Silencing FBXO7 suppresses cell growth of Hepatocellular carcinoma through autophagy

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Research Article

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Abstract

Hepatocellular carcinoma (HCC) is a common malignant tumor. However, the mechanisms of the growth, invasion and metastasis of HCC were still unclear. FBXO7 belongs to F-box protein family, which is closely related to tumorigenesis and progression, however, the role of FBXO7 in HCC is still elusive. In this study, we explored the role of FBXO7 in the progression HCC. The expression of FBXO7 in HCC cells was higher than that of normal liver cells. After FBXO7 was silenced by siRNA, the cell proliferation, colony-forming ability, migration and invasion of HCC cells were inhibited. Moreover, the cell cycle of HCC cells was also affected after FBXO7 was silenced, the proportion of cells at G1 phase was increased and the proportion of cells at S phase was decreased. In addition, autophagy of HCC cells was increased after FBXO7 was silenced. In conclusion, FBXO7 may play an oncogenic role in HCC development and progression through autophagy.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the common malignant tumors worldwide, which seriously threatens human health[1]. Risk factors for HCC include chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol consumption, and aflatoxin[2]. At present, the treatment of HCC is mainly surgery, supplemented by chemotherapy, intervention or targeted therapy[3]. However, due to the characteristics of strong invasion[4], the therapeutic effect is not ideal. Therefore, it is of great significance to explore the molecular mechanisms of the growth, invasion and metastasis of HCC, which can provide a theoretical basis for the molecular diagnosis, prognosis and treatment of HCC.

F-box protein 7 (FBXO7) belongs to F-box protein family, which participates in the formation of the Skp1-Cul1-F-box (SCF) E3 complex and plays an important role in the ubiquitin-proteasome system (UPS)[5]. Current research on FBXO7 mainly focuses on Parkinson's syndrome, FBXO7 maintains mitochondrial function through direct interaction with PINK1 and Parkin, and plays a role in Parkin-mediated mitophagy[6]. In addition, FBXO7 is also involved in the regulation of cell cycle and is closely related to tumorigenesis and progression[7, 8]. FBXO7 negatively regulates the proliferation and differentiation of primary hematopoietic stem cells (HSPC) in a p53-dependent manner, and in the absence of p53, FBXO7 expression can promote T-cell lymphoma formation[9]. In addition, FBXO7 promotes the maturation and differentiation of erythrocytes and T cells by regulating the activities of P27 and CDK6[10, 11]. However, the biological behavior and the molecular mechanism of FBXO7 in HCC are still unclear. Therefore, this study was aimed to investigate the expression and biological significance of FBXO7 in HCC.

Autophagy is an intracellular degradation process in eukaryotes under the condition of cell starvation or external stimulation, which can maintain the balance of protein metabolism and stability of cell environment[12]. Multiple proteins participate in the process of autophagy, among which P62 and LC3B could be used as molecular markers of autophagy[13]. Autophagy has a bidirectional function in tumor cells depending on types, stages or genetic context of the cancers. On the one hand, autophagy can remove damaged and senescent organelles and biological macromolecules in cells and prevent cell

malignancy before the occurrence of tumor. On the other hand, autophagy can help tumor cells fight against hypoxia, nutrient deficiency and other adverse growth environment, and promote the growth of tumor after tumorigenesis[13, 14]. A variety of antitumor drugs have been developed depending on autophagy[15]. In conclusion, we hypothesized that FBXO7 may be involved in the occurrence and development of HCC through autophagy. This study provided a new point for studying the occurrence and development of HCC, and further clarified the mechanism of FBXO7 in HCC, which may provide a new theoretical basis for blocking the development of HCC.

2. Materials And Methods

2.1 Cell culture and Cell Transfection

Human HCC cell lines (MHCC97H, SMMC7721, Huh7 and HepG2) and human normal liver cell line LO2 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) with 5% CO_2 at 37°C. The siRNA targeting FBXO7 (siRNA-FBXO7 sequence: GGAAAGAACTGTACAGGAA) was used to decrease FBXO7 expression. Lipofectamine 2000 (Invitrogen, USA) was carried out to perform the transfections.

