

VOPP1 promotes hepatocellular carcinoma proliferation through MAPK14 and RPS6KB1

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Research article

Keywords: VOPP1, hepatocellular carcinoma, proliferation, apoptosis

Posted Date: July 8th, 2019

DOI: <https://doi.org/10.21203/rs.2.11083/v1>

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Abstract

Background: The present study aimed to investigate the role of VOPP1 in hepatocellular carcinoma (HCC). **Methods:** Immunohistochemistry(IHC), Western blot and Reverse-transcription polymerase chain reaction (RT-PCR) were used to analyze the expression of VOPP1 protein, the expression of VOPP1, MAPK14, RPS6KB1, CYLD and TWIST1 and the mRNA expression of VOPP1, MAPK14, RPS6KB1, CYLD and TWIST1. The cell proliferation and apoptosis were tested using Celigo cell imaging analyzer and annexin V-APC apoptosis detection kit respectively. Colony formation and tumor xenograft assays was performed to understand their roles in tumorigenicity. **Results:** The expression of VOPP1 in HCC samples was higher than that in adjacent noncancerous tissues by immunohistochemistry. In addition, the deletion of VOPP1 using shRNA inhibited cell proliferation and tumour growth, and induced cell apoptosis in vitro and in vivo. Furthermore, VOPP1 silencing decreased the expression of MAPK14 and RPS6KB1, indicating that the MAPK and mTOR signalling pathways might be involved in VOPP1-mediated cancer cell proliferation. **Conclusion:** The present data indicates that VOPP1 may play an important role in the progression of HCC by targeting the MAPK and mTOR signalling pathways, and that VOPP1 may potentially be a candidate as a novel molecular target for HCC therapy.

Background

Hepatocellular carcinoma (HCC) is the most commonly diagnosed hepatic cancer worldwide, and has been ranked as the second leading cause of cancer-associated death [1-3]. Liver cancer is often diagnosed in the late stages, bringing about a poor survival rate in HCC patients [4-6]. At present, the main treatments for HCC include chemotherapy, hepatectomy and liver transplant [7]. Hepatectomy and liver transplantation can lead to high recurrence rates, and recurrence is correlated to frequent intrahepatic metastasis. Chemotherapy can induce severe side effects. Thus, the treatment for HCC remains unsatisfactory [8]. Based on research and clinical data, different signalling pathways are involved in the initiation and regulation of HCC, which provides a strong rationale to develop anti-cancer drugs for key components. However, the underlying molecular mechanisms of HCC progression remains unclear. Therefore, investigations involved in the molecular mechanism of HCC are necessary to develop novel avenues for targeted therapies.

Recent reports from our group have revealed that RNA polymerase II subunit 3 (Rpb3), which binds directly to Snail, promotes HCC cell proliferation, migration and tumorigenicity [9]. In order to explore the mechanism of Rpb3-dependent tumour growth, HCC-specific molecules mediated by Rpb3 were searched *via* screening genes differentially expressed in cells transfected with Rpb3 shRNA. It was found that 571 transcripts were upregulated and 768 transcripts were downregulated in shRNA-mediated Rpb3 silencing cancer cells (fold change >2 and false discovery rate [FDR] <0.01). Particularly, the most downregulated transcript in Rpb3-silenced cancer cells was VOPP1.

VOPP1, which is also perceived as EGFR-coamplified and overexpressed protein (ECOP) and glioblastoma-amplified secreted protein (GASP) [10-12], is upregulated in a quantity of human cancers,

including squamous cell carcinoma (SCC), gastric cancer, and glioblastoma [10,13-15]. Evidence suggests that ECOP is a key regulator of nuclear factor kappa B signalling, and that ECOP expression could be involved in apoptosis resistance [11]. However, the precise functional mechanism of VOPP1 in HCC remains poorly understood.

The present study examines the expression of VOPP1 in HCC cell lines and sample tissues, in order to analyse the association between the expression profile of VOPP1 and clinical characteristics of HCC. Lentivirus-mediated specific shRNA targeting VOPP1 was applied to investigate the biological role of its silencing on the proliferation, apoptosis and tumorigenesis of HCC *in vitro* and *in vivo*. Further analysis revealed that the significant inactivation of the mitogen-activated protein kinase (MAPK) and mechanistic target of rapamycin (mTOR) pathways might contribute to the VOPP1 regulation of HCC progression. These present findings reveal the potential oncogenic role for VOPP1 in HCC, which might be an effective target for HCC therapy.

Methods

Immunohistochemistry

All patients were histopathologically confirmed, and the samples were obtained with informed consent from the patients. Tissue slides (5-µm sections), which had been formalin-fixed and paraffin-embedded, were deparaffinized in xylene, and rehydrated with gradient concentrations of ethanol. Then, the slides were incubated in 3% hydrogen peroxide for 10 minutes at room temperature. Afterwards, the antigens were retrieved by microwave heating at 100°C for 20 minutes in citrate buffer. Nonspecific binding was blocked by 10% bovine serum albumin in phosphate buffer saline (PBS) for 30 minutes at room. Then, the sections were stained with VOPP1 antibody (diluted at 1:600; Bioss, Beijing, China) and the corresponding secondary antibody. After rinsing with PBS for 20 minutes, the slides were stained with 3,3'-diaminobenzidine chromogen and haematoxylin, and mounted with resinene.

Cell lines and culture

The human HCC-derived cell lines (SMMC-7721, BEL-7402 and BEL-7404) and hepatoblastoma cell line (HepG2) were grown in PRMI1640 with 10% fetal bovine serum (Gibco, MA, USA) at 37°C in an atmosphere containing 5% CO₂. Then, total RNA was extracted from the cultured HCC cell lines using TRIzol reagent (Pu Fei Biotechnology Co., Ltd., Shanghai, China). The concentration and quality of the total RNA were measured using a NanoDrop spectrophotometer, and the RNA was reverse transcribed into cDNA using a Promega M-MLV RT reagent kit (Promega, WI, USA), according to manufacturer protocols. VOPP1 expression was examined using the Light Cycler 480 Real Time System with the SYBR green reagent (TaKaRa, Beijing China). The 2^{-ΔΔCt} method was applied to quantification, and GAPDH was used as a negative control. Each experiment was performed in duplicate. The primers used for the present study were as follows: VOPP1-F: 5'-AATGGCTTTCCAGGTCCCACC-3', VOPP1-R: 5'-

CTTGGCCTTCACTACCTGTTC-3'; GAPDH-F: 5'-TGACTTCAACACGGACACCCA-3', GAPDH-R: 5'-CACCTGTTGCTGTAGCCAAA-3'. All of cell lines were identified by STR.

