

NOP7 interacts with β -catenin and activates β -catenin/TCF signaling in hepatocellular carcinoma cells

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Background: The hyperactivation of β -catenin signaling is frequently observed in clinical hepatocellular carcinoma (HCC) samples. Further understanding the mechanisms involved in activating β -catenin/TCF signaling would benefit the treatment of HCC.

Method and results: Here, it was found that NOP7 was a binding partner of β -catenin. NOP7 strengthened the interaction between β -catenin and TCF4, which led to the activation of β -catenin/TCF signaling. The upregulation of NOP7 in HCC promoted the growth (in both liquid culture and soft agar) and migration of HCC cancer cells.

Conclusion: Taken together, we have demonstrated the oncogenic functions of NOP7 in HCC, suggesting that targeting NOP7 would benefit the treatment of HCC.

Keywords: hepatocellular carcinoma, NOP7, β -catenin/TCF pathway, cell growth, cell migration

Introduction

Hepatocellular carcinoma (HCC) is a very common malignancy in Asian countries, and its outcome is still very poor.¹ Therefore, illustrating the mechanisms involved in HCC would help in designing treatments for this disease.

An inactive mutation of Axin or a constitutive mutation of β -catenin are very common in HCC, which leads to the activation of β -catenin/TCF signaling.^{2,3} Cytoplasmic and nuclear accumulation of β -catenin in HCC cells was found in approximately 80% HCC tissues, suggesting a role for the overactivation of β -catenin/TCF signaling in the development of HCC.^{3,4} In the absence of Wnt ligand, β -catenin is phosphorylated and destroyed by the destruction complex.^{5,6} The stimulation of Wnt ligand disassociates the β -catenin destruction complex, and the β -catenin protein level becomes elevated in the cytoplasm, β -catenin then translocates to the nucleus. In the nucleus, β -catenin interacts with TCF4 to promote the expression of downstream genes (N-cadherin, vimentin, Snail, and cyclinD1).^{7,8} The activity of β -catenin/TCF signaling is critical for the malignant behaviors of HCC cells.⁹⁻¹¹ The regulation of β -catenin/TCF signaling occurs at multiple levels.^{12,13} However, how the β -catenin/TCF complex is regulated remains largely unknown.

NOP7 is localized in the nucleus and includes a BRCA1 interaction domain in the C-terminus.¹⁴ NOP7, BOP1, and WDR12 form the PeBoW complex and promote cell proliferation.^{15,16} Moreover, NOP7 is essential for the proliferation and tumorigenicity of breast cancer cells.¹⁷ However, its roles in HCC remain largely unknown. In this study, the expression of NOP7 in HCC tissues and its functions and molecular mechanisms were studied.

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Materials and methods

Cell culture

All of the cell lines used in this study were purchased from the Cell Bank of Shanghai Institutes for Biological Science (Shanghai, People's Republic of China). Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 100 units/mL penicillin, and 100 g/mL streptomycin were used to culture these cell lines, which were incubated with 5% CO₂ at 37°C.

Clinical samples

The clinical samples and paired noncancerous tissues were obtained from patients at Shanghai Stomatological Hospital after obtaining their consent. The clinical samples were kept in liquid nitrogen. This study was performed after being approved by the ethics committee of Fudan University. All patients provided written, informed consent. All experiments were performed following relevant and national guidelines and regulations of Fudan University.

Western blot analysis

Cellular proteins and proteins from the tissues were extracted using RIPA buffer. After separating the proteins by SDS-PAGE, they were then transferred to PDVF membranes. The blocking was performed using 5% BSA solution at room temperature for approximately 40 minutes. Then, the membrane was incubated with primary antibodies overnight. On the second day, TBST solution was used to wash the membrane, and the membrane was then sequentially incubated with secondary antibody for 1 hour at room temperature. The proteins were visualized by an ECL kit.

Immunohistochemistry

After deparaffinizing and rehydrating fixed sections using gradually decreasing concentrations of xylene and ethanol, the sections were incubated with 0.3% H₂O₂ solution for 30 minutes at room temperature. Sodium citrate solution (pH 6.0) was used for antigen retrieval. The sections were blocked with BSA solution to diminish nonspecific binding. Then, the sections were stained with NOP7 antibody and were visualized with secondary antibody (Envision, Gene Technology, Shanghai, China). Slides were then developed with DAB and counterstained with hematoxylin.

GST pull-down

A fusion protein was obtained by cloning the coding sequence of β -catenin into the pGEX-4T-1 vector. A 7404 cell lysate

was prepared using lysis buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.1% NP40, and protease inhibitor cocktail. A total of 5 μ g of GST-NOP7 fusion protein was mixed with 500 μ g of cell lysate and was incubated at 4°C for 4 hours. Then, glutathione-Sepharose-4B beads were mixed with the samples and incubated at 4°C for an additional 1 hour. The beads were collected by centrifugation and washed with lysis buffer. Finally, Laemmli buffer was used to elute the proteins bound to the beads, and SDS-PAGE was performed.

