



Pharmacological inhibition of TRPV4 channel suppresses malignant biological behavior of hepatocellular carcinoma *via* modulation of ERK signaling pathway

Yu Fang^a, Guoxing Liu^a, Chengzhi Xie^b, Ke Qian^a, Xiaohua Lei^a, Qiang Liu^a, Gao Liu^a, Zhenyu Cao^a, Jie Fu^a, Huihui Du^a, Sushun Liu^a, Shengfu Huang^a, Jixiong Hu^a, Xundi Xu^{a,*}

^a Hunan Provincial Key Laboratory of Hepatobiliary Disease Research, Division of Hepatobiliary & Pancreatic Surgery, Department of Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China

^b Department of General Surgery, The 2nd Affiliated Hospital, Hunan University of Chinese Medicine, Changsha, Hunan 410005, China

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ABSTRACT

TRPV4 (transient receptor potential vanilloid 4), a member of the TRP superfamily, has been reported to correlate with several different forms of cancers. However, the role of TRPV4 in human hepatocellular carcinoma (HCC) remains unclear. The present study demonstrated that elevated expression of TRPV4 was shown in HCC tumor tissues when compared with paired non-tumoral livers both in protein and mRNA levels. Furthermore, the enhanced expression of TRPV4 was highly associated with histological grade ($P = 0.036$) and the number of tumors ($P = 0.045$). Pharmacological inhibition of TRPV4 channels in HCC cells with the specific antagonist HC-067047 suppressed cell proliferation, induced apoptosis and decreased the migration capability by attenuating the epithelial-mesenchymal transition (EMT) process *in vitro*. The p-ERK expression was apparently repressed after treatment with the TRPV4 antagonist, further blockade of the ERK pathway with U0126 could significantly aggravate HCC cells apoptosis. In NOD-SCID mouse xenograft models, intraperitoneal injection of HC-067047 could obviously suppress tumor growth and induce apoptosis *in vivo*. Together, our studies showed that the antitumor effects caused by TRPV4 channel inhibition in HCC cell lines might be attributed to the suppression of EMT process and inactivation of p-ERK which induced subsequent cell apoptosis. Thus, pharmacological inhibition of TRPV4 channel may be an option for HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer globally, and it is the third leading cause of cancer-related deaths worldwide [1,2]. In the past few decades, considerable advancements have been made in the diagnosis and management of HCC. Two interventions, transarterial chemoembolization (TACE) and radio-frequency ablation, are worldwide accepted as recommendations for the treatment of advanced liver cancer [3]. The poor long-term prognosis of HCC is often due to late diagnosis, high recurrence after treatment and chemotherapy-resistance [4,5]. Therefore, there is an urgent need for further exploration of more effective and safer anticancer strategies to suppress HCC progression.

Apoptosis is considered as a major mechanism of the programmed cell death. The disruption of the balance between cell proliferation and apoptosis is evidenced to contribute to the initiation of hepatic carcinogenesis, implying that antitumor strategy which specially activates

the apoptotic pathways may be an important option of treating HCC [6]. Besides, increasing evidence shows that ERK, a major member of MAPK family, is an important signaling pathway which is implicated in the regulation of cell apoptosis in hepatic cancers [7,8]. Previous studies have validated that ERK pathway mediates cell apoptosis mainly through regulating the expression of the pro-apoptotic (Bax and Bad) and anti-apoptotic (Bcl2 and Mcl-1) proteins [9]. Since the dysregulated ERK expression has a close correlation with cell apoptosis in HCC cell lines, targeting ERK may possess potential efficacy in suppressing hepatic carcinogenesis.

Transient receptor potential (TRP) channels exhibit cation permeability and therefore participate in many cellular processes controlling the fate of cancer cells, such as cell proliferation, apoptosis, and cell motility [10,11]. Numerous studies have shown that the TRPC1, TRPM1, TRPV1, TRPV2 and TRPM7 channels are expressed in nasopharyngeal carcinomas, human melanoma prostate, liver, breast, and gastric cancers [12–15]. Transient receptor potential vanilloid 4

* Corresponding author.

E-mail address: xuxundi@csu.edu.cn (X. Xu).

(TRPV4) channel, a member of the TRP superfamily, is a physiological sensor for osmotic pressure, mechanical deformation and temperature [16], which can be manipulated by mechanical stimuli and pharmacological agonists or antagonists [17,18]. Except for its more prominent expression in the nociceptors and the implication in a number of physiological processes [13,19], several studies evidence the dysregulated expression of TRPV4 in different types of tumor tissues and cancer cells. Santoni G et al. report the abnormal TRPV4 mRNA expression in glioblastoma cancer cell lines [13]. A study conducted by Lee et al. demonstrates that high TRPV4 protein levels are associated with more aggressive breast cancer types and poorer outcomes [20]. Although TRPV4 expression in tumors is preliminarily explored, rare studies are available about the effects of TRPV4 channels on the regulation of tumorigenesis and progression, especially the functional impacts of TRPV4 inhibition on HCC cells.

In the present study, we first determined the expression of TRPV4 in HCC tumor tissues and cell lines. In addition, HC-067047, a potent and selective reversible TRPV4 antagonist and GSK1016790A, a novel and potent TRPV4 channel agonist, were introduced to discover the influence of TRPV4 inhibition on HCC progression and the underlying molecular mechanisms. With the use of the selective agonist and antagonist, we aimed to prove that normal activation of TRPV4 channel was crucial for HCC cells to maintain cell viability, that inhibition of TRPV4 could cause anti-tumor effects in HCC cells, and then assessed the potential pathway involved in these processes.

2. Materials and methods

2.1. HCC specimen and clinical data collection

Primary HCC tissue and adjacent non-tumoral liver tissue were collected from patients who received curative resection from 2016–2017 at the Second Xiangya Hospital, Central South University, China. The clinical characteristics of all the patients were collected. The number of liver tumors was determined based on a combination of preoperative imaging methods: CT, MRI, PET-CT results and intraoperative findings. And two independent pathologists carefully dissected the resected HCC specimens to further confirm the number of tumors. Total RNA and proteins were obtained from these specimens. All procedures performed in studies involving human participants was approved by the Clinical Research Ethics Committee of the Second Xiangya Hospital.

2.2. Cell culture

The human immortalized hepatic cell line LO2 and human HCC cell line Huh7 were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Human HCC cell line HepG2 was obtained from the American Type Culture Collection. All the cells were cultured at 37°C in 95% air and 5% CO₂ as previously described [21].