Gene silencing transfection

Lentiviral vectors encoding VOPP1 shRNA (shVOPP1) were designed. The synthesized DNA oligonucleotides, which contained these sequences, were annealed and inserted into the GV115-GFP vector (Genechem, Shanghai, China). Then, cells were seeded in 6-well plates at a concentration of 2×10^5 cells per well. In order to facilitate the lentivirus manipulations, the GV118 vector was transfected into 293T cells, along with pHelper 1.0 and pHelper 2.0 plasmids (Genechem, Shanghai, China). At 72 hours after transduction, the cultured supernatant containing the lentiviral particles was collected and purified. For the lentivirus infection, HCC cells that experienced logarithmic growth were infected with lentivirus, according to the multiplicity of infection (MOI). The infection efficiency of the lentivirus with GFP was observed based on the number of GFP-expressing cells using a fluorescence microscope with a camera. These cells were harvested until they reached 70% confluence for mRNA and protein analysis, and for other assays.

Western blot analysis

Cells were lysed with precooled and sonicated lysis buffer. The protein concentration was determined using the BCA method (Beyotime, Shanghai, China). Cell lysates (20 µg/lane) were electrophoresed on SDS-polyacrylamide gels, and transferred onto PVDF membranes (EMD Millipore, CA, USA). Then, the membranes were blocked with TBST solution containing 5% defatted milk for one hour, and incubated overnight at 4°C with the appropriate primary antibodies. Afterwards, the blots were incubated with goat polyclonal antibody (1:2,000) for two hours at room temperature. These blots were developed by autoradiography using the ECL-Western blotting system (Thermo, MA, USA).

Annexin V-APC apoptosis assay

The cell apoptosis assay was performed using an annexin V-APC apoptosis detection kit (eBioscience, MA, USA), according to manufacturer's instructions. The cell suspensions were incubated with 10 µl of annexin V-APC in the dark at room temperature, and kept away from light for 15 minutes. Then, the stained cells were analysed by Guava easyCyte HT flow cytometry (Millipore, CA, USA).

Colony-forming cell assay

Cells infected with a lentivirus expressing shVOPP1 or scrambled shRNA were seeded in 6-well plates (1,000 cells per well) and cultured at 37°C for 14 days in a 5% CO₂ atmosphere. The culture medium was refreshed every three days. Next, cells were fixed with 4% paraformaldehyde for 30 minutes, and stained with Crystal violet dye for 10 minute. The colonies were photographed and counted.

Animal experiments

All animal experiments were conducted under the policies and protocols approved by Taizhou Hospital of Zhejiang Province. Four-week-old female BALB/c nude mice were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). BEL7404 (5×10^6) cells and their derivatives transduced with shVOPP1 (4×10^6) were suspended in 200 μ l of PBS, and subcutaneously injected in 4-week-old BALB/c nude mice. After 10 days, the animals were weighed, and the tumour sizes were measured. Tumour volume was calculated using the formula: tumour maximum diameter (L) \times the right-angle diameter (W) to that axis. Mice were anaesthetized and injected with 2 mg of D-luciferin retro-orbitally at the indicated times for bioluminescent imaging. Then, the animals were imaged in an IVIS Lumina LT (PerkinElmer, Waltham, MA, USA), and the data were recorded using the Living Image software. At day 23, all mice were sacrificed, and the tumors were harvested for further analysis. At the end of the experiment, all mice were euthanized with CO₂ asphyxiation: all mice were concentrated in the induction box, and CO₂ was introduced. After all mice died, the cervical vertebrae of each animal were dislocated to confirm their death. which is approved by Ethics Committee of Taizhou Hospital of Zhejiang Province.

Statistical analysis

The results were presented as mean \pm standard deviation (SD). The statistical analyses were performed using SPSS version 15.0 (SPSS, IL, USA). Statistical significance was determined using two-sided Student's *t*-test. For all analyses, $P < 0.05$ was considered statistically significant.

Results

VOPP1 expression is upregulated in HCC

The gene expression analysis from The Cancer Genome Atlas (TCGA) database revealed that VOPP1 expression was tightly correlated with HCC (Figure 1A). Therefore, specimens were collected and the expression of VOPP1 in HCC tissue samples and adjacent normal tissue samples were examined by immunohistochemistry (IHC) staining. The IHC results revealed the strong staining of VOPP1 in cancer tissues, when compared with adjacent liver tissues (Figure 1B). These results suggest that VOPP1 is overexpressed in patients with HCC.

The silencing of VOPP1 inhibited HCC cancer cell proliferation and tumorigenesis *in vitro*

Next, qRT-PCR was performed to detect the VOPP1 expression in liver cancer cell lines BEL-7402, BEL-7404, SMMC-7721 and HepG2. The relative expression levels of VOPP1 in the liver cancer cell lines were 9.79 ± 0.255 , 11.39 ± 0.06 , 10.07 ± 0.154 and 9.19 ± 0.049 , respectively (Figure 2A). Then, the BEL-7404 and SMMC-7721 cell lines were chosen for the further functional investigation of VOPP1 in HCC.

