptor Farnesoid X Receptor (FXR) has historically been considered primarily as a regulator of metabolism and has multifaceted functions in maintaining the homeostasis of bile acid, lipid and glucose levels in the liver [1–3]. Recent studies suggest that FXR is also a tumor suppressor in liver tissue [4–6]. FXR plays an important role in inhibiting liver carcinogenesis and progression. FXR exerts an anti-carcinogenic effect in liver tissue through diverse mechanisms [7].

Limitless replicative potential is one of the hallmarks of cancer cells, which, in human tumors, is caused by deregulated growth signaling pathways [8]. Both exogenous FXR and FXR agonists repress the proliferation of liver cancer cells in vitro and the growth of cancerous xenografts in nude mice in vivo [6–9]. This suggests that FXR may play a role in suppressing deregulated signaling pathways in cancerous cells.

The mammalian Target of Rapamycin (mTOR) is a well-conserved serine/threonine kinase that participates in the signaling network that regulates cell growth and metabolism [10]. mTOR interacts with many proteins and it is part of two distinct multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [11]. While the functions of mTORC2 remain mysterious, mTORC1 has a well-documented sensitivity to

*Abbreviations:* AP, Ammonium peroxydisulfate; Acr-Bis, Acrylamide-bisacrylamide; BCA, Bicinchoninic acid; BLAST, Basic Local Alignment Search Tool; BSA, Bovine serum albumin; DEPC, Diethypyrocarbonate; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's modified eagle medium; ECL, Enhanced Chemiluminescence; FXR, farnesoid X receptor; FBS, Fetal bovine serum; FACS, Fluorescence-activated cell sorting; GFP, Green fluorescent protein; HCC, Hepatocellular carcinoma; LV, Lentiviral vector; mTOR, Mammalian target of rapamycin; MAPK, Mitogen-activated protein kinase; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PMSF, Phenylmethanesulfonyl fluoride; PVDF, Polyvinylidene Fluoride; RT, Reverse transcription; S6K, Ribosomal Protein S6 Kinases; RNAase, Ribonuclease; SDS, Sodium dodecyl sulfate; siRNA, Small interfering RNA; TEMED, N,N,N′,N′-Tetramethylethylenediamine; Tris, Tris(hydroxymethyl)aminomethane.

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rapamycin, and it plays a central role in the promotion of cell proliferation by integrating inputs from five major signals—amino acid abundance, energy status, growth factors, hypoxia, and genotoxic stress [11,12]. The direct role of mTOR-dependent mechanisms in the proliferation and survival of many kinds of cancer cells is well documented [12–14]. Activated mTORC1 induces phosphorylation of its target kinase S6K which then drives accumulation of many oncogenic products [12].

Our previous studies indicated that FXR inhibited the growth of human hepatocellular carcinoma (HCC) cells both in vitro and in vivo [6]. To further explore the possible mechanisms by which FXR exerts this function, we used lentiviral induction of FXR overexpression in liver cancer cells, and then determined differentially expressed genes by mRNA microarray. Analysis of these data suggested that FXR is involved in regulating the mTOR/S6K signaling pathway. We then performed further investigations into the FXR-mediated suppression of mTOR/S6K signaling.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

SK-Hep-1 cells were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China). Huh7 cells were obtained from Dr. Xu Lin (Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Fujian Medical University, Fuzhou, China). Cells were routinely cultured in complete medium (DMEM supplemented with 10% heat-inactivated BSA and 1% penicillin-streptomycin) at 37 °C in a humidified atmosphere with 5% CO2. Reagents for cell culture were obtain from Millipore (Billerica, MA, USA). The FXR agonist GW4064 were purchased from Sigma–Aldrich (St.Louis, MO,USA) and mTOR inhibitor rapamycin from Cell Signaling Technologies Inc. (Danvers, MA, USA).

### 2.2. Construction of human FXR recombinant lentivirus and transduction into SK-Hep-1 and Huh7 cells

The procedures of making the human FXR overexpression or FXR knockdown Lentiviral vectors and the corresponding negative controls, and the protocols of lentivirus transduction are as previously described [1]. The FXR target sequence for interation is: GCCTCTGGATACCACTATAAT. For SK-Hep-1 cells, when transfected with lentiviral vectors that selectively overexpressed FXR or negative control were termed SK-Hep-1-FXR or SK-Hep-1-NC. For Huh7 cells, when transfected with lentiviral vectors that selectively knockdown FXR or negative control were named Huh7-siFXR or Huh7-NC.