2.3. Reagents and antibodies

GSK101 was obtained from Sigma, St Louis. HC067 and the ERK 1/2 inhibitor U0126 were purchased from Abcam. The following antibodies were used for the immunostaining: anti-Bax (1:4000, Proteintech), anti-Bcl2 (1:1000, Abcam), anti-beta-actin (1:5000, Proteintech), anti-caspase3 (1:500, Proteintech), anti-E-cadherin (1:150, Santa Cruz), anti-β-catenin (1:5000, Abcam), anti-vimentin (1:1500, Abcam), anti-N-cadherin (1:150, Santa Cruz), anti-p44/42 MAPK (Erk1/2) (1:2000, Proteintech), and anti-phospho-p44/42 MAPK(Erk1/2) (1:2000, Cell Signaling).

2.4. Treatment of HCC cells

HCC cells in the agonist group received different doses of GSK1016790A (hereafter GSK101; 0.1, 0.5, and 1 μM). In the antagonist group, HCC cells were pretreated with HC-067047 (hereafter HC067; 1 μM) for 30 min [22], then the medium was replaced with complete medium containing different concentrations of GSK101. The negative control group was treated with 0.5% dimethyl sulfoxide (DMSO). HCC cells were exposed to GSK101 treatment for 1 h to assess phospho-ERK1/2 protein levels in different groups. Cells were pretreated with 10 μM U0126 for 4 h to inhibit the expression of phospho-ERK1/2 prior to GSK101 or HC067 treatment [23].

2.5. MTT assay

HCC cells were seeded into 96-well plates (5×10^3 cells/well), and treated with different concentrations of drugs for 24, 48, and 72 h. Next, 20 μL MTT (5 mg/mL, Sigma, St Louis, USA) was added to each well, and the plates were incubated for another 4 h. Crystal violet was dissolved in 150 μL DMSO by gentle oscillation. Optical density was measured at 570 nm.

2.6. Colony-formation assay

Suspended HCC cells were seeded into six-well plates (1.5×10^3 cells/well) in 2 mL of complete medium, and the HCC cells were then cultivated under different treatments at 37 °C in air containing 5% CO₂ for 2 weeks. The medium containing different concentrations of drugs was changed every two days. The colonies were stained with 0.5% crystal violet for 20 min, and counted at a 50× magnification. The two independent experiments were performed in triplicate.

2.7. BrdU assay

The HCC cells were subjected to different treatments and cultivated for 48 h, and then incubated with 10 μM BrdU (BD, San Diego, USA) for 6 h. The procedure was performed as described previously [24]. Nuclei were stained with 1 mg/mL of 4',6-diamidino-2-phenylindole (DAPI) at a 1:1000 dilution for 3 min. BrdU-positive cells were counted across five random fields.

2.8. Wound-healing assay

For the wound-healing assay, Huh7 cells were seeded into 12-well plates and cultured until a confluent monolayer was achieved. A 10-μL pipette tip was used to create a scratch on the cell monolayer. The medium was replaced with 1 mL of serum-free medium containing different concentrations of drugs. The wound-healing process was photographed at 0, 24, and 48 h.

2.9. Migration assay

The migration assay was carried out in 6.5-mm Transwell chambers (Costar-Corning, Corning, USA). Briefly, serum-starved HCC cells were resuspended in 200 μL of serum-free medium in the upper chambers of the Transwell plate at 5×10^4 cells/well containing drugs at various concentrations. Lower chambers of the Transwell plate were filled with 500 μL of complete medium, and the cells were cultivated for 2 d. The cells that passed through the membrane were stained with 0.5% crystal violet for 15 min after fixing with methanol for 10 min.

2.10. Western blot assay

Whole cell protein (obtained from cells subjected to different treatments for 48 h) and frozen tissue protein were extracted. The

procedure of the Western blot assay was similar to those reported previously [25]. β -Actin was used as an internal reference.

2.11. RT-qPCR

Total RNA was extracted from HCC cells (subjected to different treatments for 48 h) and HCC tissues using the TRIzol reagent (Invitrogen). The procedure was performed as described previously [24]. Fold changes in mRNA expression were calculated by the relative quantification method ($2^{-\Delta\Delta Ct}$). The following primer sequences were used: TRPV4, (forward) 5'-TTTGCTCTATTCTACTCCTCCC-3' and (reverse) 5'-GCTGGCTTAGGTGACTCC-3'; caspase-3, (forward) 5'-GGA CTGTGGCATTGA

GACAG-3' and (reverse) 5'-CGACCCGTCCTTTGAATTC-3'; Bax, (forward) 5'-TCACTGAAGCGACTGATGTCCC-3' and (reverse) 5'-ACT CCCGCCACAAAGA

TGGTC-3'; Bcl2, (forward) 5'-AGCTGCACCTGACGCCCTT-3' and (reverse) 5'-ACATCTCCCGTTGACGCTCT-3'; β -actin, (forward) 5'-CATCCTGCGTCTGGA

CCTGG-3' and (reverse) 5'-TAATGTACGCACGATTTC-3'.

2.12. Immunohistochemistry

Liver tissues were fixed in formalin and then paraffin-embedded tissues were sectioned. The sections were incubated with TRPV4 antibodies (1:500, ab94868, Abcam, Cambridge, MA, USA) via a streptavidin peroxidase-conjugated method as described previously [26,27]. Evaluation of histological grade of HCC was conducted by two independent pathologists with 10 years of experience in liver pathology in the second Xiangya Hospital following the definitions of the World Health Organization: grade I, well differentiated; grade II, moderately differentiated; grade III-IV, poorly differentiated [28].

2.13. Immunofluorescence

HCC cells were seeded on coverslips and subjected to different treatments for 48 h. Cells were incubated with primary antibodies and Alexa Flour®-conjugated secondary antibodies, and DAPI was used to label the nuclei.

2.14. Flow cytometry

Cell apoptosis was measured with an Annexin V/PI assay kit I (BD, USA). Briefly, HCC cells were subjected to various treatments for 48 h, stained with 5 μ L of FITC Annexin V and 5 μ L PI for 15 min at RT in the dark, and analyzed by flow cytometry.

2.15. Animal models and drug treatment

Four-week-old female NOD-SCID mice were obtained from Vital River Laboratory Animal Technology (Beijing, China). Each NOD-SCID mouse received a single injection of Huh7 cells ($2-3 \times 10^6$ cells/per mouse) suspended in 100 μ L of Matrigel (BD Biosciences, CA) on the right flank. When the tumor size reached 100 mm³, mice were randomly distributed into three groups: negative control (100 μ L, 0.5% DMSO), agonist group (50 μ L DMSO followed by 50 μ L 10 μ g/kg/mouse GSK101), and antagonist group (50 μ L 10 mg/kg HC067 was injected into each mouse 30 min before it received 50 μ L 10 μ g/kg GSK101). Drugs were dissolved in 0.5% DMSO in a total volume of 50 μ L, and each mouse received daily intraperitoneal injection for 32 days. The tumor-bearing mice were then sacrificed after anesthesia, and tumors were removed for further study. Animal experiments were performed in accordance with the National Institutes of Guidelines for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Central South University Institutional Animal Care and Use Committee (Changsha, China).