Immunoprecipitation assay

Cell lysates were prepared with RIPA buffer. After centrifugation, the supernatant was incubated with the antibody overnight at 4°C. Then, the protein A beads were mixed with the supernatant for another 4 hours. Finally, Laemmli buffer was used to elute the proteins bound to the beads, and SDS-PAGE was performed.

Plasmids

The coding sequences of NOP7, β -catenin, and TCF4 were amplified by PCR and were inserted into pCMVTag2B (Flag tag), pcDNA3.1 (myc tag), and pCMV-HA (HA tag) plasmids, respectively.

Downregulation of NOP7 in HCC cells

RNAi lentivirus particles (sh con and sh NOP7) were provided by GeneChem (Shanghai, People's Republic of China). The same multiplicity of infection of virus was incubated with cells for 8 hours to knock down NOP7 expression. Then, the cells were incubated with medium containing puromycin for 2 weeks, and the puromycin-resistant cells were pooled.

Topflash

Then, 7404 cells were seeded in 24-well plates. On the next day, the Topflash assay was performed by cotransfection of Topflash (0.1 μ g), expression vector (0.5 μ g) and TK Renilla (0.05 μ g). Two days later, cells were incubated with Wnt3a (100 ng/mL) protein. Then, the reporter activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Cell motility assay

The upper Boyden chamber was filled with 0.05 mL of medium (1% FBS) containing 2 \times 10⁵ cells, and the lower chamber was filled with 0.15 mL of medium (10% FBS). Eight hours later, traditional hematoxylin and eosin staining

was performed to examine cells that had migrated to the lower surfaces of the filters. Cells were photographed, and statistical analysis was performed.

MTT assay

The growth rate of HCC cells was determined using MTT. Every 48 hours, the growth rate of the HCC cells was determined by incubating the cells with MTT solution (50 μ g/well) for 4 hours. Then, DMSO was used to dissolve the cells, and the OD value was measured at 540 nm.

Soft agar assay

First, a bottom layer with 0.5% agarose and 10% FBS in DMEM was used to coat the 24-well plate. An upper layer with 0.35% agarose and 10% FBS in DMEM containing HCC cells (2,000 cells/well) was then laid on the bottom layer. Two weeks later, the foci were photographed, and statistical analysis was performed.

Statistical analysis

Statistical analysis was performed using Student's *t*-test (2-tailed) with GraphPad Prism software (GraphPad

Software, La Jolla, CA, USA). Differences with $P < 0.05$ were considered statistically significant. Data are represented as the means \pm standard error of the mean.

Results

The NOP7 mRNA and protein levels were elevated in HCC

We first tested the NOP7 mRNA levels in the paired HCC tissues and noncancerous tissues. Compared with the noncancerous tissues, the NOP7 mRNA level in the HCC tissues was higher (Figure 1A), which was consistent with the observation after searching the GEPIA database (Figure 1B). We next examined the NOP7 protein levels in the HCC tissues and paired normal tissues. Both Western blot (6/6) and immunohistochemical staining revealed that the NOP7 protein level was increased in HCC samples (Figure 1C and D). Moreover, the NOP7 protein levels in normal liver cell lines (L02 and Chang) and HCC cell lines (7404, Hep3B, HepG2, QGY, MHCC97H, HLE, HLF, and Huh7) were tested. The NOP7 protein level was lower in normal liver cells and higher in HCC cells (Figure 1E). In addition, we searched the GEPIA database and correlated the expression of NOP7 with survival