### 2.3. Microarray and bioinformatics analysis

SK-Hep-1-FXR and SK-Hep-1 NC cells were pretreated with GW4064 2 μM for 24 h, then total RNA were isolated and sent to the Kangchen Bio-tech (Shanghai, China). The human gene expression microarray analysis for the two cell lines was carried out on the Agilent Array platform (The project code is H1310075).

### 2.4. Western blot analysis

Total soluble proteins from cells were extracted and the protein concentration were determined by the BCA protein assay (Beyotime, Beijing, China). Western blot analysis was performed as described previously [1]. Anti-FXR antibody was from R&D Systems (Minneapolis, MN, USA). Anti-β-actin antibody was from TransGen Biotech (Beijing, China). Primary antibodies that identified mTOR, p-mTOR, S6K and p-S6K were from Cell Signaling Technologies Inc. (Beverly, MA, USA). The chemoluminescence reagent was obtained from Millipore (Danvers, MA, USA).

### 2.5. Cell viability assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT) assay according to the manufacturer's instructions (Sigma–Aldrich,St.Louis, MO,USA). Briefly, cells were seeded at a density of $1.5 \times 10^3$/well in 96-well plates and incubated for 24 h. After treatment with 2 μM GW4064 or 20 nM rapamycin or both the two drugs for 24 h, the cells was added MTT solution (5 mg/ml) for 4 h at 37 °C. The absorbance value of each well was measured at 570 nm. Cells were incubated for 1, 2, 3, and 4 days.

### 2.6. Cell cycle analyses with flow cytometry

Cells were seeded at $5 \times 10^4$ cells/well in 6-well plates and treated with 2 μM GW4064 or 20 nM rapamycin or both the two drugs for 24 h. Then cells were harvested and fixed with 70% ethanol in PBS at −4 °C overnight. Then resuspended the cells in PBS containing 0.2% Triton X-100 for nuclear isolation. RNase A (0.5 mg/ml; Nacalai Tesque) and propidium iodide (25 μg/ml; Sigma–Aldrich) were added for RNA degradation and DNA staining. Flow cytometry analysis was performed with a FACSVerse flow cytometer. The cell cycle was analyzed with Modi-Fit software (Becton Dickinson, USA).

### 2.7. RNA isolation and quantitative real-time PCR

Total RNA from cells was isolated with TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany), and 500 ng of total RNA were reverse transcribed using the Primescript RT reagent kit (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. Quantitative real-time PCR for the quantification of mRNA was performed using SYBR Premix Ex Taq (Takara Bio, Tokyo, Japan) and an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Expression levels of target genes were normalized to the expression of β-actin [1]. The following specific forward and reverse primers were used: FXR, forward 5′-ATGCCTGTAACAAAGAAGCCCC-3′ and reverse 5′- CACACAGTTGCCCCCGTTTTTAC-3′.

S6K, forward primer 5′-AAGGGGGCTATGGAAAGGCAA-3'and reverse 5′- AATCCACGATGAAGGGATGCT-3'; mTOR, forward 5′-GAACCTCAGGGCAAGATGCT-3′ reverse 5′-CTGGTTTCCTCATTCCGGCT-3′.

### 2.8. Tumorigenicity in nude mice

Male BALB/c *nu*/*nu* mice (4–6 wk old) were obtained from Shanghai SLAC Laboratory Animal (Shanghai, China). Mice were injected subcutaneously into the flank with $5 \times 10^6$ SK-Hep-1-FXR or SK-Hep-1-NC. Tumor growth was observed as previously described [1]. Animals were sacrificed on day 21 after injection, and tumors were harvested for the analysis of protein expression. All experimental procedures involving animals were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Animals, and were approved by the Animal Welfare Committee of Fujian Medical University.

### 2.9. Immunohistochemistry

Immunohistochemical staining was performed with the Envision Plus System according to the manufacturer's instructions. Primary antibodies against p-S6K were used to detect the