2.16. Statistical analysis

Either Pearson's χ^2 test or Fisher's exact test was used for comparison between TRPV4 expression and clinicopathological parameters. The student's *t* test was used for two-group comparisons. One-way analysis of variance followed by Bonferroni adjustment was used to compare more than two groups. All statistical analyses were performed using SPSS 19.0. Differences were considered significant at a *P* value of < 0.05 .

3. Results

3.1. TRPV4 overexpression in HCC tumor tissues and cells

To examine whether TRPV4 was involved in HCC progression, we first immunohistochemically analyzed TRPV4 expression in 45 different histopathological types of HCC. TRPV4 was detected in the cytoplasm and membrane of most HCC cancer cells, and the majority of poorly differentiated HCC tumor tissues strongly stained positive for TRPV4 (16/19, 84.2%). In contrast, high TRPV4 expression was only detected in 9 of 26 highly or moderately differentiated HCC tumor tissues (34.6%; Fig. 1A). TRPV4 protein and mRNA expression levels were dramatically upregulated in HCC tumor tissues relative to non-tumoral liver tissues (Fig. 1B–D), indicating that TRPV4 was overexpressed in HCC.

Forty-five patients were divided into TRPV4^{low} (*n* = 20) and TRPV4^{high} (*n* = 25) groups based on protein levels. Correlative analysis revealed a significant association between increased TRPV4 expression and histological grade (*P* = 0.036) and the number of tumors (*P* = 0.045, Table 1). The results of the Western blotting indicated that TRPV4 protein levels were obviously higher in the HCC cells relative to the normal cell line, and the TRPV4 level was almost undetectable in LO2 cells (Fig. 1E).

3.2. TRPV4 channel inhibition suppressed HCC cells proliferation in vitro

Cell viability *in vitro* was determined via the MTT, BrdU incorporation, and colony-formation assays. The results of the MTT assay revealed that treatment with 0.1 and 0.5 μ M GSK101 did not affect HCC cells viability. Therefore, a concentration of less than 0.5 μ M GSK101 resulted in no cell toxicity. Treatment with 1 μ M GSK101 significantly reduced cell growth of Huh7 cells, which was not found in HepG2 cells. Pretreatment with 1 μ M HC067 for 30 min obviously suppressed the growth of HCC cells at 48 and 72 h relative to the untreated negative control (Fig. 2A). The results of the BrdU assay revealed that TRPV4 inhibition markedly reduced DNA synthesis in HCC cells (Fig. 2B). Furthermore, inhibition of TRPV4 dramatically decreased the size and number of colonies (Fig. 2C and D). In the previous studies, some researchers chose to incubate cells with antagonist alone to investigate the functional impacts of TRPV4 inhibition [20,29]. In our study, we found that HC067 treatment alone could also markedly attenuate cell proliferation in HCC cells, and there was no obvious difference in cell viability when compared with HC067 pretreatment group (Supplementary Fig. 1A and B).

3.3. TRPV4 channel inhibition promoted HCC cell apoptosis and modulated the expression of apoptosis-related molecules

We further investigated whether TRPV4 channel inhibition could induce apoptosis in HCC cells. Annexin V/PI assays indicated that TRPV4 channel blockade significantly induced a higher level of end-stage apoptosis compared with the other two groups, and there was no significant difference between the agonist and NC group (Fig. 3A and B). As illustrated by the results of the Western blot, TRPV4 channel inactivation-induced apoptosis was further confirmed by the upregulation of Bax and caspase-3 cleavage, and downregulation of Bcl2 and