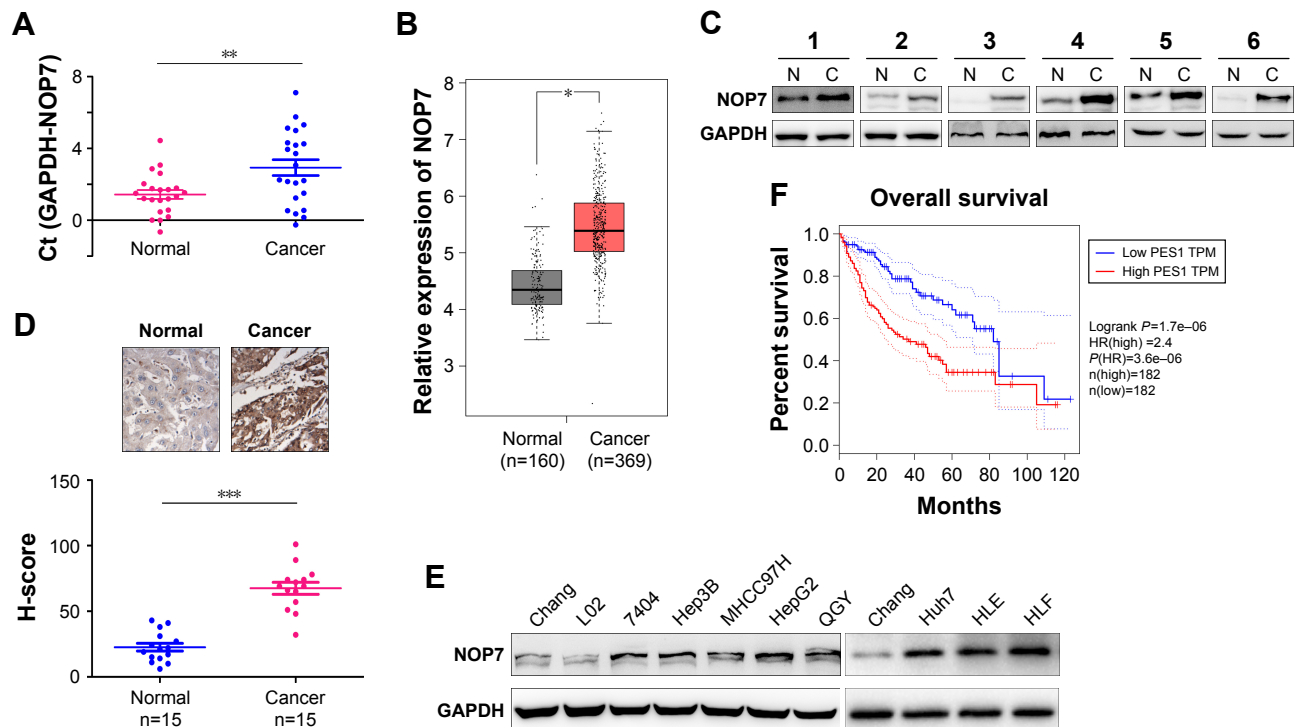


Figure 1 NOP7 was overexpressed in HCC.

Notes: (A) The mRNA levels of NOP7 in 22 HCC tissues and paired noncancerous tissues were examined via q-PCR in triplicates. GAPDH was used as the internal control. (B) Mining the GEPIA database to show the expression of NOP7 in HCC tissues. A total of 160 normal tissues and 369 HCC tissues were included in this study. (C) Western blot was performed to examine the protein levels of NOP7 in 6 HCC tissues and paired noncancerous tissues. (D) IHC was performed to examine the protein levels of NOP7 in HCC tissues and paired noncancerous tissues. Statistical analysis was performed. (E) Western blot was performed to examine the protein levels of NOP7 in normal liver cells (Chang) and HCC cells. (F) Mining the GEPIA database to show the correlation between the expression of NOP7 and survival. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Abbreviations: HCC, hepatocellular carcinoma; IHC, immunohistochemistry; q-PCR, quantitative PCR; N, normal tissues; C, cancer tissues; TPM, transcripts per kilobase million.

and found that NOP7 expression predicted poor survival (Figure 1F). Collectively, these data revealed that NOP7 was upregulated in HCC.

NOP7 accelerated the growth and enhanced the migration of HCC cells

To further investigate the functions of NOP7 in HCC, NOP7 (Flag-NOP7) vectors were transfected into 7404 and Hep3B cells, and the expression of Flag-NOP7 was confirmed by Western blot analysis (Figure 2A). Then, we used the MTT assay, the Boyden chamber assay and the soft agar assay to examine the growth and motility of HCC cells. Forced expression of NOP7 in 7404 and Hep3B cells accelerated cellular growth in the MTT assay (Figure 2B), enhanced migration (Figure 2C) in the Boyden chamber

assay, and promoted colony formation in the soft agar assay (Figure 2D).

To test the endogenous functions of NOP7 in HCC, NOP7 expression in 7404 and Hep3B cells was knocked down using 2 independent sequences (Figure 3A). Knocking down NOP7 impaired the growth (Figure 3B), migration (Figure 3C), and colony formation (Figure 3D) of 7404 and Hep3B cells. In summary, these results demonstrated that NOP7 is important for the growth and migration of HCC cells.