2.2 Quantitative PCR (qPCR)

Total RNA was extracted using RNAiso Plus (Takara, Japan) following the manufacturer's protocol. Total RNA samples were incubated with RNase-free DNase I to eliminate contaminating DNA. The cDNA was synthesized using a specific reverse transcription kit (Takara, Japan) following the manufacturer's protocol. QPCR was performed with SYBR Green (Takara, Japan). GAPDH was uded as an internal control. The relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ method[16]. Primers used in this study are listed as follows: FBXO7-F: TGCTCTGTAGTGAATCGGA, FBXO7-R: CTTCGGTGCCCTGAGGTATG; GAPDH-F: GAGTCCACTGGCGTCTTCA, GAPDH-F: TCTTGAGGCTGTTGTCATACTTC.

2.3 Western Blot Assay

Cultured cell lysates were prepared by lysis buffer supplemented with protease inhibitor. The protein concentration was determined with the BCA Kit (Beyotime, China). Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membrane. Afterwards, the membranes were blocked with 5% skim milk for 2 h at room temperature and incubated with primary antibodies overnight at 4°C. After incubating with a secondary antibody for 2 h at room temperature, immunoreactive bands were visualized by automatic chemiluminescence image analysis system (Vilber Lourmat, France)[17].

2.4 Cell Proliferation Assay

Cells were seeded in 96-well plates at a density of 5000 cells per well. CCK8 assay was used to measure the number of viable, proliferating cells after transfection. The absorbance was measured at 450 nm[3].

2.5 Colony Forming Assay

Cells were plated in 6-well plates and incubated in DMEM containing 10% fetal bovine serum. Two weeks later, the cells were fixed in methanol for 30 minutes and stained with 1% crystal violet dye and counted using microscope[3].

2.6 Wound healing and Transwell assay

Cell migratory capacity was analyzed using the wound-healing assay. Cells were cultured in 6-well plates and cultivated until 95% confluent. Wounds were incised in the cell monolayer using a sterile pipette tip. At 0 h and 48 h after the wounding, the distance between the two wounds were measured under microscope[16].

For the Transwell assay, cells were seeded into the upper chamber of a transwell insert. For the invasion experiment, the upper chamber was coated with Matrigel, and the lower chamber contained a 20% FBS medium. After incubation for 48 h, the cells were fixed, stained, and then counted under a microscope[18].

2.7 Cell cycle analysis

DNA content in cells was measured by flow cytometry of cells stained with DAPI. Cells were fixed with alcohol overnight, and stained with DAPI (Beyotime, China) for 30 min and cell populations in the G0/G1, S, and G2/M phases were measured by a flow cytometry (BD Biosciences, USA)[19].

2.8 Luciferase Reporter Gene Assay

The sequence of LC3 and GFP 3′ untranslated region (3′-UTR) was cloned into the adenovirus vector. After 24 h of transfection, luciferase activity was tested by a dual luciferase assay system (Olympus, Japan)[16].

2.9 Statistical Analysis

Independent repetitions of experiments were performed three times. The data were presented as means ± standard deviation (SD). The differences were analyzed using Student's *t*-tests. Statistical analysis was performed using SPSS Statistics 20.0 software. $P < 0.05$ was considered statistically significant[18].

3. Results

3.1 FBXO7 was highly expressed in HCC cells

To evaluated the expression of FBXO7 in HCC, qPCR and western blot were performed. The expression of FBXO7 in HCC cell line SMMC7721 and HepG2 was higher than that of normal liver cell line L02, however, the expression of FBXO7 in Huh7 was lower than L02, and there was no significant difference between 97H and LO2 (Fig. 1A and B).

3.2 Silencing FBXO7 inhibited proliferation and colony-forming of HCC

To investigate the role of FBXO7 in HCC, FBXO7 was silenced by siRNA. The efficiency was tested by qPCR. In the siRNA silenced groups, the expression of FBXO7 mRNA in HepG2 and SMMC7721 was significantly lower compared with the negative control groups (NC) (Fig. 1C and D).