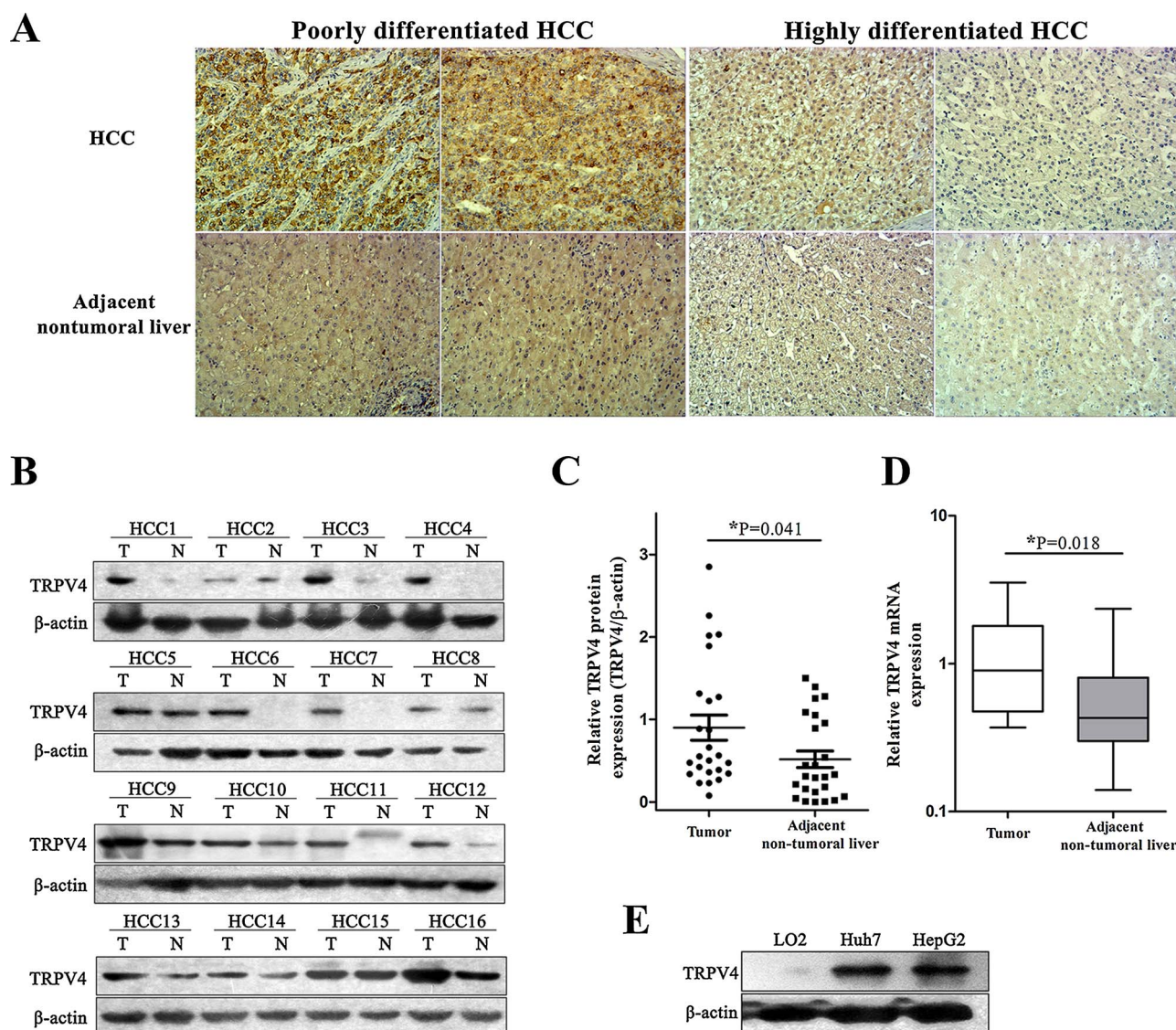


Fig. 1. TRPV4 expression in hepatocellular carcinoma (HCC) tissues and cell lines. (A) Immunohistochemical examination of TRPV4 in HCC tissues and adjacent non-tumoral liver tissues. Magnification, $\times 100$. (B and C) Western blot analysis of TRPV4 levels in HCC tissues (T) and adjacent non-tumoral liver tissues (N). (D) RT-PCR analysis of TRPV4 mRNA levels normalized to β -actin in tumor tissues and paracancerous tissues. (E) TRPV4 protein levels in three cell lines. *P < 0.05.

caspase-3 protein levels (Fig. 3C). And similar alterations of Bcl2 and Bax protein levels were also detected when cells were exposed to HC067 treatment alone (Supplementary Fig. 1C). RT-PCR analysis revealed that HC067 pretreatment increased the transcriptional level of Bax and decreased the level of Bcl2 in HCC cells (Fig. 3D and E).

3.4. Involvement of the ERK signaling pathway in TRPV4 channel inhibition related apoptosis

To explore the pathway in apoptosis induced by TRPV4 channel inhibition, we investigated ERK and phospho-ERK1/2 protein levels after 1h treatment of GSK101 in different groups. As expected, pretreatment with the TRPV4 antagonist resulted in an apparent downregulation of phospho-ERK1/2 compared with the other two groups, whereas no obvious changes were found in the total ERK (Fig. 4A). Therefore, we used U0126, the ERK1/2 inhibitor, to define the potential role of the ERK1/2 pathway in cell apoptosis. As there were no significant correlations between agonist concentration and apoptosis, we selected 0.1 μ M GSK101 as the agonist group. Pretreatment with U0126 for 4 h significantly increased the number of apoptotic cells both in the agonist and antagonist groups (Fig. 4B and C). And the further

reduction of p-ERK1/2 expression induced by U0126 led to the upregulation of Bax and caspase-3 cleavage, and downregulation of Bcl2 and caspase-3 protein levels (Fig. 4D). Together, these findings strongly indicated that TRPV4 inhibition induced HCC cells apoptosis by suppressing ERK1/2 activation, and further inhibition of ERK1/2 activity enhanced cell apoptosis in HCC cells.

3.5. TRPV4 channel inhibition repressed HCC cells migration by attenuating the EMT process in vitro

Transwell and wound-healing assays were used to assess the impact on cell migration capacity with the blockade of TRPV4 channels. Huh7 and HepG2 cells exhibited an enhanced migration capability when treated with 0.1 and 0.5 μ M GSK101 compared to the NC group, while this increase was markedly reversed with HC067 pretreatment (Fig. 5A and B). Consistent with these results, in the wound-healing assay, Huh7 cells showed slightly increased migration in the agonist group, and TRPV4 channel inhibition also significantly repressed this increase (Fig. 5C and D).

We further examined the expression of some important epithelial and mesenchymal markers in HCC cells. The results of the Western blot

Table 1
Correlative analysis of TRPV4 expression with clinicopathological parameters.

Parameter	n	Expression of TRPV4		P-value
		Low (n = 20)	High (n = 25)	
Gender				
Male	35	17	18	0.496
Female	10	3	7	
Age (years)				
≥ 50	24	11	13	0.841
< 50	21	9	12	
HBsAg				
Positive	41	19	22	0.770
Negative	4	1	3	
Serum AFP (μg/l)				
≥ 20	37	17	20	0.965
< 20	8	3	5	
Tumor size (cm)				
≥ 5	32	15	17	0.607
< 5	13	5	8	
Portal vein invasion				
Yes	23	7	16	0.053
No	22	13	9	
Number of tumors				
Multiple	21	6	15	0.045
Solitary	24	14	10	
Histological grade				
Poorly differentiated	19	5	14	0.036
Well/moderately differentiated	26	15	11	

Higher TRPV4 protein level was considered > 1.5-fold upregulation relative to the adjacent nontumoral liver.

analysis indicated that HCC cells incubated with 0.1 and 0.5 μM GSK101 alone showed a decreased expression of E-cadherin and β-

catenin, and an enhanced expression of N-cadherin and vimentin compared with the NC group, while TRPV4 channel inhibition in advance exerted the opposite effects on the protein levels (Fig. 4E). Similar alterations could be observed by immunofluorescence staining (Fig. 4F). Together, these findings strongly demonstrated that TRPV4 channel inhibition suppressed the migration of HCC cells by attenuating the EMT process.

3.6. TRPV4 channel inhibition suppressed tumor growth and induced apoptosis in vivo

We established an Huh7 cell xenograft tumor model in NOD-SCID mice to investigate the effect of TRPV4 channel inhibition on tumor growth *in vivo*. We found that TRPV4 channel blockade markedly reduced both tumor size and weight compared with the NC group, while agonist treatment did not affect tumor growth (Fig. 6A and B). Further immunostaining analysis of the tumor tissues exhibited the increased Bax and cleaved caspase-3 expression, and decreased Ki67 expression with HC067 treatment (Fig. 6C and E). And a higher percentage of necrosis and collapse of tumor cells were detected in the tumor sections of the antagonist group when compared with the other two groups (Fig. 6C). Ki67-positive rate was further calculated to estimate cell proliferation *in vivo* (Fig. 6D). Additionally, western blot analysis confirmed the reduced expression of p-ERK1/2 in tumor tissues subjected to TRPV4 inhibition (Fig. 6F).