NOP7 elevated the transcriptional activity of the β -catenin/TCF complex in HCC cells

Next, we screened the pathways regulated by NOP7 using a reporter assay. Downregulation of NOP7 impaired the

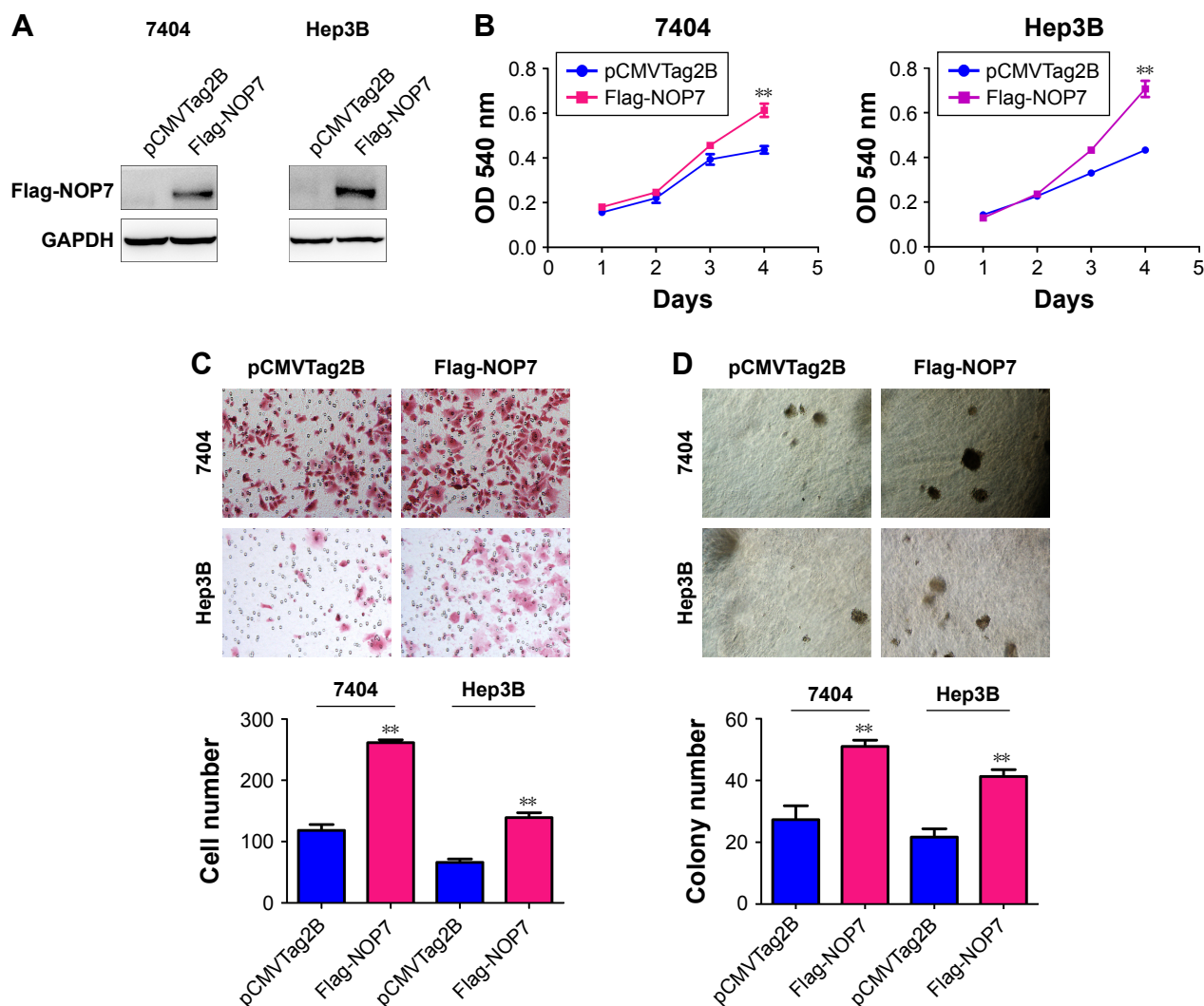


Figure 2 NOP7 promoted the growth and migration of 7404 and Hep3B cells.

Notes: (A) Western blot was performed to confirm the expression of exogenous NOP7 (Flag-NOP7) in 7404 and Hep3B cells. (B) An MTT assay was performed to confirm the effects of NOP7 on the growth of 7404 and Hep3B cells. (C) A migration assay using a Boyden chamber was performed to confirm the effects of NOP7 on the migration of 7404 and Hep3B cells. (D) A colony formation assay was performed to confirm the effects of NOP7 on the anchorage-independent growth of 7404 and Hep3B cells. ** $P < 0.01$.