The positive correlation between VOPP1 expression and HCC suggests that VOPP1 may be involved in HCC progression. In order to further study the biological function of VOPP1 in HCC, two shRNAs that were cloned into a GFP-expressing lentivirus vector in SMMC-7721 cells were screened. On account of the GFP

expression, the lentivirus infection efficiency was above 88% (Figure 2B). The qRT-PCR analysis revealed that cells infected with the shVOPP1 lentivirus exhibited a significantly reduced VOPP1 expression, when compared with that in cells infected with the NC lentivirus ($P<0.05$, Figure 2C). The deletion of VOPP1 in SMMC-7721 cells was also observed by western blot analysis using the corresponding antibodies. Among the analysed shRNAs, shVOPP1-1 appeared to have a better knockdown efficiency and inhibitory effect on cell proliferation, when compared shVOPP1-2 (Figure 2D, 2E and 2F). Therefore, shVOPP1-1 was selected for the subsequent experiments. In order to explore the effect of VOPP1 on cell proliferation, SMMC-7721 and BEL-7404 cells with either the shVOPP1 lentivirus or scrambled-shRNA lentivirus were seeded in 96-well plates, and cell proliferation was monitored using an Celigo cell imaging analyzer for five days. The cell proliferation rate slowed down following the shVOPP1 lentivirus treatment in both cell lines (Figure 3A and 3B).

VOPP1 promoted cell colony formation

Next, the role of VOPP1 on cell tumorigenesis was analysed by colony formation assay. The results revealed that the colony formation ability drastically declined following VOPP1 knockdown in both SMMC-7721 and BEL-7404 cells. The number of colonies were 111 ± 2 in the control group vs. 18 ± 3 in the shVOPP1 group in SMMC-7721 cells, and 168 ± 11 in the control group vs. 22 ± 4 in the shVOPP1 group in BEL-7404 cells ($P<0.01$, Figure 4A and 4B). These results indicate that VOPP1 is necessary for cell proliferation *in vitro*.

VOPP1 silencing inhibited tumour growth in BALB/c nude mice

In order to examine the role of VOPP1 on primary tumour growth *in vivo*, an orthotopic xenograft model was used. The BEL-7404 xenograft tumor efficiency rate in nude mice was 100%. Hence, these nude mice were randomly selected and subcutaneously injected with BEL-7404 cells transfected with shVOPP1 or NC shRNA for 23 days. In accordance with the results *in vitro*, the tumors harvested from mice that received shVOPP1 cells were smaller than those harvested from mice that received NC shRNA cells. Both the volumes and weights of tumors from the shVOPP1 group were significantly smaller, when compared to those from the control group ($P<0.001$). VOPP1 knockdown significantly decreased tumor growth, as shown by the remarkable decrease in tumor size and weight, when compared with those from the control treatment (Figure 5A, 5B and 5C). These results indicate that VOPP1 is vital for HCC cell tumorigenicity.

Silencing of VOPP1-induced apoptosis in HCC

Cell proliferation is usually affected by cell cycle change or cell apoptosis. Therefore, annexin V-APC staining to was further performed determine whether the programmed apoptosis was triggered by VOPP1 downregulation. As presented in Figure 6, the apoptosis rate of cells treated with shVOPP1 significantly increased, when compared to that of the scrambled shRNA. The percent of apoptotic cells detected in the SMMC-7721 cell line was $4.48 \pm 0.15\%$ in the control group vs. $12.47 \pm 0.29\%$ in the shVOPP1 group ($P<0.001$), while the apoptosis rate in the BEL-7404 cell line was $2.24 \pm 0.22\%$ in the control group vs.

23.66 ± 0.55% in the shVOPP1 group ($P < 0.001$). In summary, these results revealed that the apoptosis of HCC cells was mediated by the downregulation of VOPP1.

VOPP1 exerts its oncogenic activity *via* the MAPK14 and mTOR signalling pathway

In order to investigate the potential molecular mechanism by which VOPP1 promotes HCC cell proliferation, microarray analysis was performed using cells that were either transfected with shVOPP1 or scrambled shRNA. A total of 330 upregulated genes and 679 downregulated genes were authenticated along with the VOPP1 knockdown. Based on the bioinformatics analysis, tumor necrosis factor-mediated signalling pathways were significantly activated. Therefore, the present study focused on genes that might be correlated to the tumor necrosis factor. First, the expression of four genes (MAPK14 [16] RPS6KB1 [17], CYLD [18] and TWIST1 [19]) was analysed using qRT-PCR. The qRT-PCR data revealed that the mRNA levels of MAPK14, RPS6KB1 and CYLD were upregulated in VOPP1-silenced cells (Figure 7A). The western blot results also revealed that the levels of MAPK14, RPS6KB1 and CYLD increased (Figure 7B). These results indicate that the activation of MAPK and mTOR might be responsible for the VOPP1-regulated HCC proliferation and apoptosis.

Discussion

The molecular mechanisms of HCC have gradually become clearer in recent years [20-22], while the survival rate of HCC patients remains low. Cell proliferation and migration are significant characteristics in the development and progression of most cancers, including HCC. Many studies have shown that the balance between cell proliferation and apoptosis is influenced by tumor coding oncogenes or suppressor genes [23,24].

Recent studies have implicated VOPP1 in the regulation of tumorigenesis [25]. The role of VOPP1 has been found in various cancers, including squamous cell carcinoma (SCC), gastric cancer and glioblastoma [10,13-15]. However, there is little knowledge about its expression and role in HCC. In the present study, the expression of VOPP1 in HCC tissues and adjacent liver tissues was investigated, and the association between VOPP1 expression and clinical relevance was analysed. In addition, the role of VOPP1 on HCC cell proliferation, colony formation tumorigenesis, and apoptosis *in vitro* and tumorigenesis *in vivo* was further evaluated.

These present results indicate that VOPP1 was upregulated in HCC samples by IHC. VOPP1 knockdown dramatically inhibited HCC cell proliferation and tumorigenesis *in vitro*. These results highlight that VOPP1 may play an vital role in the promotion of HCC progression. Furthermore, evidence has shown that VOPP1 enhanced HCC cell tumorigenesis in a nude mouse model.