4. Discussion

TRPV4 channel function has been clarified in different normal cells or tissues, like hypothalamic 4B cells, epithelial cells, skin and so on [22,30,31]. However, evidence about the effects of TRPV4 channels on the regulation of tumorigenesis and progression is scarce and controversial. Fusi et al. demonstrated that overnight exposure to GSK101 did not affect cell viability of HaCaT cells [22]. While in the study of

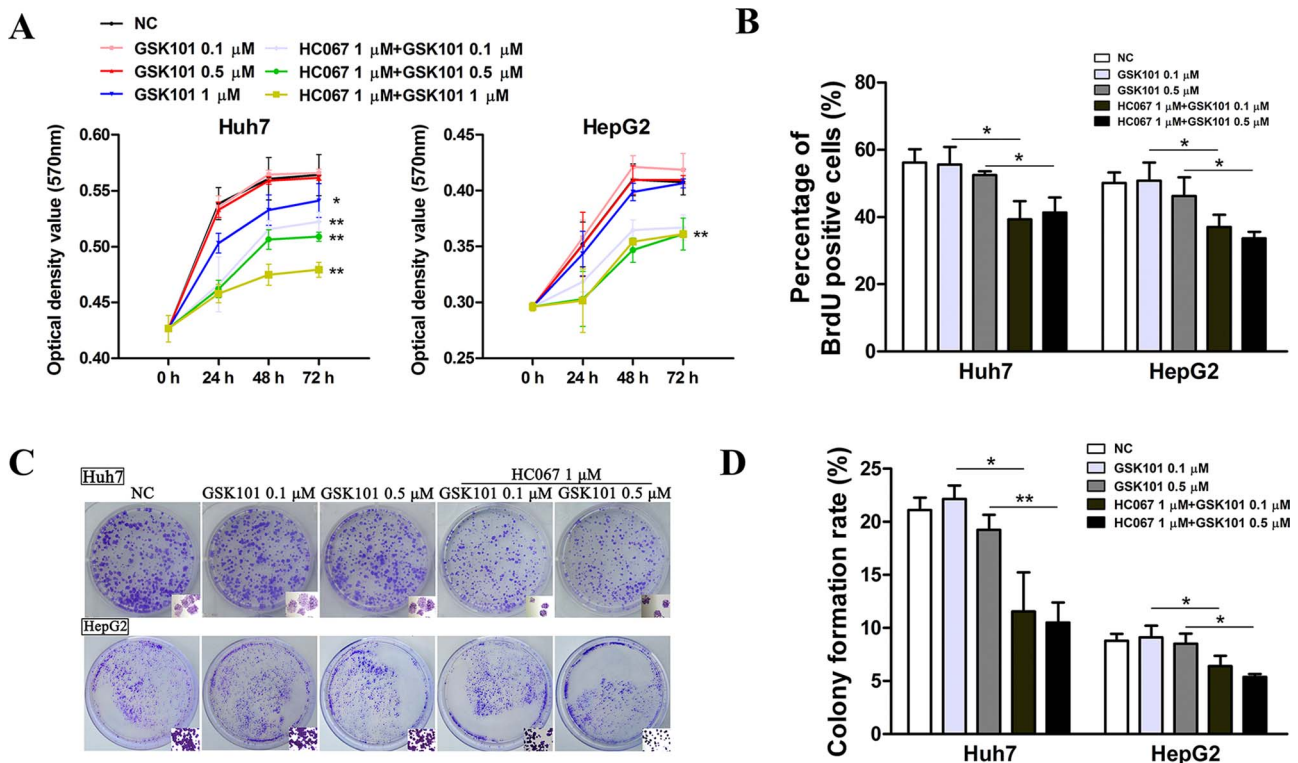


Fig. 2. TRPV4 channel inhibition suppressed HCC cell viability *in vitro*. (A) HCC cells viability subjected to different treatments was evaluated by the MTT assay. (B) DNA synthesis of HCC cells was evaluated using the BrdU incorporation assay. (C and D) Colony-forming abilities of HCC cells were assessed by the colony-formation assay. Data are shown as means ± SDs of three (C and D) to five (A and B) independent experiments. *P < 0.05, **P < 0.01, antagonist group vs NC.