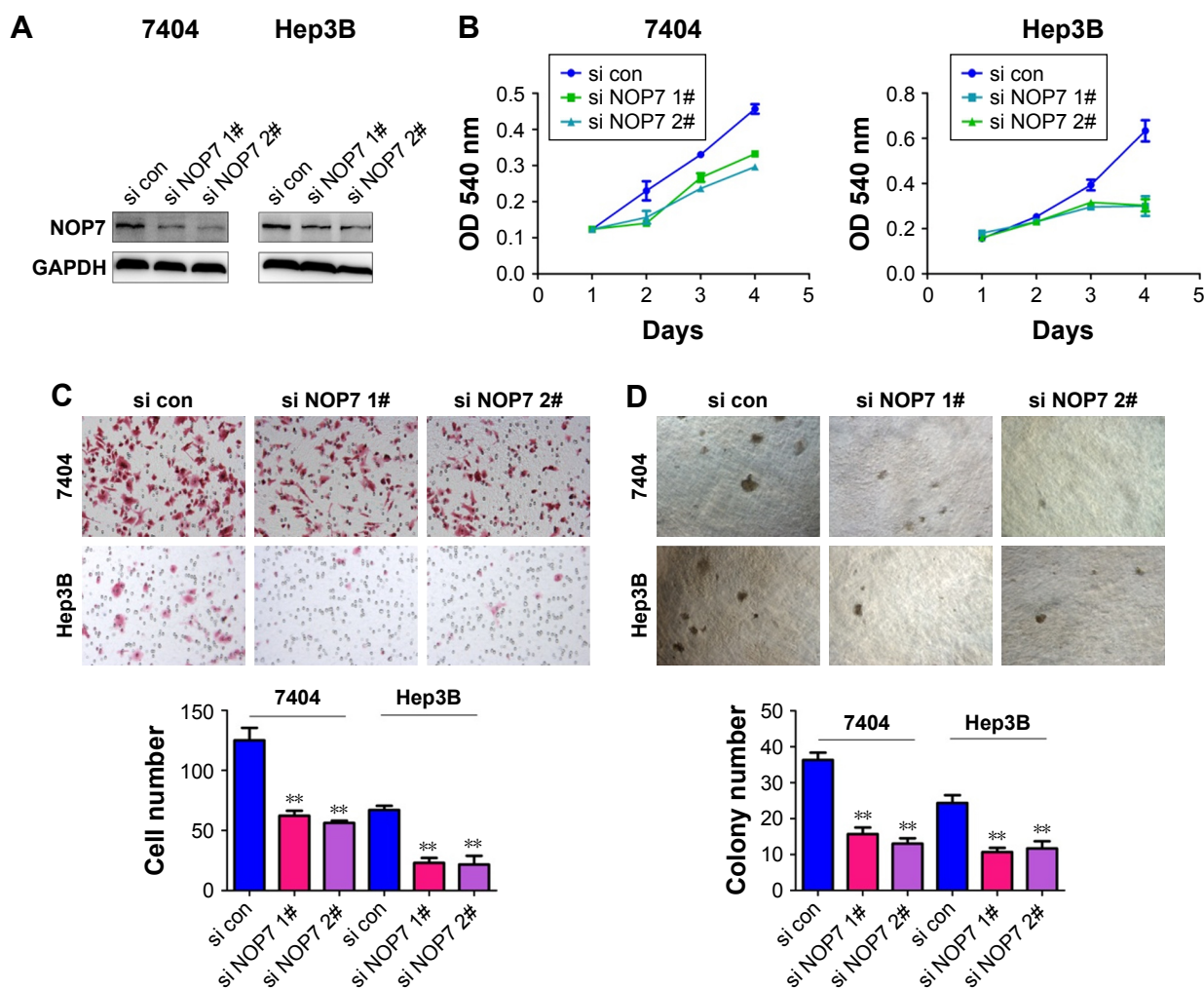


Figure 3 Knocking down NOP7 inhibited the growth, migration, and metastasis of 7404 and Hep3B cells.

Notes: (A) Western blot was performed to confirm the downregulation of exogenous NOP7 (Flag-NOP7) in 7404 and Hep3B cells. (B) An MTT assay was performed to confirm the effects of NOP7 downregulation on the growth of 7404 and Hep3B cells. (C) A migration assay using a Boyden chamber was performed to confirm the effects of NOP7 downregulation on the migration of 7404 and Hep3B cells. (D) A colony formation assay was performed to confirm the effects of NOP7 downregulation on the anchorage-independent growth of 7404 and Hep3B cells. ** $P < 0.01$.

Abbreviation: con, control.

activity of Topflash (Figure 4A). Furthermore, downregulation of NOP7 in 7404 and Hep3B cells decreased the protein expression levels of N-cadherin, Snail, vimentin, and CyclinD1 (β -catenin/TCF complex downstream genes) (Figure 4B). These results indicated that NOP7 was essential for the activation of β -catenin/TCF signaling. Moreover, downregulation of β -catenin rescued the functions of NOP7, such as the migration and colony formation of HCC cells (Figure 4C and D).

NOP7 bound to β -catenin

Next, we examined the position of NOP7 in the β -catenin/TCF signaling pathway. A GST pull-down assay showed that NOP7 interacted with β -catenin (Figure 5A). Immunoprecipitation using 7404 cell lysate demonstrated that exogenously expressed NOP (Flag-NOP) and β -catenin (myc- β -catenin)

formed a complex (Figure 5B). Moreover, endogenously expressed NOP7 and β -catenin were in the same complex (Figure 5C). These observations demonstrated that NOP interacted with β -catenin. Furthermore, NOP7 bridged β -catenin and TCF4 together (Figure 5D and E). Collectively, these findings indicated that NOP7 activated β -catenin/TCF4 by strengthening the binding between β -catenin and TCF4.