To evaluated the role of FBXO7 on cell proliferation of HCC, CCK8 was performed. The OD450nm of siRNA silenced HepG2 and SMMC7721 was significantly lower than negative control (NC) (Fig. 2A and B), suggesting that cell proliferation of HCC was inhibited by silencing FBXO7. Consistent with the CCK8 assay, the colony forming ability was also inhibited in siRNA silenced HepG2 and SMMC7721 cells (Fig. 2C and D).

3.3 Silencing FBXO7 inhibited cell migration and invasion of HCC

The capacity of cell migration was determined using wound healing assay. After 48 h, the wound was almost filled in the NC groups of HepG2 and SMMC7721, while there was still a gap in siRNA silenced groups (Fig. 3A and B). Moreover, the capability of cell invasion was detected using Transwell assay, the cell invasion of siRNA silenced HepG2 and SMMC7721 were both inhibited (Fig. 3C and D). These results indicated that silencing FBXO7 impaired the capacity of cell migration and invasion of hepatocellular cancer cells.

3.4 Silencing FBXO7 affected the cell cycle of HCC

To explore the potential mechanism of FBXO7 on cancer cell proliferation, we detected the proportion of cells in each phase of cell cycle after FBXO7was silenced by siRNA. Flow cytometry analysis showed that silencing FBXO7 inhibited SMMC7721 and HepG2 cell growth by causing progressive cell cycle arrest, as siRNA silenced HepG2 and SMMC7721 have more cells at G1 phase, but less cells at S phase than negative control (Fig. 4A and B).

3.5 Silencing FBXO7 induced autophagy of HCC

To investigate the role of autophagy on HCC through FBXO7, we detected the expression of autophagy related protein P62 and LC3 using western blot and detected the localization of LC3 using immunofluorescence. After FBXO7 was silenced by siRNA, the expression of FBXO7was down-regulated, confirming that FBXO7 was successfully silenced. Meanwhile, the expression of P62 was down-regulated and LC3 was up-regulated (Fig. 5A), these results indicated that autophagy was induced after FBXO7 was silenced. Immunofluorescence assay showed that LC3 was mainly located in the cytoplasm and was fine granular and vesicular, and the fluorescence of LC3 was increased in the silenced group compared with negative control (Fig. 5B), further confirming that silencing FBXO7 enhanced autophagy of HepG2 and SMMC7721 cells.

Discussion

Hepatocellular carcinoma (HCC) is a common malignant tumor, and the risk factors of HCC is including chronic HBV and HCV infection, alcohol consumption, and aflatoxin[20]. HCC have the character of strong

invasion[4], as a result, it is a great threat to human health. Collectively, it is of great significance to explore the molecular mechanisms of the growth, invasion and metastasis of HCC, which can provide a theoretical basis for the molecular diagnosis, prognosis and treatment of HCC.

FBXO7 belongs to F-box protein family, which participates in the ubiquitin-proteasome system[21]. In addition, FBXO7 is also involved in the regulation of cell cycle and is closely related to tumorigenesis and progression[22, 23], however, the role of FBXO7 in HCC is still elusive. In this study, the expression of FBXO7 in HCC cells was higher than that of normal liver cells, indicating that FBXO7 may play a role in the progression of HCC. In order to investigate the function of FBXO7 in HCC, FBXO7 was silenced by siRNA in this study. The results showed that silencing FBXO7 inhibited the cell proliferation, migration and invasion of hepatocellular carcinoma. Collectively, FBXO7 may play an oncogenic role in HCC development and progression. Moreover, the proportion of cells at G1 phase was increased and the proportion of cells at S phase was decreased after FBX07 was silenced, indicating that silencing FBXO7 in HCC cells might inhibit cell proliferation through promoting cell cycle arrest.

Autophagy is an intracellular degradation process in eukaryotes, among which ubiquitination plays an important role in the process of autophagy[24]. As FBXO7 participates in the ubiquitin-proteasome system[25], we speculated that FBXO7 may play a part in the progression of HCC through autophagy. To verify this hypothesis, we detected the status of autophagy in HCC cells, the results showed that autophagy was enhanced after FBXO7 was silenced. However, autophagy has a bidirectional function in tumor cells, on the one hand, autophagy can prevent cell malignancy before the occurrence of tumor; on the other hand, autophagy can promote the growth of tumor after tumorigenesis[26]. As a result, the role of autophagy induced by FBXO7 in HCC deserves further investigation.