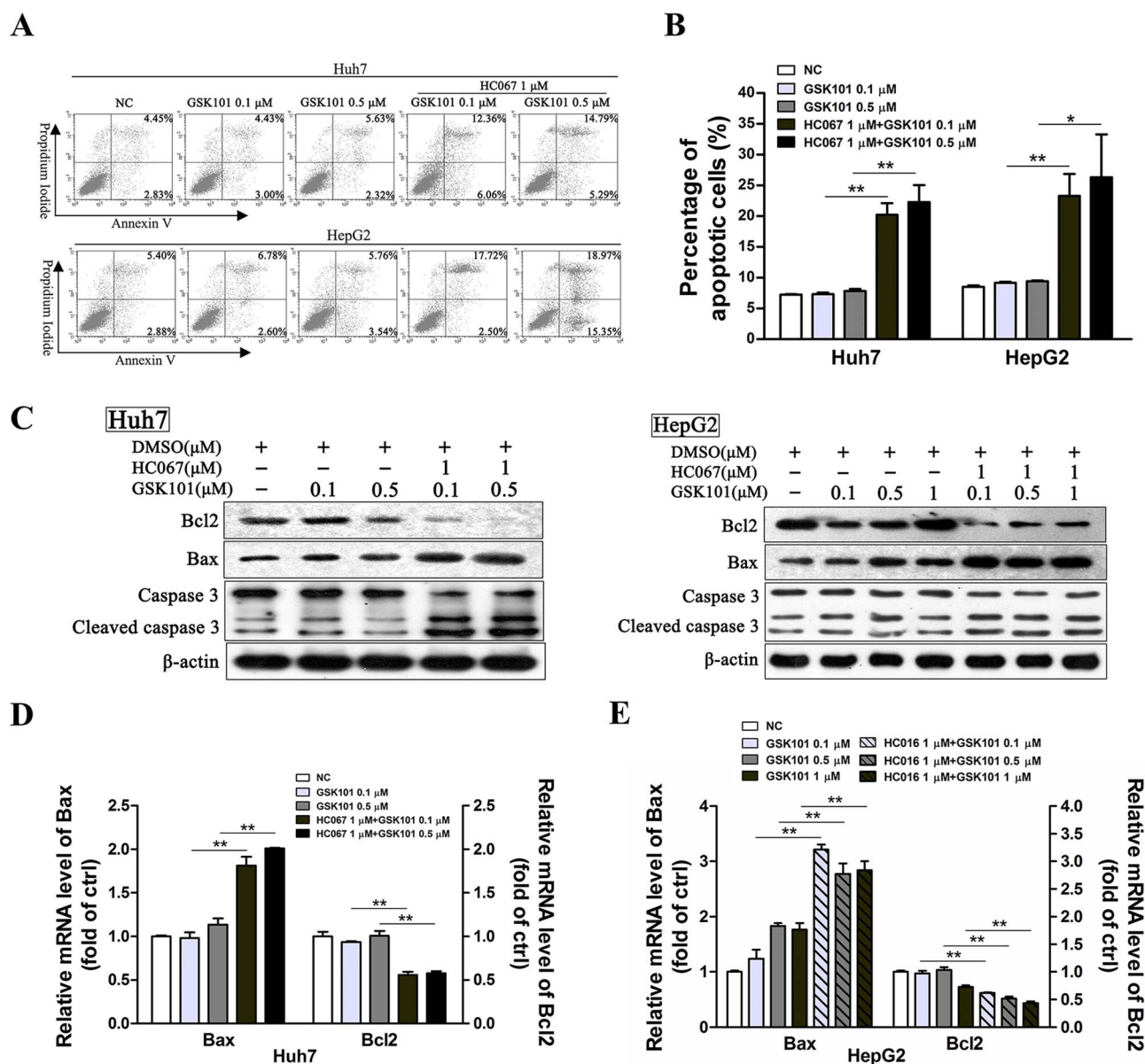


Fig. 3. TRPV4 channel inhibition promoted HCC cells apoptosis. (A) Cell apoptosis was analyzed by flow cytometry with Annexin V/PI. (B) An increased level of end stage apoptosis was induced by TRPV4 channel inhibition. (C) Bcl2, Bax, caspase-3, cleaved caspase-3 protein levels were analyzed in HCC cells subjected to different treatments. (D and E) RT-PCR analysis of Bax and Bcl2 mRNA levels in HCC cells. β -actin was used as the internal loading control. Data are shown as means \pm SDs of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Viguera et al., pharmacological activation of TRPV4 with GSK101 could suppress the proliferation of human melanoma cells and HaCaT keratinocytes [32]. To explore whether TRPV4 could be used as a potential target for pharmacological treatment of HCC, we introduced a highly selective TRPV4 agonist, GSK101, and the TRPV4 antagonist HC067 in the present study. Several studies have confirmed that 100 nM–1 μ M GSK101 is sufficient to activate TRPV4 channel [33], and pretreatment with 1 μ M HC067 can completely inhibit TRPV4 channel which is activated by various stimuli [22,29]. In our study, it was found that agonist stimulation did not alter cell viability either *in vitro* or *in vivo*, and the slightly enhanced migration ability was detected when HCC cells were subjected to the agonist treatment. However, TRPV4 channel blockade with HC067 pretreatment or treatment alone could break the steady state, induce apoptosis and attenuate HCC cells migration capability. These findings were partly consistent with the study conducted by Thoppil et al, in which TRPV4 activation caused by GSK101 administration, dramatically inhibited tumor endothelial cell proliferation, but had no effect on tumor cells viability [31]. Lee et al. also

reported that TRPV4 inhibitors could effectively suppress the migration and invasion of the human breast cancer cells 4T07 which exhibited high levels of TRPV4 expression [20]. Therefore, we supposed that the normal activation of TRPV4 channel might be essential for HCC cells to maintain physiological function. Hence, pharmacological inhibition of TRPV4 could induce the anti-tumor effects in HCC cells.

Apoptosis can be induced in response to various stimuli, mainly through the intrinsic (mitochondrial) pathway, which is normally activated by endogenous stress signals, and the extrinsic pathway, which is associated with death receptors [34]. Bcl2 family members play an essential role in regulating the intrinsic pathway, which includes anti-apoptotic and pro-apoptotic proteins that control the release of apoptogenic cytokines [35]. All of these processes can eventually lead to caspase-3 activation. Our study found that TRPV4 channel blockade might influence the transcription and translation of the apoptosis-related genes Bax and Bcl2, resulting in the induction of caspase 3 activation, preliminarily illustrating the pro-apoptotic effect of TRPV4 inhibition.

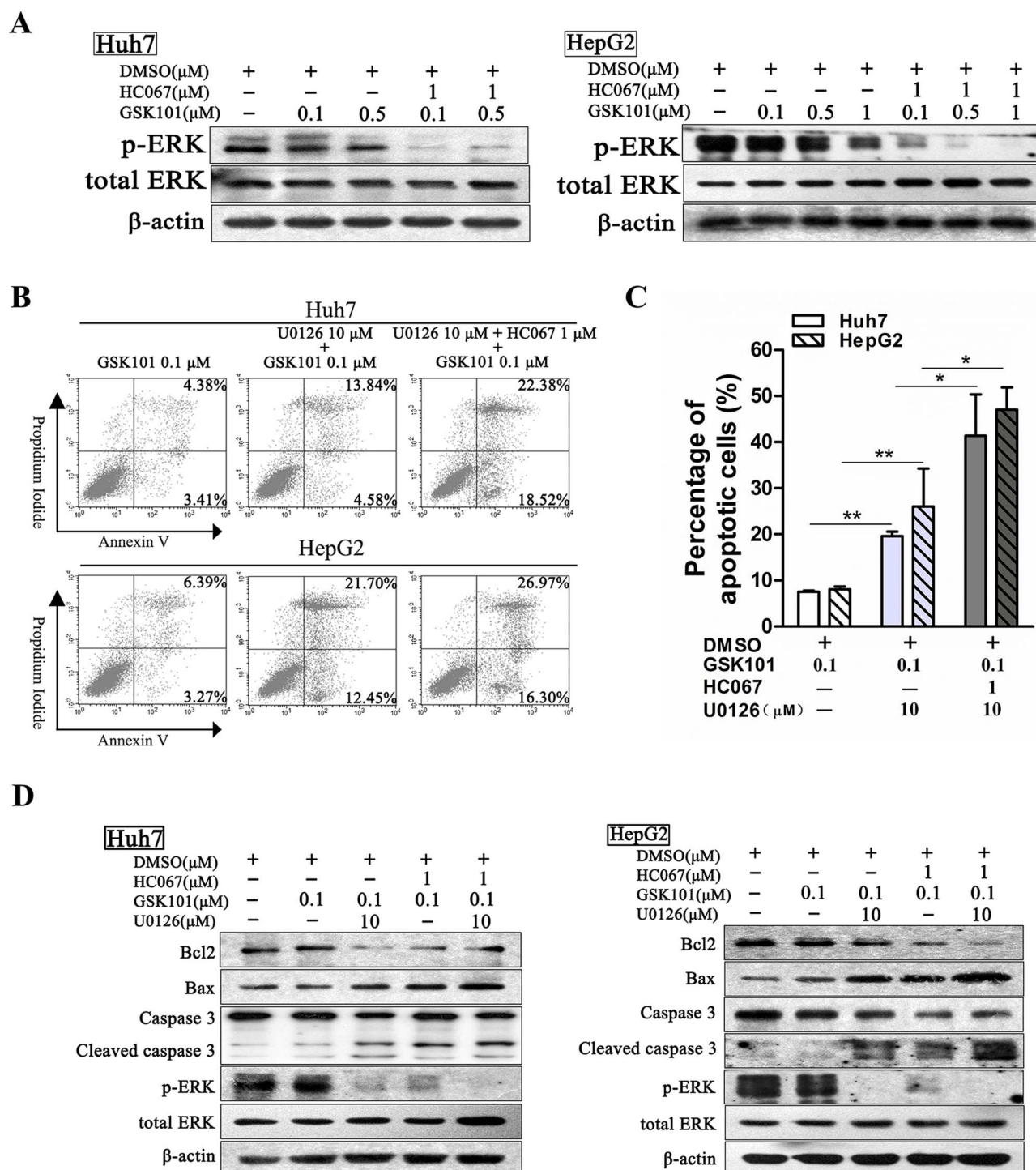


Fig. 4. Involvement of ERK signaling pathway in TRPV4 channel inhibition related apoptosis. (A) ERK and phospho-ERK1/2 protein levels of HCC cells were determined after 1 h treatment of GSK101 by Western blotting. (B and C) HCC cells were pretreated with U0126 (10 μM) for 4 h prior to agonist or antagonist treatment, and cell apoptosis was analyzed by flow cytometry with Annexin V/PI. (D) Immunoblot analysis of Bcl2, Bax, caspase-3, cleaved caspase-3, phospho-ERK1/2, and total ERK in HCC cells with the pretreatment of U0126. Data are shown as means \pm SDs of three independent experiments. *P < 0.05, **P < 0.01.