NOP7 promoted the tumorigenesis of 7404 cells

To examine the functions of NOP7 in vivo, we subcutaneously injected 7404 cells into nude mice. The growth of tumors was represented by the tumor volume. As shown in Figure 6A, NOP7 overexpression promoted the tumorigenesis of 7404 cells, which was impaired by knocking down β -catenin (Figure 6A). After the mice were sacrificed, the metastatic foci in the lung tissues were examined. More metastatic

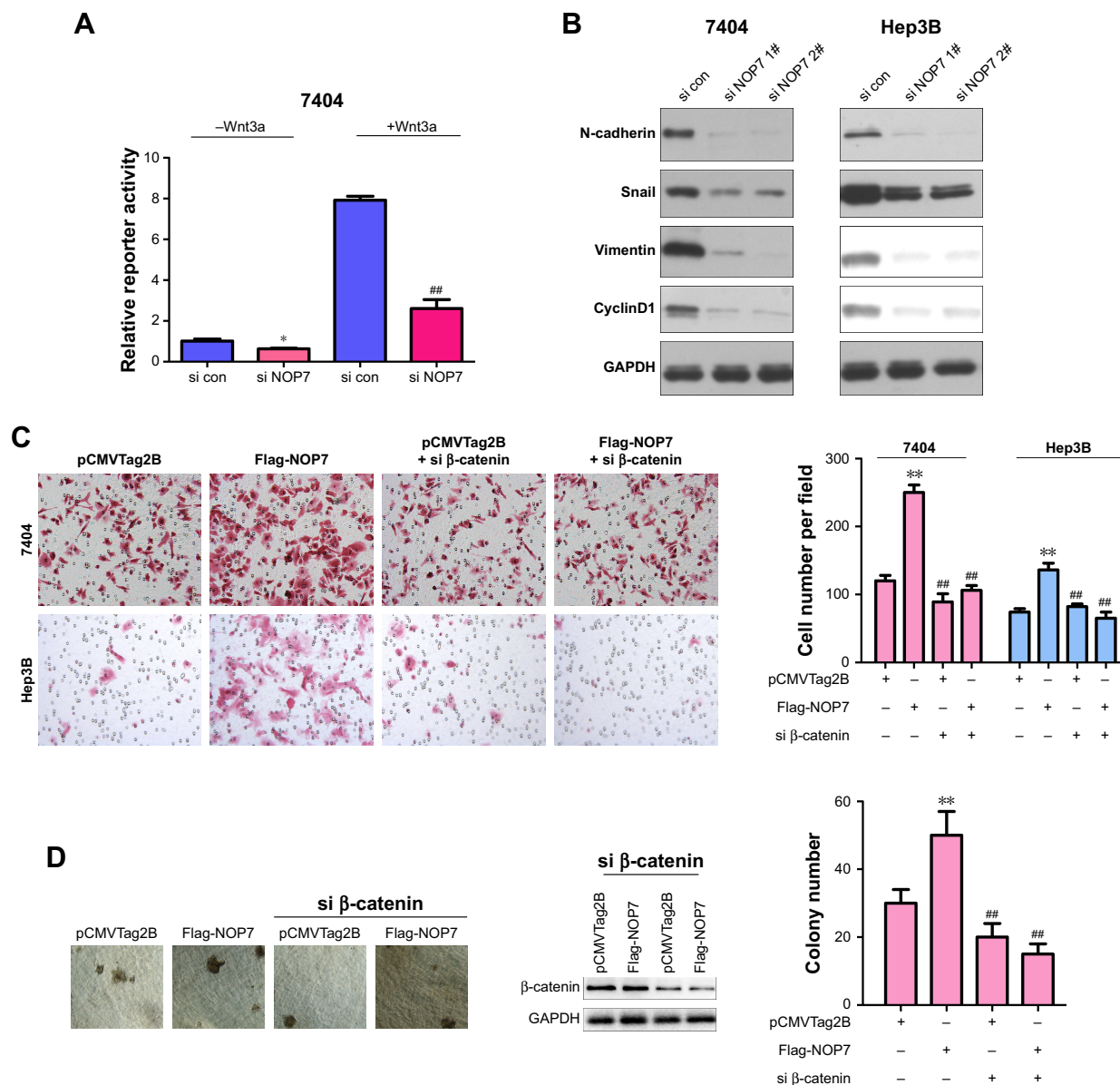


Figure 4 Knocking down NOP7 inhibited the growth, migration, and metastasis of 7404 and Hep3B cells. **Notes:** (A) A Topflash reporter assay was performed. (B) Western blot was performed to examine the expression of β-catenin target genes. (C) Knocking down β-catenin abolished the promoting effects of NOP7 on cell migration. Statistical analysis was performed. (D) Knocking down β-catenin abolished the promoting effects of NOP7 on colony formation. The expression of β-catenin was examined, and statistical analysis was performed. ##*P*<0.01; ***P*<0.001. **Abbreviation:** con, control.

foci were observed in the lungs of the mice injected with 7404 cells overexpressing NOP7 (Figure 6B and C). Taken together, these data suggested that NOP7 promoted the tumorigenesis and metastasis of 7404 cells.