In conclusion, FBXO7 may play an oncogenic role in hepatocellular carcinoma development and progression through autophagy. As a result, FBXO7 can be used as a biomarker for hepatocellular carcinoma, and further studies on the function and mechanism of FBXO7 will help to find new diagnostic and therapeutic methods of hepatocellular carcinoma. However, there are some limitations in this study that we only investigated the biological functions and mechanism of FBXO7 in vitro. The functional role of FBXO7 in vivo is required to explore in future.

Declarations

Conflict of Interest

None

Funding

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

HY and JW designed the project and wrote the manuscript. HY, ZL, WX and CH carried out the experiments. HY and SW analyzed the data. HL and JW reviewed the manuscript.

Data Availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

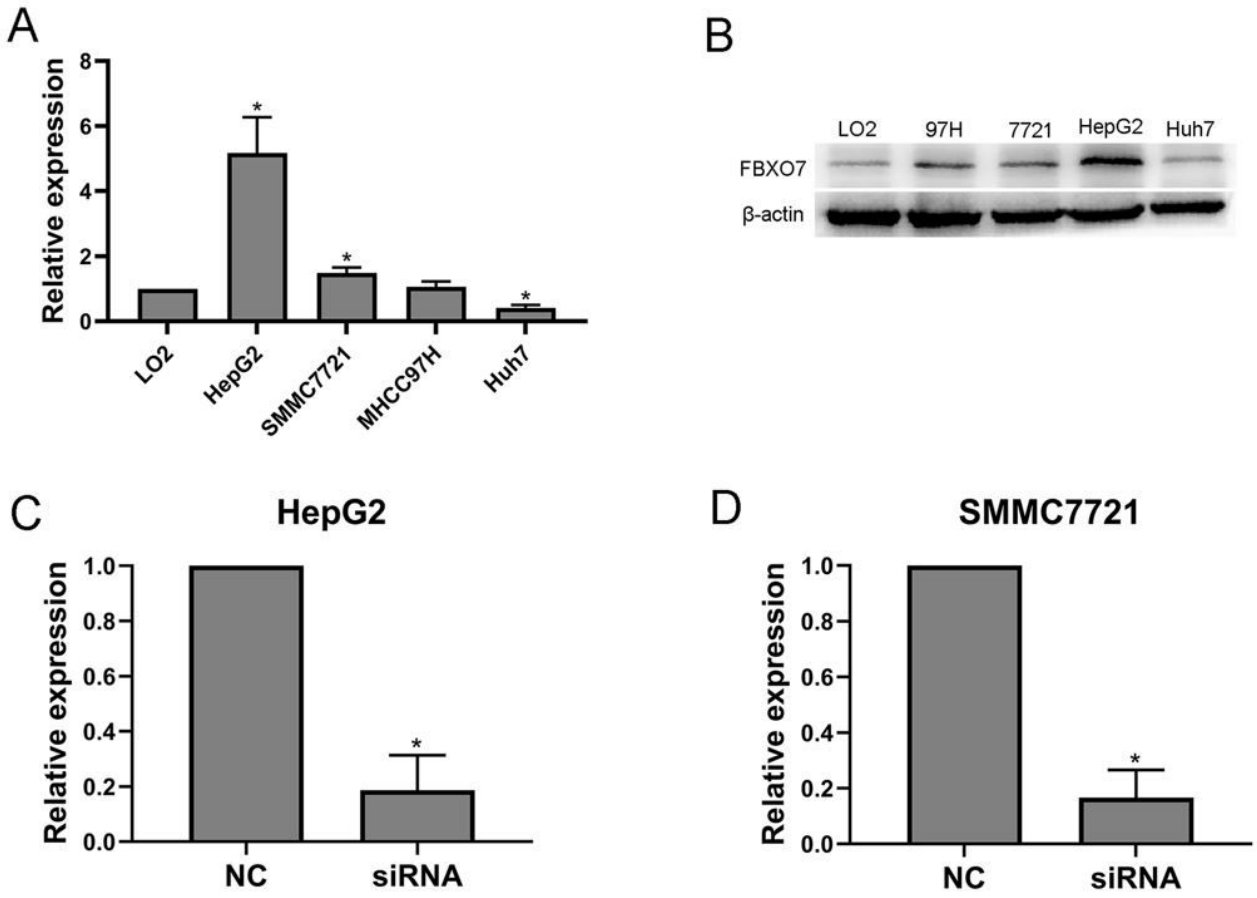

Figure 1

Expression of FBXO7 in hepatocellular carcinoma (HCC) cells. Expression of FBXO7 at (A) mRNA level by qPCR and (B) protein level by western blot in HCC cell lines and normal liver cell. Expression of FBXO7 at mRNA level in (C) HepG2 cell and (D) SMMC7721 cell after FBXO7 was silenced by siRNA. Results presented represent the mean of triplicate experiments ± SD. *$P < 0.05$ compared with negative control (NC).

