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ORIGINAL ARTICLE

Inhibition of Csn3 expression induces growth arrest and apoptosis of hepatocellular carcinoma cells

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Abstract
Purpose Csn3 (or CSN3) encodes the third subunit of an eight-subunit complex, the COP9 signalosome (CSN), which acts as a protein kinase and a deneddylase in mammalian cells. Previous studies have shown that Csn3 is essential for maintenance of cell proliferation in the mouse embryonic epiblast and associated with the tumorigenesis process in osteosarcoma. However, its correlation with hepatocellular carcinoma (HCC) has not been explored yet.
Methods The expression of Csn3 in HCC ($n = 30$), cirrhosis ($n = 30$), and normal tissues ($n = 30$) was detected using immunohistochemical analysis. The impacts of lentivirus-mediated inhibition of Csn3 on HCC cells were detected using MTT, BrdU incorporation assay, and flow cytometric analysis. In addition, the colony formation and tumor growth ability in nude mice were detected to define the role of Csn3 in tumorigenesis.
Results Knockdown of Csn3 expression in HCC cell lines (SMMC-7721 and Hep3B) significantly inhibits the tumor growth both in vitro and in vivo. Further investigation indicates that this growth inhibition effect may be mediated through cell cycle arrest in G0/G1 phase and inductions of pro-apoptotic proteins BIK and Caspase-8. In addition, knockdown of Csn3 expression evidently suppresses tumor growth in a xenograft nude mice model.
Conclusion Collectively, this study demonstrates Csn3 as an oncogene that regulates the tumorigenesis process in HCC cells.

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Keywords Csn3 · Hepatocellular carcinoma · Tumor growth · Apoptosis

Introduction

Hepatocellular carcinoma (HCC) represents the sixth most common malignancy and the third most common cause of cancer related death worldwide [1]. Most cases of HCC are secondary to either a viral hepatitis infection (hepatitis B or C) or cirrhosis [2]. Surgical resection (HR), local ablation therapies, and liver transplantation (LT) are regarded as potentially curative treatment modalities depending on the size and number of tumors [3]. However, surgical prognosis for many patients with HCC is not favorable due to a highly likelihood of intrahepatic and remote recurrences [3]. Targeted therapy has shown a promising future in treating HCC patients [4]. Nevertheless, because of the high heterogeneity of HCC patients, one targeting strategy may be only effective to certain subgroup of HCC patients. Therefore, to identify novel targets that regulate the tumorigenesis process of HCC will be a way to fulfill the goal of the individualized therapy against HCC.

Csn3 (or CSN3) encodes the third subunit of an eight-subunit complex, the COP9 signalosome (CSN) that was first identified in *Arabidopsis thaliana* in 1996 as a negative regulator of constitutive photomorphogenesis (COP) [5, 6]. The CSN is conserved between plants and mammals and is related to the 26S proteasome regulatory complex [7]. Two activities associated with the CSN have been identified so far: a protein kinase and a deneddylase. The CSN-associated kinase phosphorylates transcription factors, which determines their stability toward the ubiquitin system. The associated deneddylase regulates the activity of specific SCF (Skp1, Cullins, F-box proteins) E3 ubiquitin ligases. The CSN thus appears to be a platform

connecting signaling with proteolysis, which regulates the stability of many signaling proteins, including c-Jun, p53, ICSBP, and p27 [8].

Human Csn3 maps to the Smith-Magenis syndrome common deletion interval. Smith-Magenis syndrome, associated with del (17) (p11.2p11.2), is a multiple congenital anomaly/mental retardation syndrome associated with developmental delay, anatomical developmental defects, and neurobehavioral abnormalities. Reduction-of-function mutations of the *Arabidopsis thaliana* Csn3 gene decreased the COP9 complex level and caused defects in diverse aspects of plant development [9]. Also, Csn3 is essential for maintenance of cell proliferation in the mouse embryonic epiblast [5]. These results indicate that Csn3 plays an important role in mammalian development. In addition, amplification and overexpression of Csn3 in osteosarcomas potentially target TP53 for proteasome-mediated degradation and break down the anti-tumor pathway [10]. RNAi-mediated Csn3 gene silencing inhibits metastasis of osteogenic sarcoma cells [11]. However, the correlation between Csn3 and other tumors has not been studied yet.

Here, we report that lentivirus-mediated knockdown of Csn3 expression in SMMC-7721 and Hep3B HCC cell lines significantly inhibits the proliferative ability of the tumor cells, which may be caused by the cell cycle arrest at G0/G1 phase and an enhanced apoptosis. Molecular analysis shows that knockdown of Csn3 expression leads to the upregulation and accumulation of pro-apoptotic molecules BIK and Caspase-8. Finally, knockdown of Csn3 expression evidently suppresses tumor growth in a xenograft nude mice model. Taken together, these data demonstrate Csn3 as an important regulator of cell cycle and cell survival mediating the proliferation of HCC cells and highlight that targeting Csn3 or COP9 signalosome might be a promising strategy for anti-HCC therapy.