We next investigated the potential signal pathways by which TRPV4 inhibition could induce HCC cells apoptosis. Previous studies have shown that the ERK signaling pathway is deeply involved in the regulation of apoptosis in HCC cells [36,37]. And several researches report that there is a close correlation between decreased p-ERK levels and TRPV4 inhibition in the meditation of inflammation and cell differentiation [29]. In the present study, we found that pretreatment with the TRPV4 antagonist downregulated the p-ERK levels. And further blockade of ERK pathway with U0126 markedly enhanced cell

apoptosis. These data indicated that TRPV4 inhibition induced cell apoptosis was, at least partially, *via* the inactivation of p-ERK in HCC cell lines. However, the activation of TRPV4 did not cause increased level of p-ERK, as had been reported before [38]. Since, the expression p-ERK was evaluated 1h after GSK101 treatment, we supposed the possibility that the activation of p-ERK in HCC cells exposed to GSK101 might have happened earlier, less than 1 h. Subsequently, the increased p-ERK might waned towards the basal levels after 1h treatment. In consistency with our results, Nayak et al. also found that TRPV4 agonist did

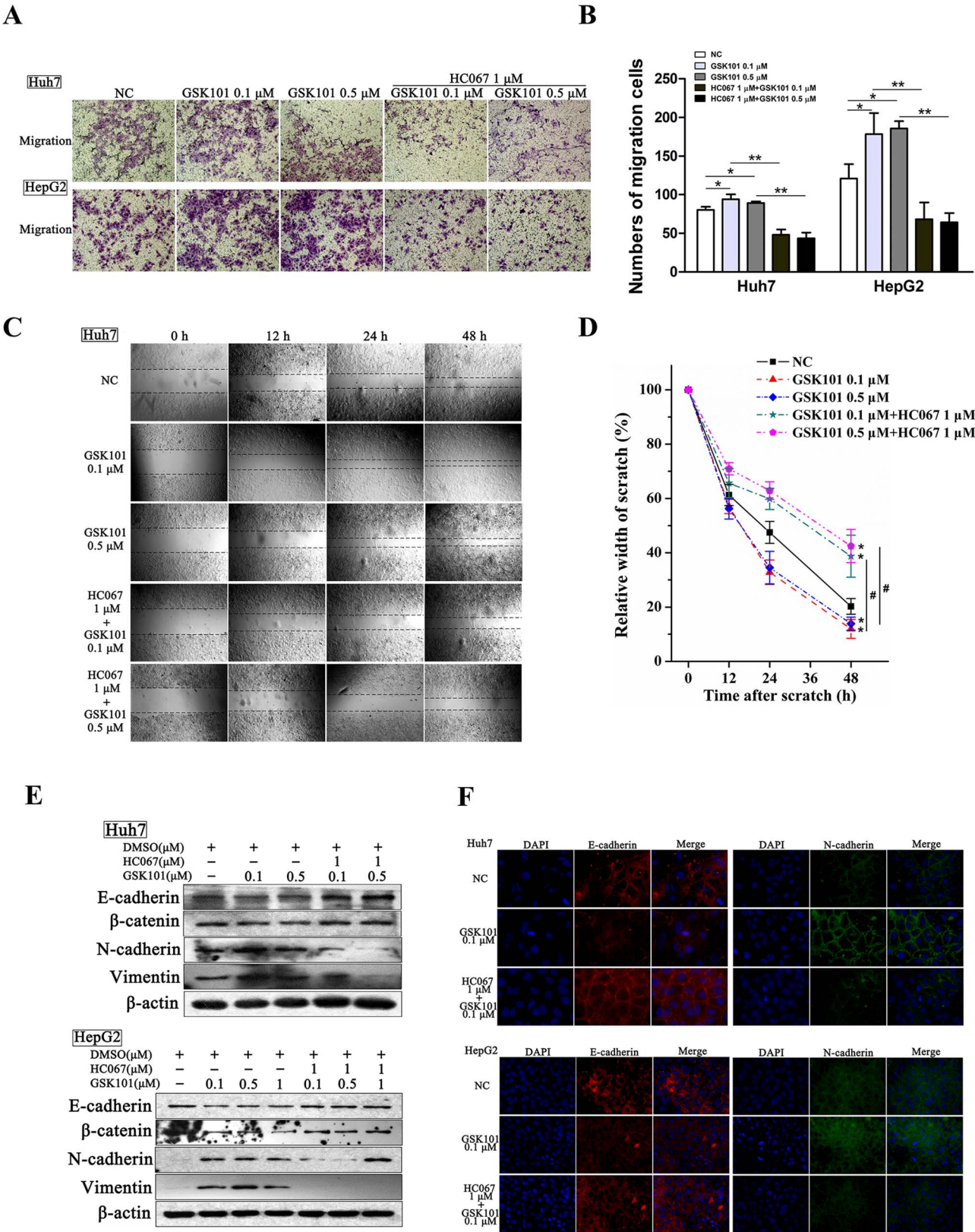


Fig. 5. TRPV4 channel inhibition suppressed HCC cell migration by reversing EMT. (A and B) Transwell migration assays of HCC cells subjected to different treatments. Cells were counted in three randomized fields at a magnification of $\times 100$. ((C and D)) The migration ability of Huh7 cells was analyzed by wound-healing assay. Magnification, $\times 50$. $^{\#}P < 0.05$, antagonist group vs agonist group. (E and F) Western blot and Immunofluorescence staining of E-cadherin, β -catenin, N-cadherin and vimentin with different treatments. Data are shown as means \pm SDs of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, antagonist group vs NC.