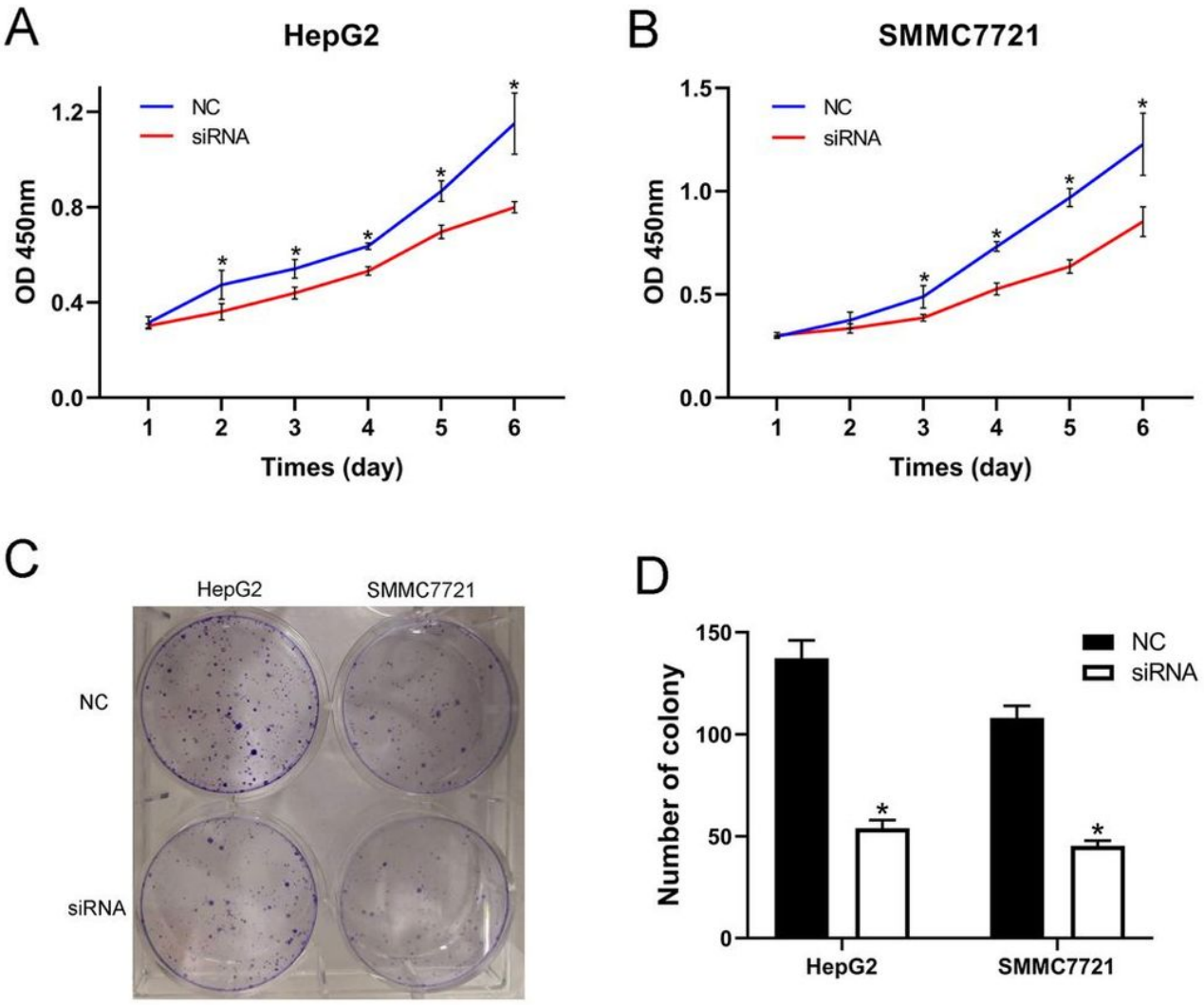

Figure 2

Silencing FBXO7 inhibited cell proliferation of hepatocellular carcinoma cells. CCK8 assay showed that the cell proliferation of (A) HepG2 cell and (B) SMMC7721 cell was inhibited after FBXO7 was silenced. (C-D) Colony forming assay showed that the colony-forming ability of HepG2 cell and SMMC7721 cell was inhibited after FBXO7 was silenced. Results presented represent the mean of triplicate experiments ± SD. *$P < 0.05$ compared with negative control (NC).