Materials and methods

Cell lines and patient samples

HEK293T, SMMC-7721, and Hep3B cells were purchased from Shanghai Institute of Biochemistry and Cell biology. The cell lines were routinely maintained in DMEM supplemented with 10% FBS at 37°C in a humidified incubator with a constant air flow of 5% CO_2 and 95% O_2.

Surgically removed liver specimens from a total of 30 HCC patients were used in this study. They were diagnosed as primary liver cancer by clinical, imaging, and pathological examinations. For pathological diagnosis, the surgically removed liver tissues were hematoxylin–eosin (HE) stained and analyzed by pathologists as hepatocellular carcinoma. For comparison, another 30 cases of cirrhosis and 30 cases of normal liver tissues were set as controls.

Immunohistochemical staining

Specimens were routinely fixed in 10% buffered formalin and embedded in paraffin. The samples were incubated with the Anti-Csn3 (Abcam, Cat #: ab10463) antibody and goat anti-rabbit secondary antibody (Abcam, Cat #: ab6721) and visualized by the EnVision method (DAKO; Hamburg, Germany) according to the manufacturer's instructions. Results were analyzed according to the intensity of the brown staining and the percentage of the immune-positive cells to total cancer cells. Ten high-magnification fields were randomly chosen and interpreted by three pathologists using double-blind method. Each slice was scored according to the staining intensity of the positive cells and the percentage of positive cells to total cancer cells. In each slice, light yellow, brownish-yellow, and dark-brown were scored as 0, 1, 2, and 3; and the positivity of 0, <1/3, 1/3–2/3, and >2/3 were scored as 0, 1, 2, and 3. The two scores were combined and the total score of 0 was defined as negative, and 1–6 as positive.

Lentivirus packaging and transduction of HCC cells

The shRNA sequences targeting Csn3 were inserted into the lentiviral vector pLVTHM and purified with the Qiagen plasmid purification kit. The transfection was carried out using MISSION Lentiviral Packaging Mix kit according to the manufacturer's instruction. Briefly, shRNA containing pLVTHM vector was mixed with 20 µl Packing Mix (PVM),and 12 µl PEI in 400 µl serum-free DMEM medium. After 15 min of incubation at RT, the transfection mixture was added into HEK293T cell at a confluency of 70–80%. After 48 h of incubation, the cell culture medium was collected, and the lentiviral particles were concentrated with the Centricon Plus-20 Centrifugal Filter Device.

SMMC-7721 and Hep3B cells were seeded onto the 6-well plates at a concentration of 5×10^4/well and transduced with lentivirus at MOI of 20 and 10 for SMMC-7721 and Hep3B cells, respectively. The knockdown efficiency was validated with real-time PCR at day 3 post-transduction and with Western blot at day 4. After confirming the knockdown efficiency, the cells were seeded onto the 96-well plates for MTT assay, and onto the 6-well plates for colony formation assay, and apoptotic and cell cycle analysis.

Analysis of tumorigenicity

The tumorigenesis ability of HCC cells was analyzed in BALB/c nude mice (No. 20, female, 4–6 weeks old, 20 ± 2 g, purchased from Shanghai SLAC laboratory Animal Co., Ltd.). Lentivirus-transduced SMMC-7721 cells of more than 80% transduction efficiency were resuspended in serum-free DMEM medium to a concentration of 2×10^7/ml. Mice were randomized into two groups

(10 mice in each group): LV-con group and LV-shCsn3 group, and 0.25 ml cell suspension (5×10^6 cells/mouse) was subcutaneously injected into the right rear flank of the mice. Tumor size was recorded every other day with a precision caliper as the maximum diameter (a, mm) and vertical short diameter (b, mm). The tumor volume was calculated using the formula $V\,(\mathrm{mm}^3) = 1/2ab^2$. After 27 days of observation, mice were killed and the xenograft tumors were dissociated, weighed, and photographed.

Statistical analysis

All statistical calculations were carried out with the Prism software. The χ^2 test or Fisher's exact test were used to compare qualitative variables, while continuous variables were compared using Student's t test or Mann–Whitney test for variables with an abnormal distribution. Receiver operating characteristic curve analysis was used to determine the optimal cutoffs of continuous variables. $P < 0.05$ was considered statistically significant.