The MAPK and mTOR signalling pathway plays important roles in tumor progression [16,26,27]. In the present study, MAPK14 and RPS6KB1 were found to be upregulated following VOPP1 knockdown, suggesting that VOPP1 knockdown mediated cell proliferation, apoptosis and migration *via* the MAPK and mTOR signalling pathways, and the multi-targeting regulation of VOPP1.

Conclusions

Taken together, the present study provides a novel perspective on the function of VOPP1 in HCC. VOPP1 may be deemed as a potential target for the therapy of HCC. However, the additional mechanisms of VOPP1 function in HCC require further exploration. Recently, increasing evidences have also indicated that small noncoding RNAs, such as miRNAs, are linked with apoptosis through the negative regulation of oncogenes or tumor suppressor genes [28-31]. Future studies would clarify whether miRNA participates in the regulation of VOPP1.

Abbreviations

Hepatocellular carcinoma (HCC)

RNA polymerase II subunit 3 (Rpb3)

false discovery rate (FDR)

EGFR-coamplified and overexpressed protein (ECOP)

glioblastoma-amplified secreted protein (GASP)

mitogen-activated protein kinase (MAPK)

mechanistic target of rapamycin (mTOR)

phosphate buffer saline (PBS)

multiplicity of infection (MOI)

standard deviation (SD)

The Cancer Genome Atlas (TCGA)

immunohistochemistry (IHC)

Declarations

Ethics approval and consent to participate

I confirm that I have read the Editorial Policy pages. This study was conducted with approval from the Ethics Committee of Taizhou Hospital of Zhejiang Province. This study was conducted in accordance with the declaration of Helsinki. All applicable international guidelines for the care and use of animals were followed.

Consent for publication

Not applicable

Availability of data and material

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We are particularly grateful to all the people who have given us help on our article.

Funding

This study was supported by grants from the National Natural Science Foundation of China (Grant No. 81872237), the Science Technology Program of Zhejiang Province on the Scientific Research Project (Grant No. GF19H160059, Y17H160289 and Q18H160117) , Zhejiang Provincial Health Department Project (Grant No. 2017KY161 and 2015KYB434). The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Author contributions

HJD, PH and BFW designed experiments; HJD, PH, BFW, QYH and YFX carried out experiments; YZ and MFG collected data; SDL analyzed data; ZPF and WLZ wrote the manuscript; WZ reviewed manuscript.

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Figures

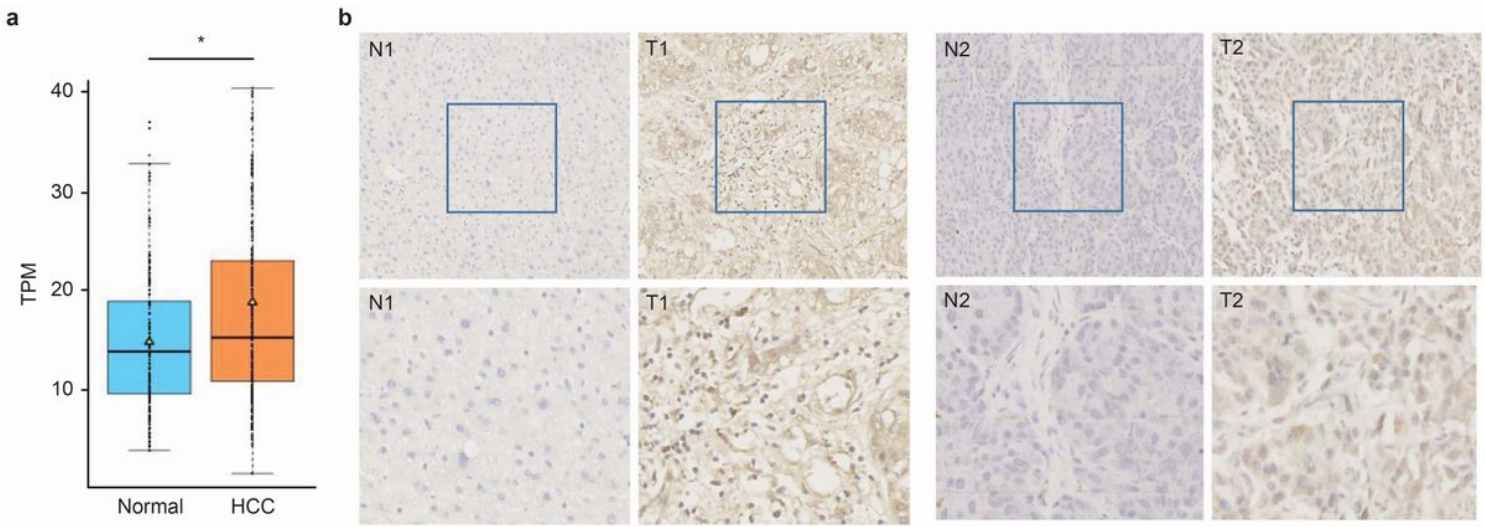


Figure 1

The expression of VOPP1 is upregulated in human HCC surgical specimens: (A) A boxplot showing the relative expression of VOPP1 in normal and HCC specimens. (B) The immunohistochemical staining of VOPP1 in HCC tissue samples (T) and matched adjacent normal tissues (N). Two representative clinical cases of immunohistochemical staining are shown (upper panel with 200× magnification; lower panel with 400× magnification).