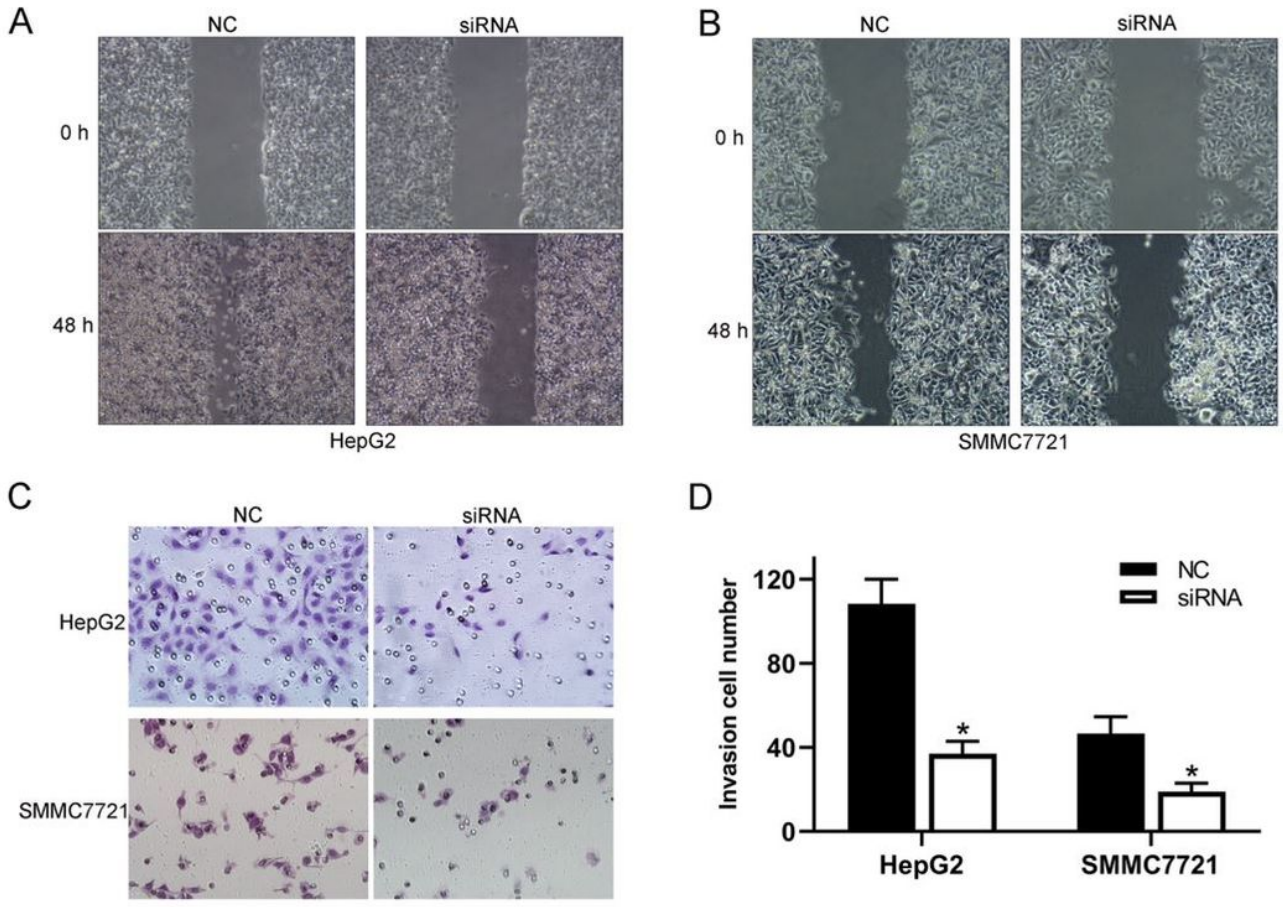

Figure 3

Silencing FBXO7 inhibited cell migration and invasion of hepatocellular carcinoma cells. Cell migration of (A) HepG2 cell and (B) SMMC7721 cell was inhibited after FBXO7 was silenced. (C-D) The invasion of HepG2 cell and SMMC7721 cell was inhibited after FBXO7 was silenced. Results presented represent the mean of triplicate experiments ± SD.*$P < 0.05$ compared with negative control (NC).