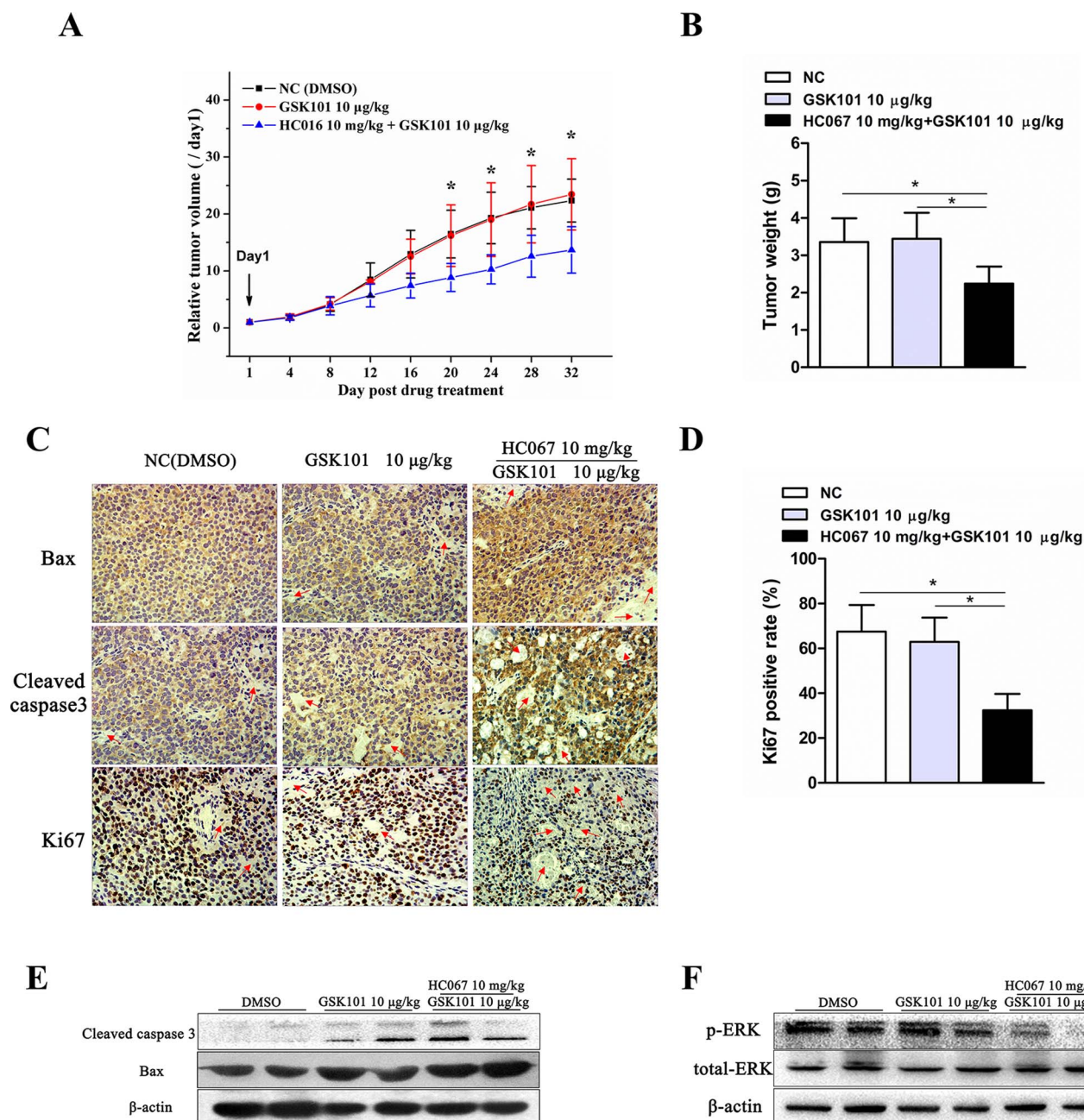


Fig. 6. TRPV4 inhibition suppressed proliferation of HCC cells and induced apoptosis *in vivo*. (A) Relative tumor volume was calculated to evaluate the viability of Huh7 cells subjected to different treatments *in vivo*. * $P < 0.05$, antagonist group vs negative control. (B) Xenograft tumor weights were measured after dissection of the NOD-SCID mice. (C) Immunohistochemical examination of Bax, cleaved caspase-3 and Ki67 in xenograft tumor sections. Red arrows, necrosis and collapse of tumor cells. Magnification, $\times 200$. (D) Ki67-positive rates of different groups. (E) Immunoblot analysis of cleaved caspase-3 and Bax in frozen xenograft tumor tissues. (F) Western blot analysis of total and phosphorylated ERK1/2 in xenograft tumor tissues. β -actin was used as a loading control. Data are shown as the means \pm SD of six NOD-SCID mice. * $P < 0.05$.

not affect p-ERK levels in lung epithelial cells, while cells exposed to 1 μ M HC067 exhibited 40% reduction of p-ERK expression relative to agonist treatment [29]. Together, the precise mechanism through which TRPV4 inhibition induced cell apoptosis might be obscure, but it was clear that p-ERK inactivation played a critical role in this process.

Recent research has reported that TRPV4 dysfunction diminishes migration and invasion capability in the human breast cancer cell line [20]. As the TRPV4 protein level was associated with number of tumors and histological grade, we supposed whether TRPV4 inhibition could suppress the migration of HCC cells. We observed that treatment with TRPV4 agonist alone slightly increased cell migration, whereas TRPV4 inhibition dramatically reduced the migration capacity and affected the expression of several EMT-related markers in HCC cells. EMT is deeply

involved in cancer cell metastasis, and downregulation of E-cadherin and β -catenin and upregulation of mesenchymal markers, such as N-cadherin and vimentin, are the hallmarks of EMT. Thus, when these factors are altered, HCC cells gain the ability of intrahepatic migration or extensive distant metastasis. The variations in the EMT-related proteins in our study indicated that TRPV4 channel dysfunction inhibited the migration of HCC cells by suppressing the EMT process.

Some limitations still needed to be further addressed in our study. Since, we mainly explored the impact on the malignant biological behavior in HCC cells, one constraint of the present study was the limited number of patients analyzed. To clearly illuminate the significance of TRPV4 expression in HCC, larger numbers of tissue specimens, more detailed clinical characters and prolonged follow-up time are helpful to

provide more accurate correlational analyses. Furthermore, our results only examined the dysregulated expression of p-ERK after TRPV4 inhibition in HCC cell lines. Whether there are other potentially involved signal molecules and the more precise role of the ERK1/2 signaling pathway in HC067-induced cell apoptosis still need to be studied further.

In summary, our study illustrated the essential role of the TRPV4 channel in HCC development and that its pharmacological inhibition inhibited proliferation and induced apoptosis in HCC cells, which was at least in part, ascribed to inactivation of the ERK pathway. TRPV4 channel blockade could also attenuate the migration capacity of HCC cells by suppressing the EMT process. Therefore, our findings provided a theoretical basis for inhibiting the TRPV4 channel in the treatment of HCC.

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Appendix A. Supplementary data

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