Discussion

Although the functions of NOP7 in the assembly of Pre-60S ribosomal subunit have been investigated,^{18,19} the functions of NOP7 in cancer have been rarely studied. This study has clearly shown that NOP7 expression was elevated in HCC tissues. NOP7 promoted cellular growth, migration, and colony formation and positively regulated the growth, migration, and

colony formation of cancer cells. Moreover, NOP7 was determined to be a binding protein of β-catenin and bridged the interaction of β-catenin and TCF4. These observations clearly demonstrated the tumor-promoting roles of NOP7 in the progression of HCC. In addition, these observations suggested that NOP7 regulated β-catenin/TCF signaling. Based on the finding that β-catenin/TCF signaling is aberrantly active in HCC, targeting NOP7 might be a promising strategy.

The most interesting observation in this study is the identification of NOP7 as the binding protein for β-catenin. The regulation of β-catenin/TCF signaling remains largely unknown. Several proteins have been reported to modulate

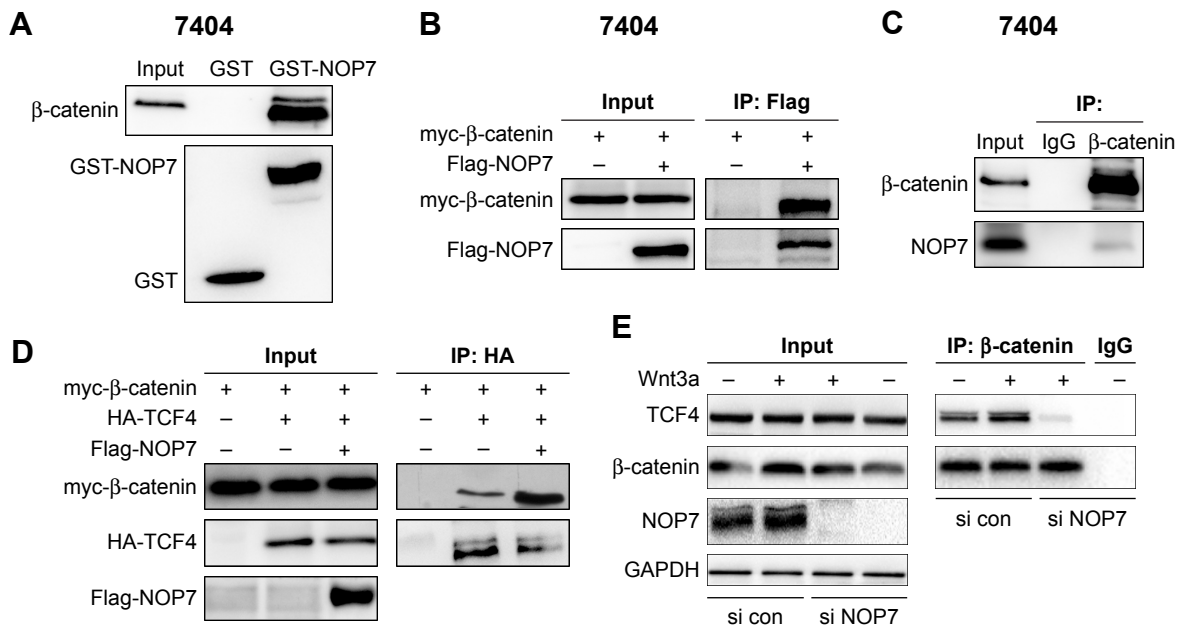


Figure 5 NOP7 interacted with β -catenin.

Notes: (A) A GST pull-down assay was performed to examine the interaction between a GST-NOP7 fusion protein and β -catenin. (B) Immunoprecipitation was performed to examine the interaction between Flag-NOP7 and myc- β -catenin. (C) Immunoprecipitation was performed to examine the interaction between endogenous NOP7 and myc- β -catenin. (D) Immunoprecipitation was performed to examine the interaction between exogenous NOP7 and myc- β -catenin. (E) IP was performed to examine the interaction between endogenous NOP7 and myc- β -catenin.

Abbreviation: con, control.