Results

Upregulation of Csn3 in hepatocellular carcinoma tissues

Csn3 expression was detected in normal liver and HCC tissues, including 30 normal liver tissue samples, 30 cirrhosis samples, and 30 HCC samples. As shown in Table 1, 7 normal liver tissue samples, 11 cirrhosis samples, and 14 HCC samples were positive for Csn3 staining. Although there were 7 samples of normal liver tissues positive for Csn3 staining (23.3%), only 2 samples showed Csn3 expression in the nucleus (6.7%). As shown in Fig. 1, in comparison with normal liver tissues, cirrhosis samples had a higher rate of positivity for Csn3 expression: 4 samples in the cytoplasm (13.3%) and 7 samples in the nucleus (23.3%, $P < 0.05$); while HCC tissues presented the highest rate of positivity for Csn3 expression, especially in the nucleus: 2 samples in the cytoplasm (6.7%) and 12 samples in the nucleus (40.0%, $P < 0.05$). These results indicated a correlation between Csn3 expression and the pathological status of liver tissue and suggested that Csn3 might be upregulated in tumorigenesis process of HCC.

Table 1 Expression pattern of Csn3 in HCC, cirrhosis, and normal liver tissues revealed in immunohistochemistry analysis

Type of tissues	Case	Csn3 negative	Csn3 positive		*P* value
			Nucleus	Cytoplasm	
HCC	30	16 (53.3%)	12 (40.0%)	2 (6.7%)	0.046
Cirrhosis	30	19 (63.3%)	7 (23.3%)	4 (13.3%)	
Normal	30	23 (76.7%)	2 (6.7%)	5 (16.7%)	

Knockdown of Csn3 expression inhibits cell proliferation in HCC cells

The transduction efficiencies is of around 80% in SMMC-7721 and Hep3B cells transduced with LV-con or LV-shCsn3 as checked according to GFP expression (Fig. 2a). LV-shCsn3 effectively knocked down Csn3 expressions at the mRNA and protein levels as confirmed by the qPCR method (Fig. 2b, $P < 0.05$) and Western blot analysis (Fig. 2c), respectively.

After confirming the knockdown efficacy of LV-shCsn3 transduction, HCC cells were examined for their growth rate by MTT method. As shown in Fig. 3a, b, LV-shCsn3 significantly reduced the growth rate of SMMC-7721 and Hep3B cells ($P < 0.05$). To further study the anti-proliferative effect of LV-shCsn3, lentivirus-transduced cells were seeded onto the 6-well plates and cultured for 10 days. The colony formation was observed under microscope and photographed (Fig. 3c, d, upper panel). LV-shCsn3 drastically inhibited the colony formation of SMMC-7721 and Hep3B cells (Fig. 3c, d, lower panel, $P < 0.05$). These results implied that Csn3 is an important protein regulating cell proliferation in HCC cells.

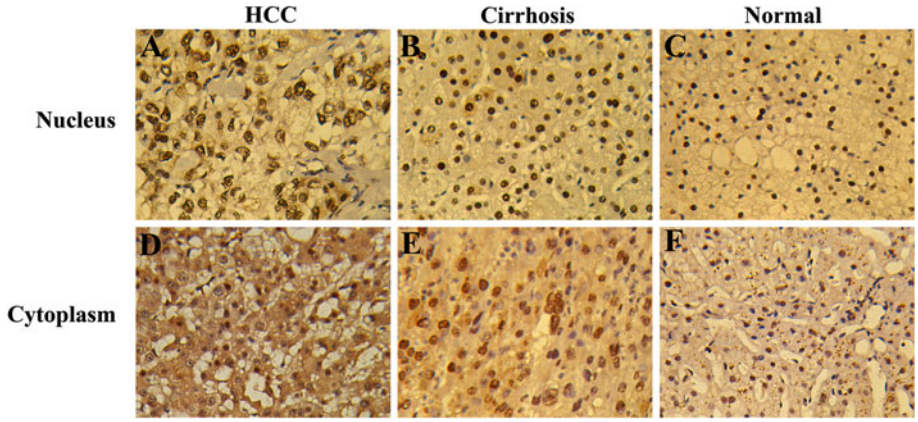

Fig. 1 Expression of Csn3 protein in liver tissues. Expression of Csn3 was localized in the nucleus and cytoplasms of the hepatocytes, and the pattern was different among the HCC (**a**, **e**), cirrhosis (**d**–**f**), and normal liver tissue (**a**–**c**). The magnification is ×400