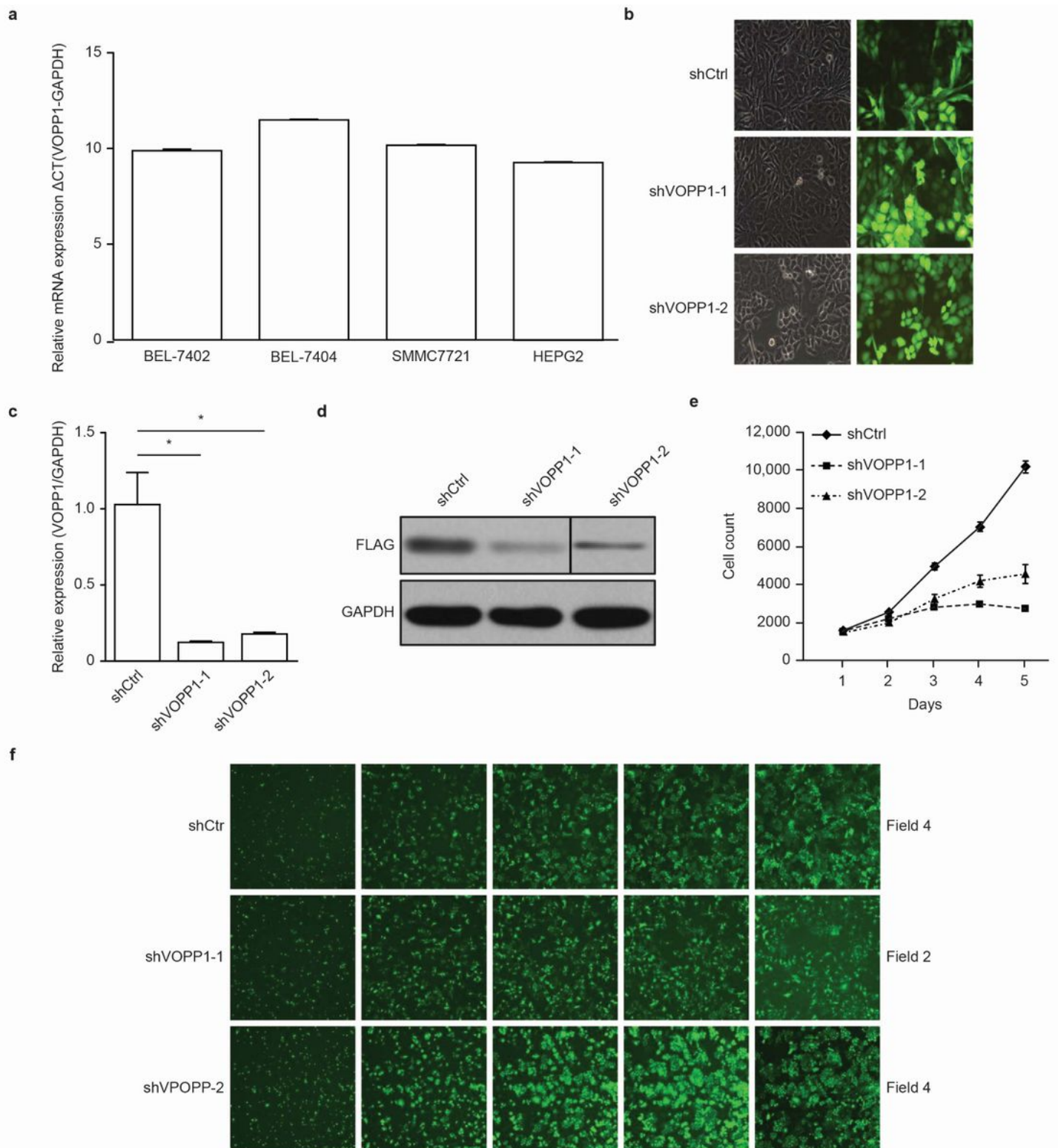


Figure 2

The analysis of VOPP1 expression and its lentivirus-mediated shRNA knockdown: (A) The real-time PCR analysis of VOPP1 expression in HCC cell lines. (b-d) The transfection and knockdown efficiency of lentivirus shRNA using fluorescence microscopy (B), real-time PCR (C), and western blotting (d) in SMMC-7721 cells. (e-f) The effect of VOPP1 knockdown on cell growth potential in SMMC-7721 cells. Cell

growth was measured using a Celigo cell imaging analyzer for five days. The data was presented as mean \pm standard deviation (SD); *P<0.05.