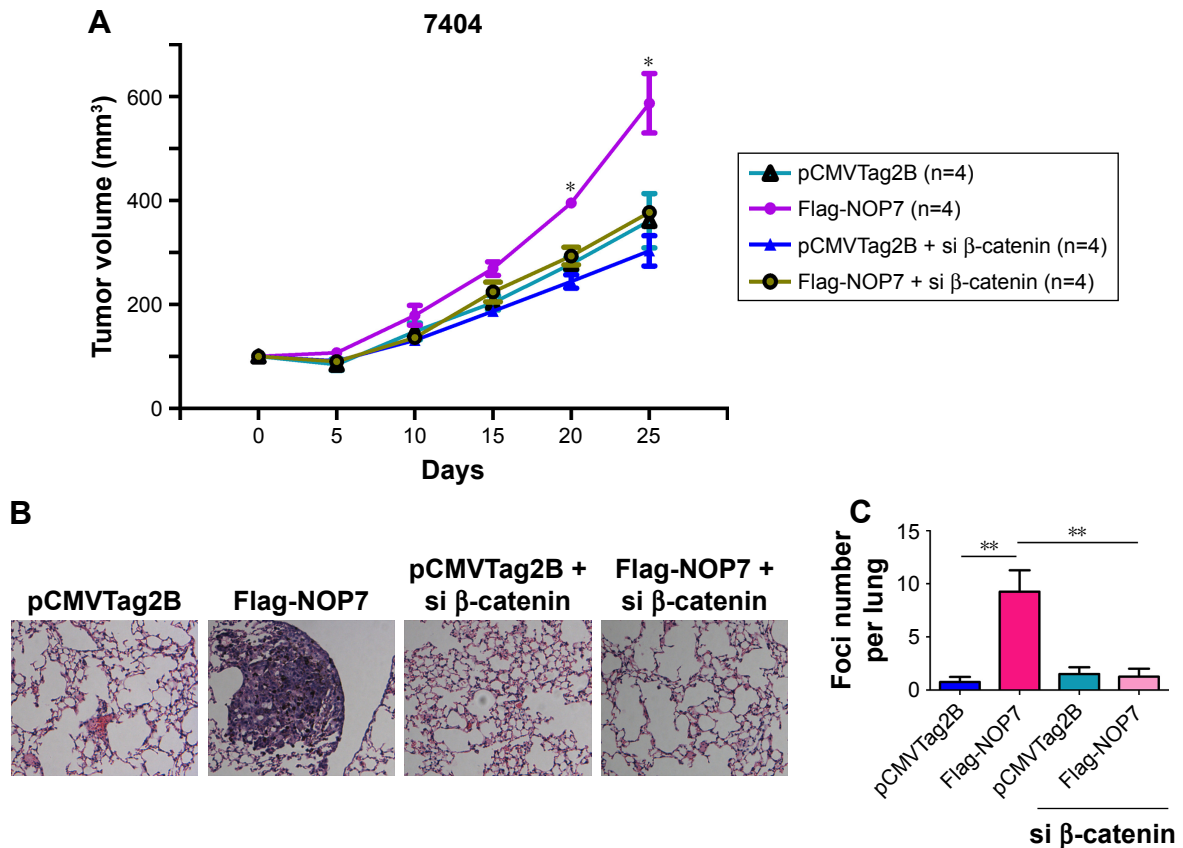


Figure 6 NOP7 promoted the tumorigenesis and metastasis of 7404 cells.

Notes: (A) The effects of NOP7 on the tumorigenesis of 7404 cells were examined. Each group includes 4 mice. The width and length of the tumors were measured every 5 days, and the volumes of the tumors were calculated. (B) The metastatic foci in the lungs were examined using HE staining. (C) The statistical analysis of (B). * $P < 0.05$; ** $P < 0.01$.

Abbreviation: HE, hematoxylin and eosin.

the interaction between β -catenin and TCF4. For example, ICAT has been found to destroy the binding between β -catenin and TCF4 and, thus, to inhibit β -catenin/TCF signaling.²⁰ Therefore, further determination of the binding domain between β -catenin and NOP7 would provide novel insights.

In summary, we have shown that NOP7 enhanced the motility of HCC cancer cells. NOP7 also activated β -catenin/TCF signaling, a major modulator of the epithelial–mesenchymal transition. Moreover, knocking down NOP7 expression inhibited the expression of mesenchymal markers (N-cadherin and vimentin). These observations indicated that NOP7 might promote the epithelial–mesenchymal transition of HCC cells.

In conclusion, NOP7 promoted the progression of HCC, suggesting that NOP7 might be a potential target for the treatment of this disease.

Ethical approval

Ethical and legal approval was obtained prior to the commencement of the study. Fudan University gave the approval. Written confirmation has been given, and all experiments were performed following relevant and national guidelines and regulations of Fudan University.

Author contribution

Nan Wu designed this project. Jing Zhao, Youhua Yuan, Chuanjia Lu, Wenjing Zhu, and Qun Jiang did the assay. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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