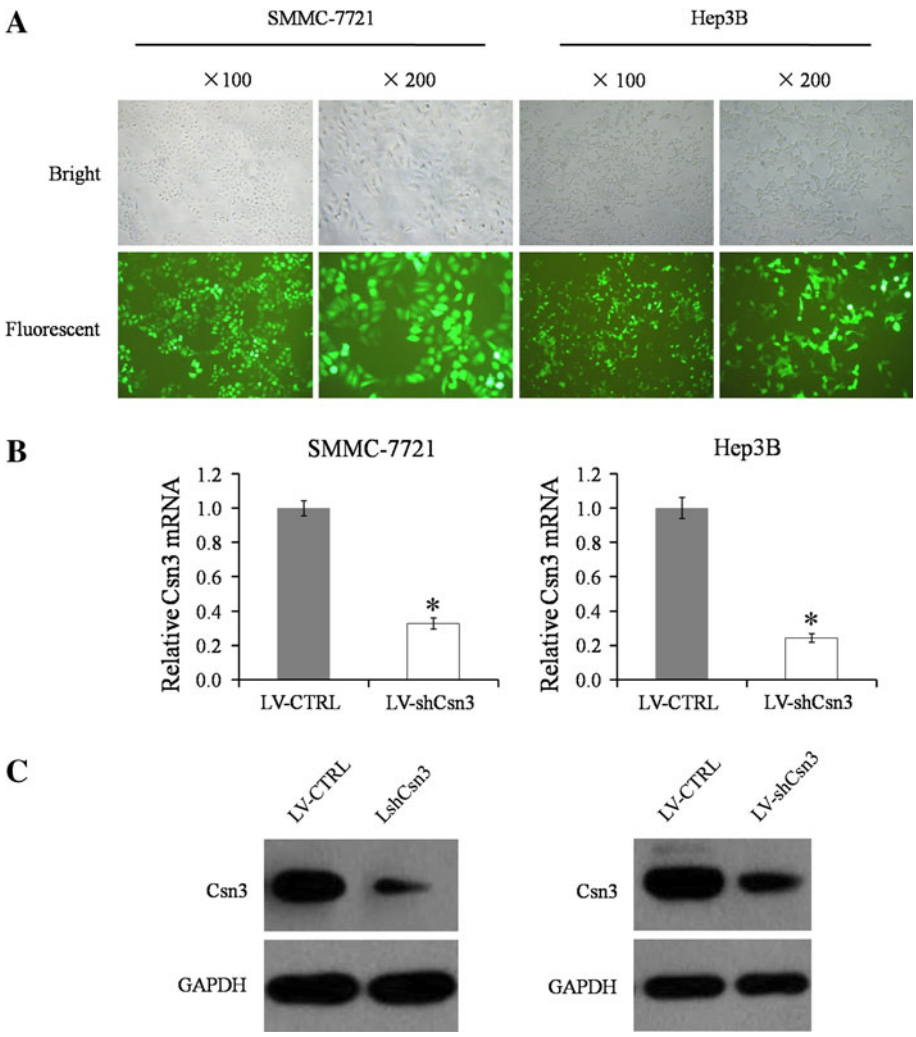

Fig. 2 Lentivirus-mediated knockdown of Csn3 in hepatocytes. **a** Efficient transduction of LV-shCsn3 in HCC hepatocellular carcinoma cells. HCC cells were transduced with lentivirus LV-Control or LV-shCsn3. The MOIs for SMMC-7721 and Hep3B were 20 and 10, respectively. **b** LV-shCsn3 knockdown Csn3 mRNA expression in SMMC-7721 and Hep3B cells (*$P < 0.05$). **c** LV-shCsn3 knockdown Csn3 protein level in SMMC-7721 and Hep3B cells. Lentivirus-transduced cells were lysed and subjected to Western blot analysis of protein expression with anti-Csn3 and GAPDH antibodies (*$P < 0.05$)

Knockdown of Csn3 expression leads to G0/G1 arrest in HCC cells

In order to study the mechanism how LV-Csn3 inhibited cell growth, the lentivirus-transduced cells were stained with PI and analyzed by the flow cytometry. LV-shCsn3 induced cell cycle arrest at G0/G1 phase and reduced the cell population in S phase and G2/M phase (Fig. 4a, b, $P < 0.05$), which indicated that Csn3 might be a cell cycle regulator maintaining the normal cell cycle progression. In addition, as analyzed by the BrdU incorporation assay, LV-shCsn3 dramatically inhibited the DNA synthesis activity in SMMC-7721 and Hep3B cells (Fig. 4c, d, $P < 0.05$). These results suggested that knockdown of Csn3 expression might reduce DNA synthesis, inhibit cell division, and put cells into the quiescent stage (G0/G1).

Knockdown of Csn3 expression induces apoptosis in HCC cells

Except for the cell cycle modification, an increase in cell population in the sub-G1 phase could also be found in LV-shCsn3-transduced Hep3B cells, implying that Csn3 might be involved in the apoptosis of liver cells. Therefore, the apoptotic rate of lentivirus-transduced HCC cells was examined by flow cytometry with Annexin V and 7-AAD staining. As shown in Fig. 5a, b, LV-shCsn3 transduction potently induced apoptosis in both SMMC-7721 and Hep3B cells ($P < 0.05$). To further investigate the underlying molecular mechanism, a series of apoptosis-associated proteins were detected with Western blot analysis. In accordance with the flow cytometry result, the cleavage of PARP, the DNA repair enzyme, and a marker of cell apoptosis were significantly enhanced