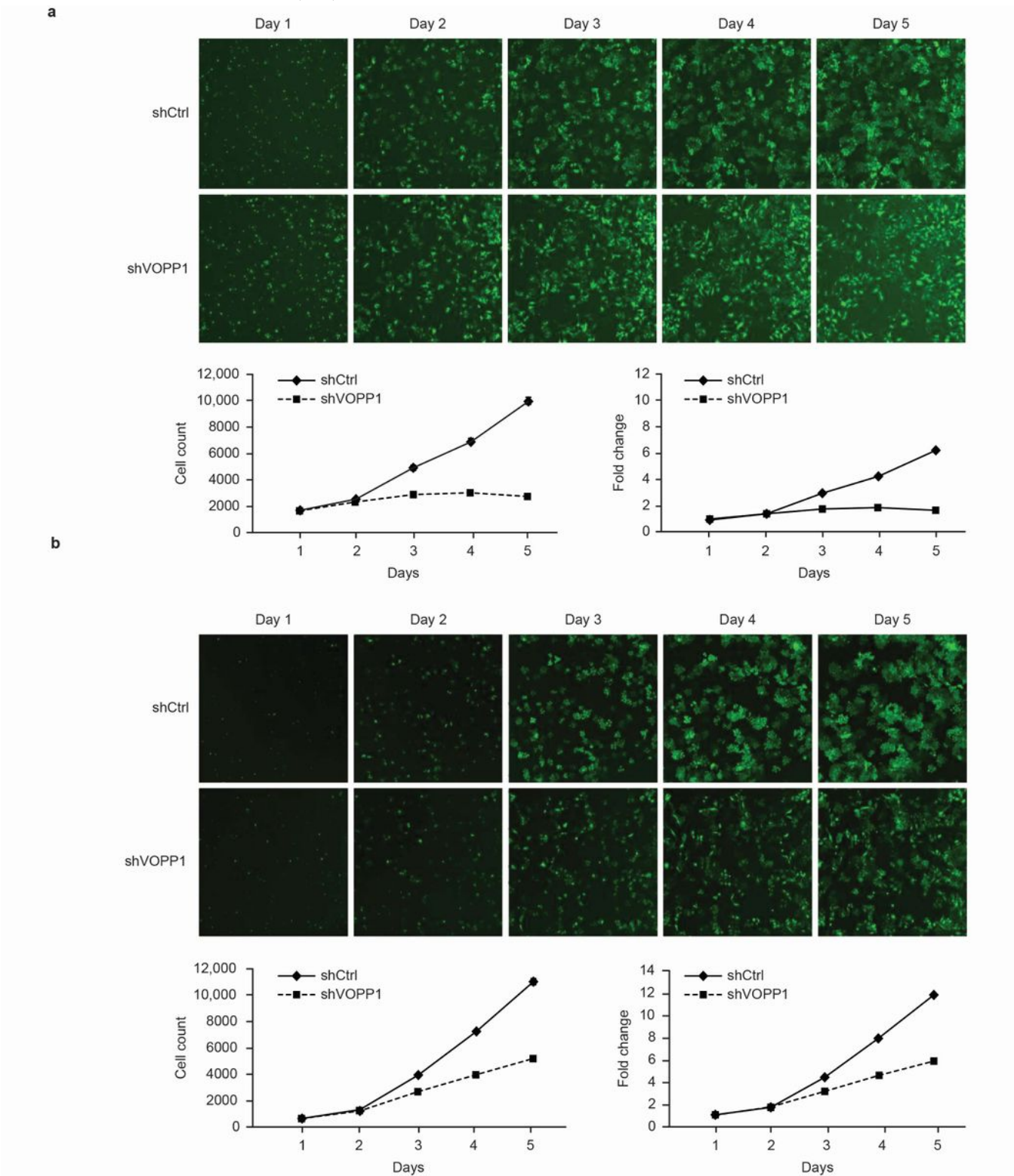


Figure 3

VOPP1 knockdown inhibited the cell proliferation in HCC cell lines: VOPP1 knockdown attenuated the cell growth potential in SMMC-7721 (A) and BEL-7404 (B) cells. Cell growth was measured using a Celigo cell imaging analyzer for five days. The data was presented as mean \pm standard deviation (SD).

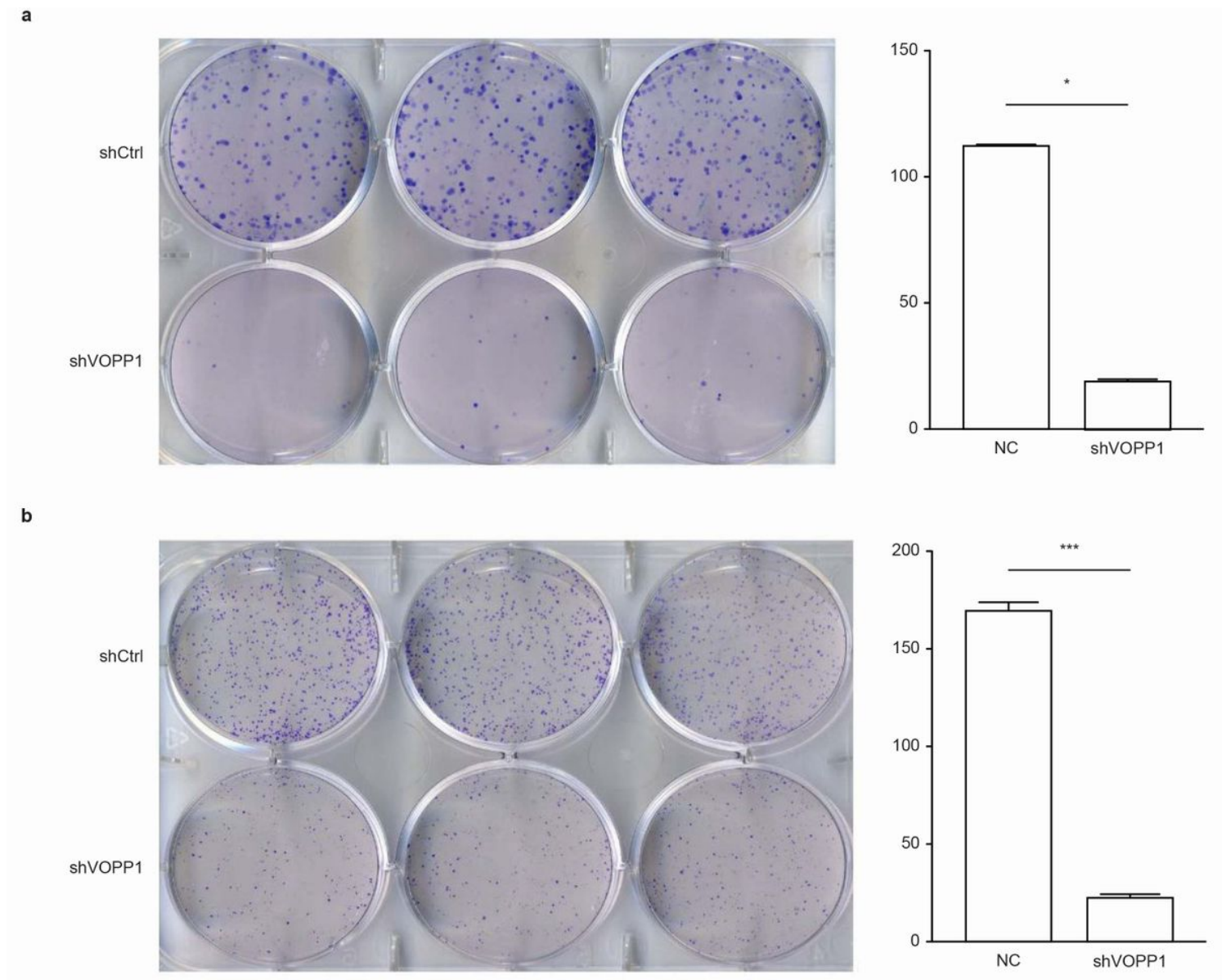


Figure 4

VOPP1 knockdown reduced HCC cell colony formation: VOPP1 knockdown reduced the quantity of SMMC-7721 (A) and BEL-7404 (B) colonies. The left panel presents the crystal violet dye-stained colonies in well plates; * $P < 0.05$. *** $P < 0.001$.