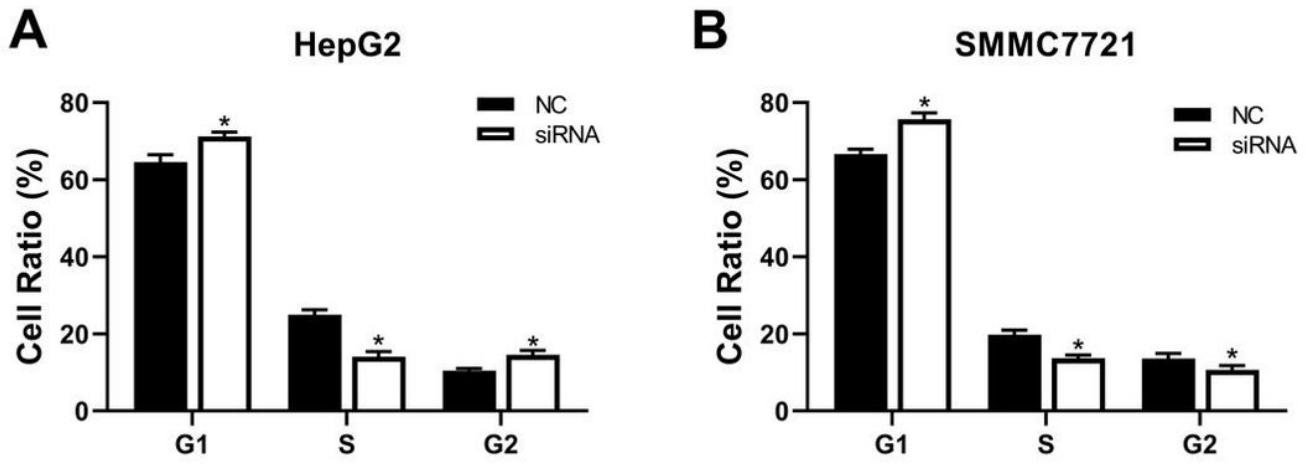

Figure 4

Silencing FBXO7 affected cell cycle of hepatocellular carcinoma cells. The proportion of cells at G1 phase was increased in (A) HepG2 cell and (B) SMMC7721 cell after FBXO7 was silenced. The proportion of cells at S phase was decreased in (A) HepG2 cell and (B) SMMC7721 cell after FBXO7 was silenced. Results presented represent the mean of triplicate experiments ± SD. *$P < 0.05$ compared with negative control (NC).