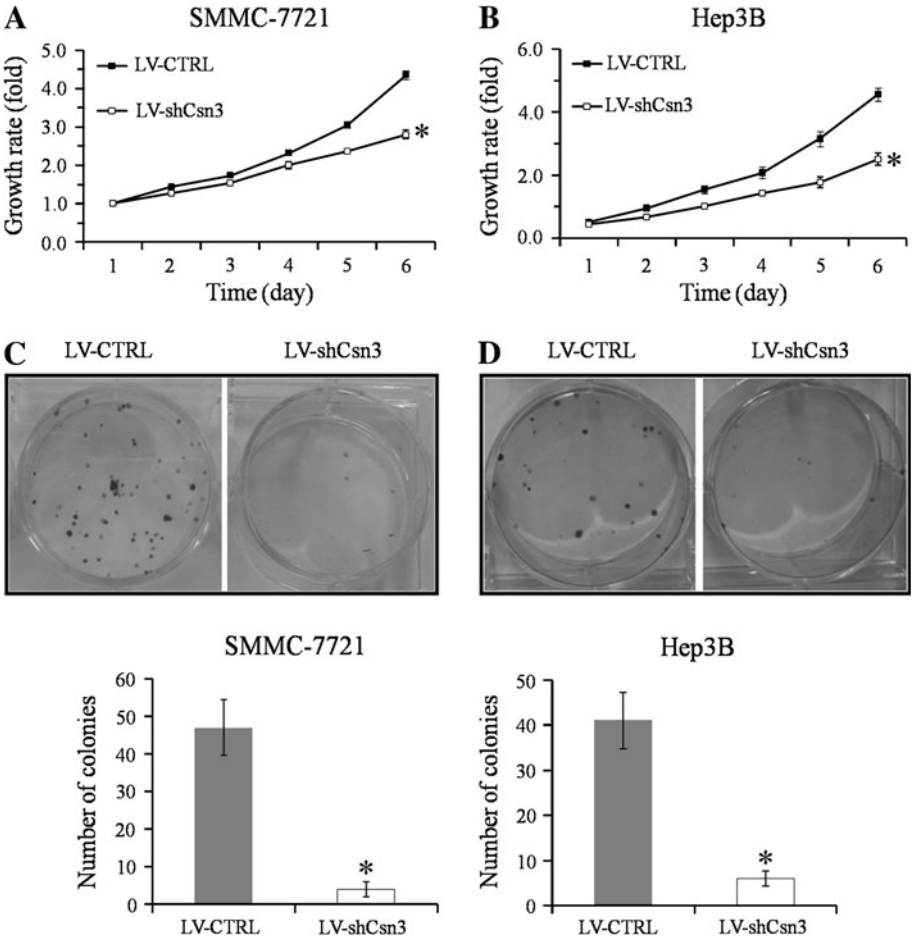

Fig. 3 LV-shCsn3 suppresses the proliferations of HCC cell lines. **a**, **b** LV-shCsn3 inhibited cell growth rates of HCC cells. SMMC-7721 and Hep3B cells were transduced with LV-con and LV-shCsn3, seeded onto the 96-well plates, and measured with MTT method. The result showed that knockdown Csn3 expression with LV-shCsn3 significantly inhibited the cell growth rate of HCC cell lines. **c**, **d** LV-shCsn3 suppressed the colony formation ability of SMMC-7721 and Hep3B cells. Cells were seeded onto the 6-well plates at a concentration of 2,000 cells/well in triplicates. After cultured for 10 days, the cells were stained with Giemsa solution and visualized. The number of colonies were calculated and statistically analyzed (*$P < 0.05$)

(Fig. 5c). Meanwhile, LV-shCsn3 obviously increased the protein levels of BIK and Caspase-8, indicating the activations of the mitochondrial and death receptor pathways.

Knockdown of Csn3 expression inhibits tumor growth in nude mice

As LV-shCsn3 significantly suppressed cell cycle progression and potently induced apoptosis in HCC cells, we investigated whether it has an impact on the tumorigenesis of HCC cells in nude mice. LV-CTRL- or LV-shCsn3-transduced SMMC-7721 cells were subcutaneously injected into the nude mice. The mice injected with LV-CTRL-transduced SMMC-7721 cells produced large tumors, whereas the mice injected with LV-shCsn3-transduced SMMC-7721 cells showed a remarkable reduction in tumor size (Fig. 6a, $P < 0.05$). Injection of LV-shCsn3-transduced SMMC-7721 cells demonstrated 59.6% reduction in tumor weight compared to that of LV-CTRL-transduced cells (Fig. 6b, $P < 0.05$).