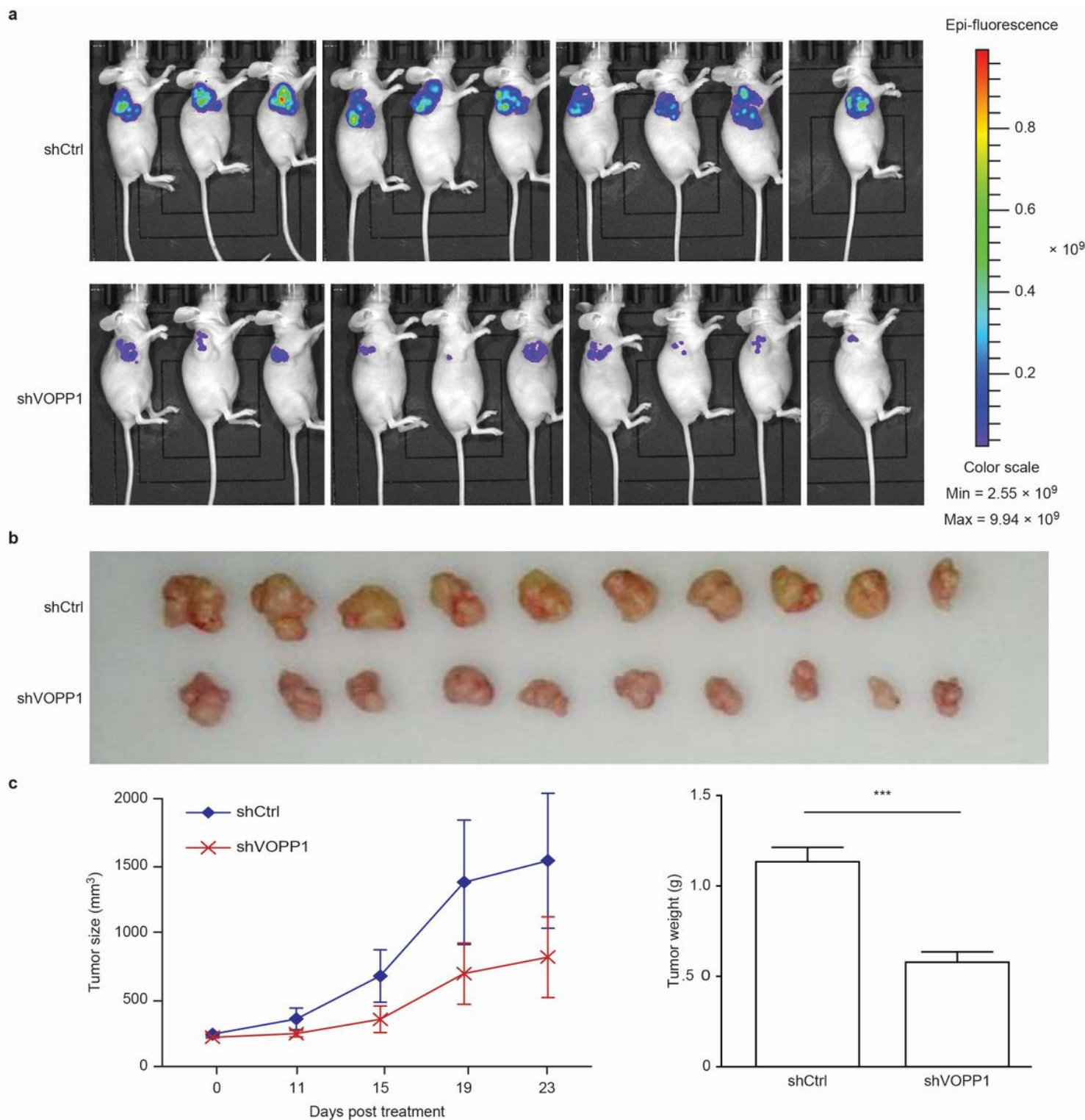


Figure 5

The knockdown of VOPP1 inhibited HCC cell growth in the orthotopic xenograft model: (A) BEL-7404 cells infected with lentiviral vectors encoding a control shRNA (NC shRNA) and shRNAs targeting VOPP1-1 (shVOPP1) were subcutaneously injected into nude mice. The representative bioluminescent imaging captured from subcutaneous tumors are presented. (B) The representative images of tumors are shown. (C) The growth curves of tumours in nude mice injected with cells are presented; * $P < 0.05$. (d) At 23 days

after shRNA treatment, the tumors were isolated, and the tumor weights were measured; *** $P < 0.001$. The data was presented as mean \pm standard deviation (SD, $n = 10$).