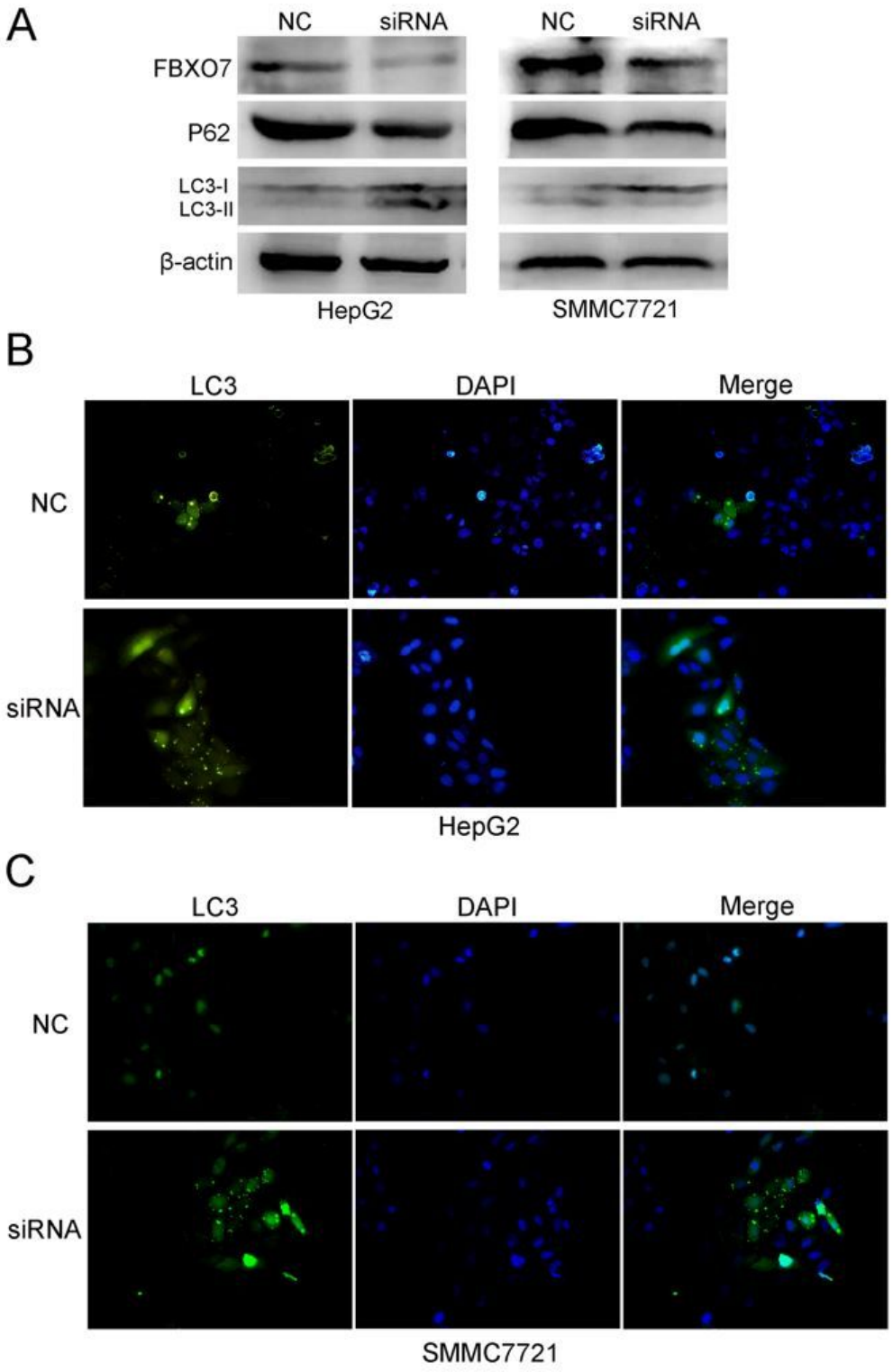

Figure 5

Silencing FBXO7 induced autophagy of hepatocellular carcinoma cells. (A) Western blot showed the expression of FBXO7 and autophagy-related protein P62 and LC3 after FBXO7 was silenced. Immunofluorescence assay showed the expression level and location of LC3 in (B) HepG2 cell and (C) SMMC7721 cell after FBXO7 was silenced.