Discussion

In this report, we show that lentivirus-mediated knockdown of Csn3 expression in SMMC-7721 and Hep3B cells dramatically represses the proliferation of tumor cells. Colony formation assay shows that knockdown of Csn3 expression significantly reduces tumor growth in vitro. Subcutaneous injection of lentivirus-transduced tumor cells in nude mice demonstrates that knockdown of Csn3 expression inhibit tumor growth in vivo. Cell cycle analysis shows that knockdown of Csn3 expression evidently induces cell cycle arrest in G0/G1 phase and reduces the cell numbers in S phase and G2/M phase. Moreover, knockdown of Csn3 expression induced apoptosis, which may be associated with an upregulation of BIK and Caspase-8 expression in SMMC-7721 cells. The data here suggest Csn3 as an oncogene, maintaining the survival and proliferation of HCC cells.

Regulation of subcellular localization, such as cytoplasma-nuclear shuttling, of signaling proteins is a key strategy for mammalian cells to regulate the intracellular signal

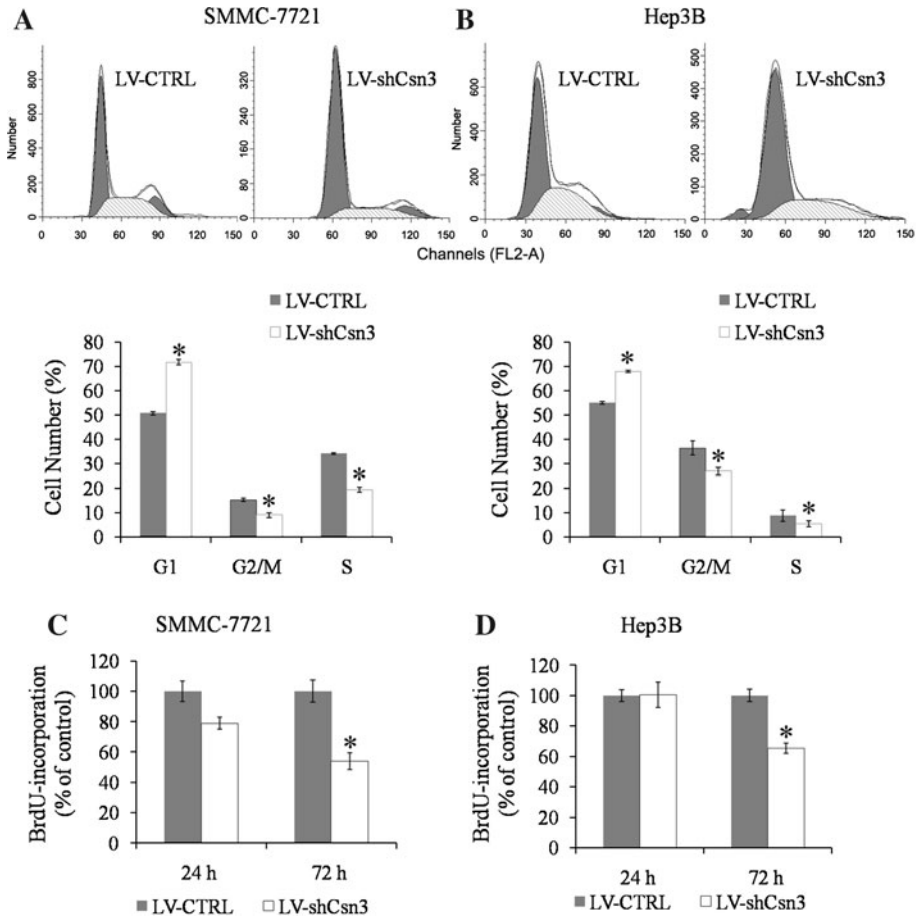

Fig. 4 LV-shCsn3 induces cell cycle arrest at G0/G1 phase. **a** LV-shCsn3-induced G0/G1 phase arrest in SMMC-7721 cells. **b** LV-shCsn3-induced G0/G1 phase arrest in Hep3B cells. Lentivirus-transduced SMMC-7721 and Hep3B cells were stained with PI solution and analyzed using the flow cytometer (*$P < 0.05$). **c**, **d** LV-shCsn3 inhibited SMMC-7721 and Hep3B cell growth by decreasing DNA synthesis. Lentivirus-transduced cells were analyzed with BrdU cell proliferation ELISA as indicated in the "Materials and methods" (*$P < 0.05$)