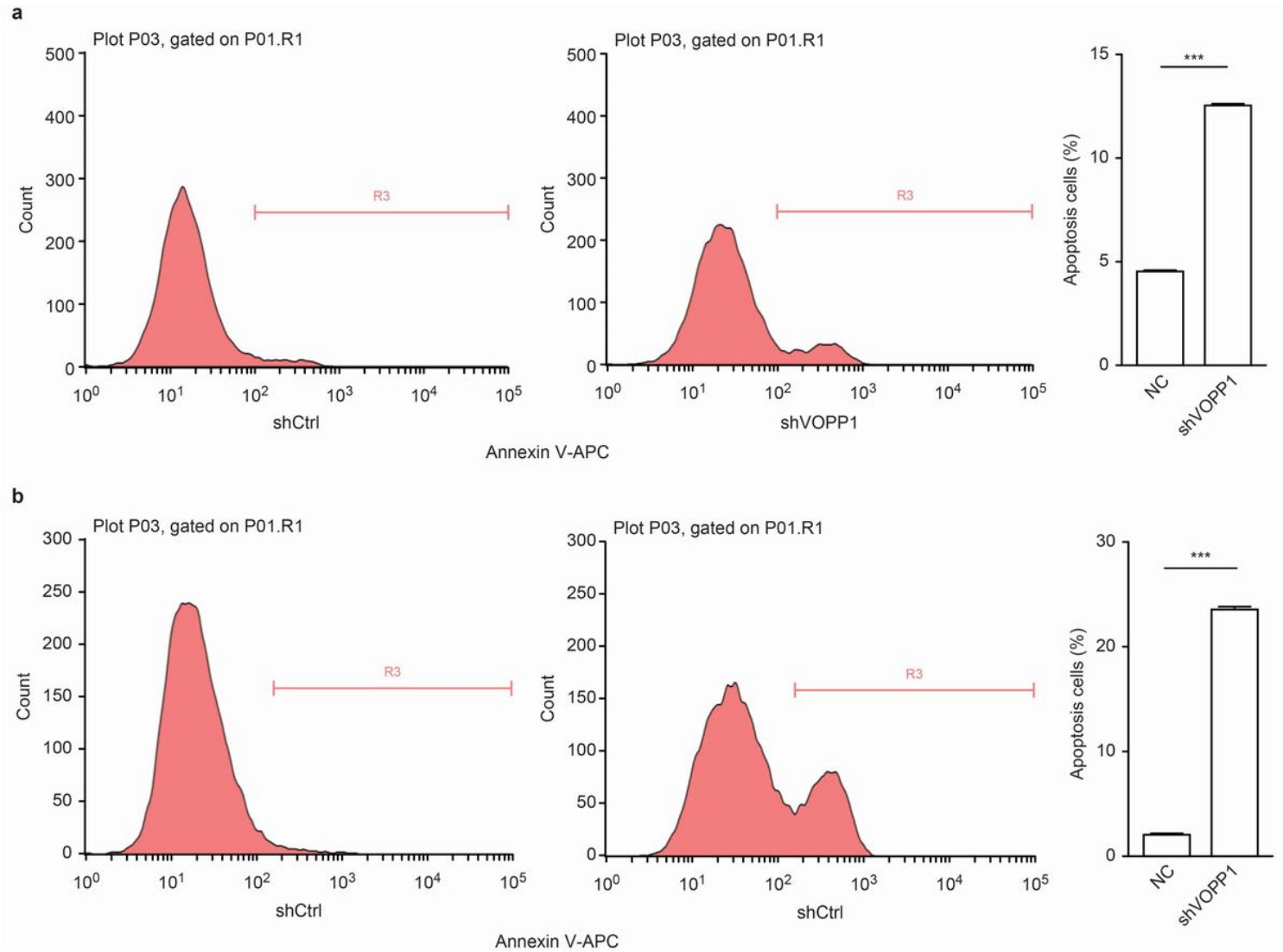


Figure 6

VOPP1 knockdown induced apoptosis in HCC cells: VOPP1 knockdown increased the incidence of apoptosis in SMMC-7721 (A) and BEL-7404 (B) cells. The percentage of apoptosis was calculated based on the triplicate experiments. The results were expressed as mean \pm standard deviation (SD, $n = 3$); *** $P < 0.001$.

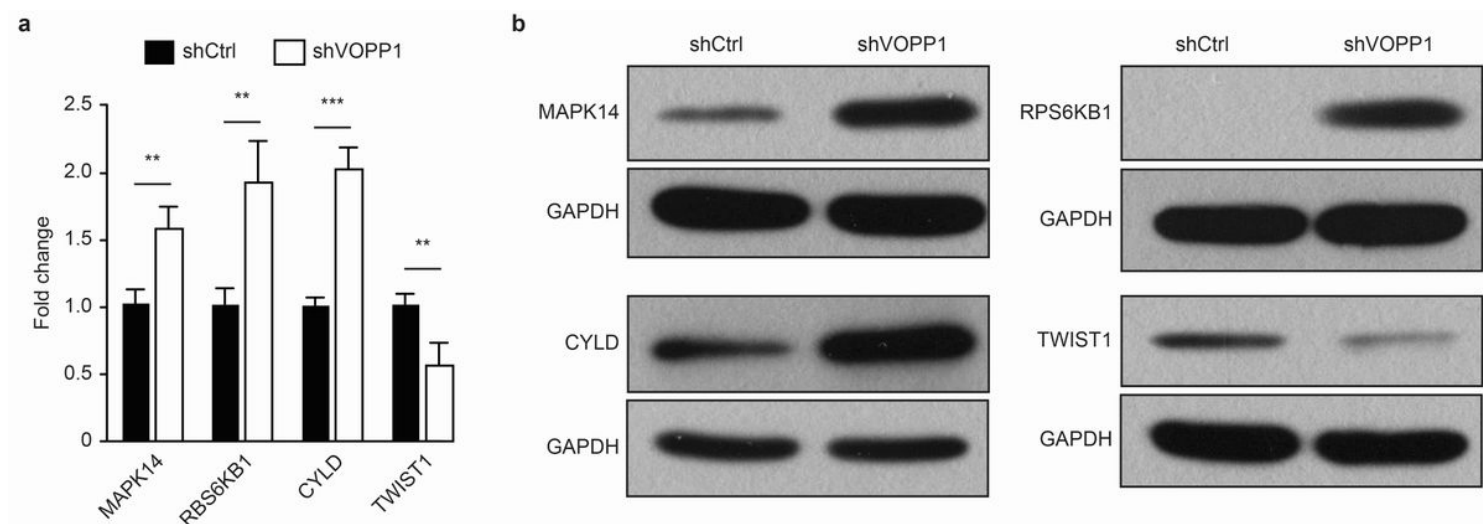


Figure 7

VOPP1 regulates the expression of MAPK14 in HCC cells: (A) The quantitative reverse transcription PCR analysis of MAPK14, RPS6KB1, CYLD and TWIST1 expression following VOPP1 knockdown in HCC cells. (B) The western blot analysis of MAPK14, RPS6KB1, CYLD and TWIST1 expression following VOPP1 knockdown in HCC cells. GAPDH served as the loading control (n=3); **P<0.01, ***P<0.001.

Supplementary Files

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