transduction and gene expression. Nuclear translocation of steroid receptors, including estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR), is triggered by ligand binding and initiate the transcription of downstream target genes [12]. The rapid regulations and activations of such proteins are normally mediated through the post-translational modifications, for example, phosphorylation, ubiquitination, acetylation, sumoylation, etc. [13]. CSN regulates the nucleocytoplasmic partitioning of COP1, an E3 ligase, between cytoplasm and nucleus in response to light stimuli [14]. It has been reported that CSN is localized in the nucleus and serves as a platform for protein ubiquitination in *Arabidopsis* [15–18]. However, the subcellular localization of different CSN subunits and how CSN is assembled in mammalian tissues are poorly understood. In the present study, immunohistochemical staining shows that Csn3 can localize either in the cytoplasm or in the nucleus in the liver tissues (Fig. 1). A comparison between normal liver tissues, cirrhosis, and HCC tissues reveals that Csn3 expression level and subcellular localization pattern are correlated with the malignant status of the liver tissues (Table 1). More HCC samples are positive for Csn3 staining compared with cirrhosis samples and normal liver samples. As for the subcellular localization patterns of Csn3 is concerned, significantly more HCC samples show a nuclear localization of Csn3, whereas only few normal liver samples have nuclear localization. The results imply that Csn3 can shuttle between the cytoplasm and nucleus, and the nuclear localization is related to the tumorigenesis process in HCC. The nuclear localization of Csn3 may be vital for the assembly of CSN in liver tissues.

Mutation of different CSN subunits may disrupt the entire complex in plants [19], and CSN stability is decreased in CSN3$^{-/-}$ mice [5], suggesting Csn3 as an important component maintaining the integrity of CSN. In this study, we use lentivirus to knockdown Csn3 expression in SMMC-7721 and Hep3B cells, which may also break down the CSN complex in HCC cells. Csn3 is essential for maintenance of cell proliferation in the mouse embryonic epiblast [5]. The present study demonstrates that knockdown of Csn3 expression prominently suppresses the proliferation of HCC cells and inhibits the tumor growth in the nude mouse model. These results highlight the potential of Csn3 as an oncogene supporting tumor growth of HCC.

Two major biochemical functions have been reported for the CSN. One is the protein kinase function, and the other is E3 ubiquitin ligase deneddylase, through which CSN maintains the stability of a wide variety of proteins, including

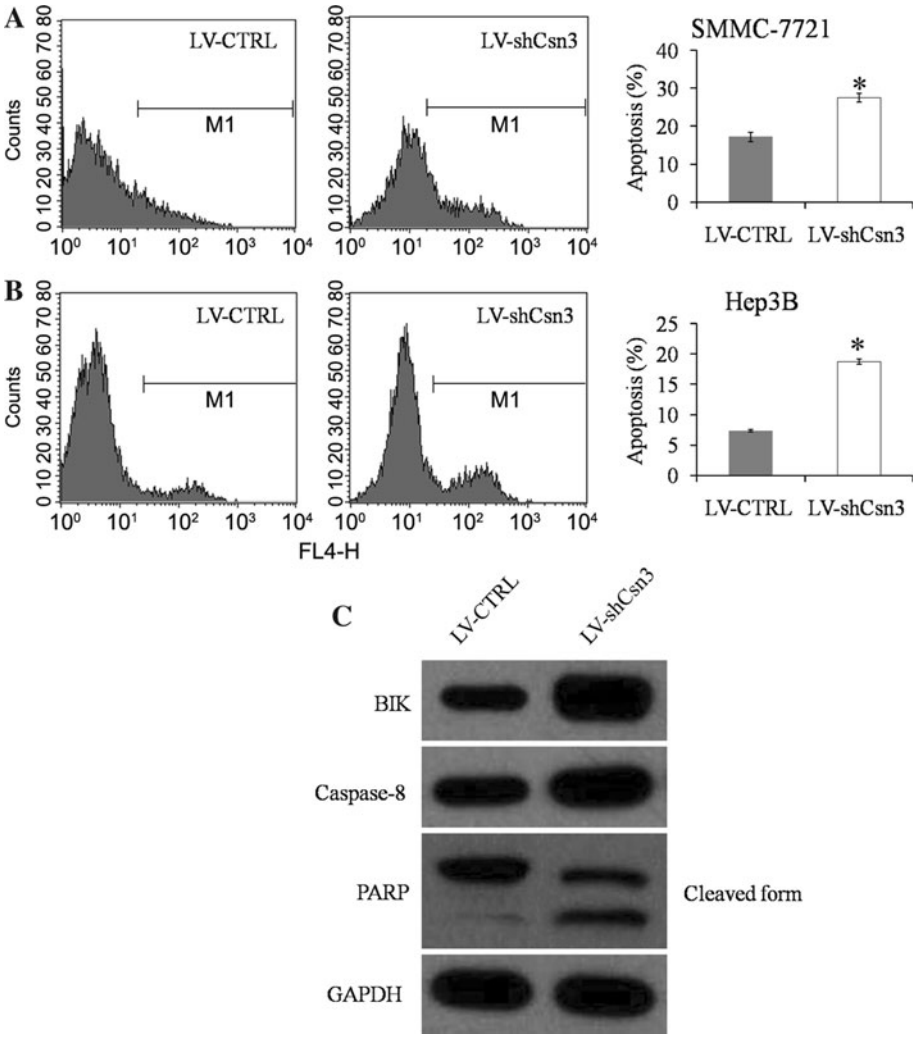

Fig. 5 LV-shCsn3 induces apoptosis through upregulating BIK and Caspase-8 expressions in HCC cell lines. **a** LV-shCsn3-induced cell apoptosis in SMMC-7721 cells (*$P < 0.05$). **b** LV-shCsn3-induced cell apoptosis in Hep3B cells (*$p < 0.05$). **c** LV-shCsn3 upregulated BIK and Caspase-8 expression and induced PARP cleavage in SMMC-7721 cells. Lentivirus-transduced cells were subjected to Western blot analysis with anti-BIK, anti-Caspase-8, anti-PARP, and anti-GAPDH antibodies

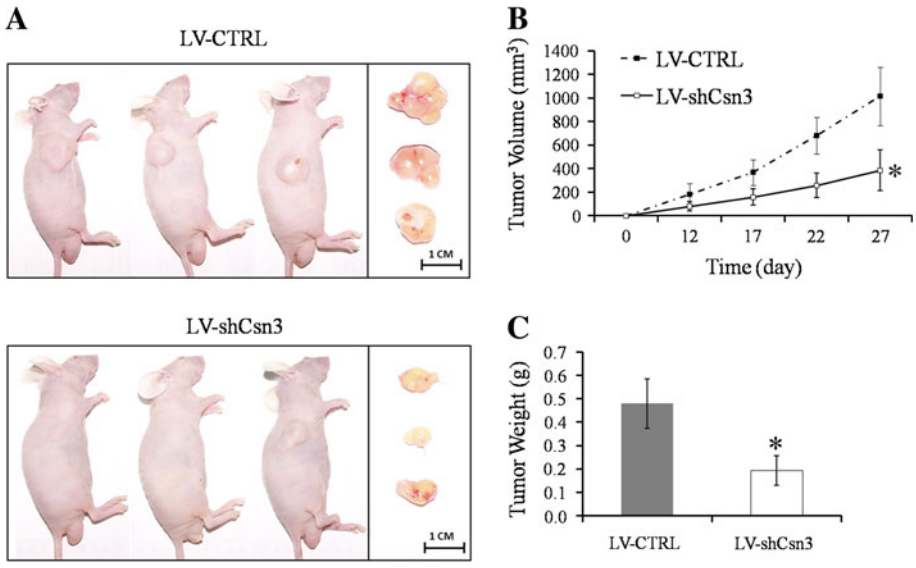

Fig. 6 LV-shCsn3 inhibits HCC growth in nude mice. **a** Tumor growth curve in nude mice. LV-con- and LV-shCsn3-transduced cells were s.c. injected into the right rear flank of nude mice. Tumor volume was calculated every other day after s.c. injection of SMMC-7721 cells as indicated in the "Materials and methods." **b** Twenty-seven days after injection, the mice were killed and the tumors were dissociated. The dissociated tumors were weighed and statistically analyzed (*$P < 0.05$)

those involved in the cell signaling pathway, such as c-Jun, and those in cell cycle control and apoptosis, such as p53 and $p27^{kip1}$. Therefore, CSN subunits are important regulators of cell cycle progression. CSN8-deficient T cells showed defective entry into the cell cycle from the G0 quiescent state. This phenotype was associated with a lack

of signal-induced expression of cell cycle-related genes, including G1 cyclins and cyclin-dependent kinases, and with excessive induction of p21 (Cip1) [20]. This finding is in consistent with our observation that knockdown of Csn3 expression in HCC cells arrests the cell cycle at G0/G1 phase and dramatically reduces the population of replicating cells (during the S phase of the cell cycle). These results indicate that the presences of CSN subunits are vital for cell cycle progression, underscoring the importance of CSN complex in cell proliferation and tumorigenesis.

Cell cycle progression and apoptosis are two closely connected processes in eukaryocytes, which develop a mechanism called checkpoints to quality control the normal cell division. In addition to cell cycle control, CSN complex also functions in the cell apoptotic pathway, due to its ability to regulate the tumor suppressor p53. Disruption of the COP9 signalosome Csn2 subunit in mice causes deficient cell proliferation, accumulation of p53 and cyclin E, and early embryonic death [21]. Csn6 plays an important role in regulating DNA damage–associated apoptosis and tumorigenesis through control of the MDM2-p53 signaling pathway [22]. In the present study, we found that targeted knockdown of Csn3 expression in HCC cells significantly upregulates pro-apoptotic proteins BIK and Caspase-8, and induces apoptotic cell death. Accordingly, CNS family subunits are important cell survival regulator, which may function through influencing the formation of CSN complex.

Conclusions

In summary, this study demonstrates that targeted knockdown of Csn3, a CSN subunit, suppresses cell cycle progression, induces apoptosis, and inhibits the proliferation of HCC cells. The results suggest that Csn3 plays an important role in the tumorigenesis process of HCC and that Csn3 might be a target for anti-HCC therapy.

Conflict of interest The authors have no conflict of interest.

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