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Capsaicin combined with cisplatin inhibits TGF-β1-induced EMT and TSCC cells migration via the Claudin-1/PI3K/AKT/mTOR signaling pathway

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Abstract

Tongue squamous cell carcinoma (TSCC) is one of the most common malignant tumors among oral cancers, and its treatment is based on radio-chemotherapy and surgery, which always produces more serious side effects and sequelae. Traditional medicine can compensate for the shortcomings of modern medical treatments and play a better therapeutic role. Currently, active ingredients derived from plants are attracting the attention of researchers and clinical professionals. We examined capsaicin (CAP), an active ingredient isolated from Capsicum annuum (*family Solanaceae*), and explored the effect of CAP combined with cisplatin (DDP) on epithelial-mesenchymal transition (EMT) and TSCC cells migration. Our results demonstrated that Transforming growth factor-β1(TGF-β1) induced EMT and promoted cell migration in TSCC cells. CAP combined with DDP inhibits non-TGF-β1-induced or TGF-β1-induced EMT and migration. Mechanistically, the inhibition of non-TGF-β1-induced EMT and migration by CAP combined with DDP was mediated by the AMPK/mTOR pathway, whereas TGF-β1-induced EMT and migration were regulated by the Claudin-1/PI3K/AKT/mTOR pathway. A nude lung metastasis mouse model was established for in vivo validation. These results support our hypothesis that the combination of CAP and DDP inhibits TSCC metastasis. These data set the stage for further studies aimed at validating CAP as an effective active ingredient for enhancing chemotherapy efficacy and reducing the dosage and toxicity of chemotherapeutic drugs, ultimately paving the way for translational research and clinical trials for TSCC eradication.

Keywords Tongue squamous cell carcinoma, Capsaicin, Cisplatin, Epithelial-mesenchymal transition, TGF-β1, Migration

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Background

Tongue squamous cell carcinoma (TSCC) is one of the most common oral cavity carcinomas with high malignancy, metastasis, invasiveness, and poor prognosis [1]. Due to the numerous blood and lymphatic vessels distributed around the tongue and frequent movement of the tongue, tumor cells are prone to metastasize to adjacent tissues and organs. Most TSCC patients already have local infiltration or even lymph node metastasis during diagnosis [2]. However, their therapeutic efficacy against metastatic TSCC is poor.

As a classic chemotherapeutic drug, cisplatin (DDP) is a platinum-based chemotherapeutic agent extensively used to treat a variety of solid tumors and significantly improves the survival rates of cancer patients [3]. However, it has several side effects, including nephrotoxicity, peripheral neuropathy, and ototoxicity [4], and long-term application can lead to drug resistance [5]. Therefore, reducing DDP dosage, alleviating its toxic side effects, and improving its therapeutic efficacy are urgently required. In lung cancer-related studies, TGF-β1 was found to promote EMT by enhancing N-cadherin and Vimentin proteins and decreasing E-cadherin, during which drug-resistant proteins, including ERCC1 and p-gp, were also upregulated. Conversely, TGF-β1 inhibition increases cancer cells sensitivity to DDP while suppressing TGF-β1-induced EMT and drug resistance-associated protein expression [6]. Similarly, other studies have found that TGF-β1 inhibition reverses EMT and tumor metastasis, making cancer cells in chemotherapy more sensitive to DDP [7, 8]. Therefore, TGF-β1 inhibition can be considered an effective strategy to enhance DDP efficacy and sensitivity. The combination of DDP with other natural products has been extensively studied in oncological research and has yielded some success. For example, curcumin in combination with DDP synergistically inhibits thyroid cancer metastasis, and fresh ginger juice attenuates DDP-induced testicular alterations and toxicity [9].

Traditional Chinese medicines have played a significant role in treating diverse diseases. Better efficacy was achieved in combination therapy. Capsaicin (CAP) is the major component responsible for the unfair taste of chili peppers, and recent studies have found it to be more effective in the treatment of conditions, including osteoarthritis [10], pain [11], and tumors [12]. For example, capsaicin attenuates cholangiocarcinoma carcinogenesis by modulating Hh signaling and inhibiting EMT in vivo [13]. It has been found that CAP attenuates the TGF-β2-induced EMT process in lens epithelial cells in vivo and in vitro [14]. These results suggested that capsaicin induces cell-specific effects on EMT. Additionally, CAP in combination with chemotherapeutic drugs had a better antitumor effect. CAP synergizes with sorafenib to exert anti-hepatocellular carcinoma activity by inhibiting the epidermal growth factor receptor and phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT)/ mammalian target of rapamycin (mTOR) signaling [15]. However, the effects of the combination of CAP and DDP on EMT in TSCC cells and its downstream mechanisms remain to be elucidated.

Transforming growth factor-β (TGF-β) is an important cytokine that regulates diverse biological processes, including cell growth, differentiation, apoptosis, immunity, angiogenesis, migration, and invasion [16]. TGF-β induces EMT in a variety of malignant tumors (lung cancer, gastric cancer, and breast cancer) [17–19]. During EMT, epithelial tumor cells lose intercellular junctions and parietal polarity, resulting in extracellular matrix remodeling, acquisition of a mesenchymal-like phenotype, and a significant increase in cell motility and invasiveness [20]. Thus, TGF-β has a significant impact on tumor invasion and metastasis. However, little is known about how CAP affects TGF-β1-induced EMT in TSCC cells and what the specific mechanisms are.

Epithelial-mesenchymal transition (EMT) is a phenomenon, wherein epithelial-type cells lose polarity and intercellular junctions, acquire a mesenchymal phenotype with increased motility [21] and is critical for tumor migration, invasion, and metastatic colonization [22]. Claudin-1 belongs to the claudin superfamily and is an important component of tight junctions, which are predominantly distributed on the cell membrane surface [23]. Studies have demonstrated that Claudin-1 is an EMT-related marker associated with cellular transformation regulation, Matrix metalloproteinases (MMPs) activation, and tumor metastasis [24, 25]. Claudin-1 is differentially expressed in normal and malignant tissues [26]. Additionally, claudins undergo delocalization, which plays a crucial role in the invasive ability of tumors. Claudin-1 has been extensively studied, but its role as a tumor promoter or suppressor has not yet been elucidated [27]. In some cancers, low Claudin-1 expression promotes cancer progression and invasion [28], whereas in others, Claudin-1 deficiency improves patient survival [29, 30]. However, how CAP affects Claudin-1 and whether it will affect EMT remains obscure.

Adenosine 5'-monophosphate-activated protein kinase (AMPK) is an energy regulator in vivo and is always activated by altered intercellular pressure, glucose depletion, hypoxia, and local ischemia [31]. CAP inhibits the migration, invasion, and EMT of renal cancer cells by regulating AMPK/mTOR signaling-mediated autophagy [32]. It was found that AMPK activation by antitumor drugs inhibited the metastatic potential of cancer cells [33]. For example, KDM5B inhibits breast cancer cell proliferation and metastasis by reversing EMT and lipid metabolism reprogramming in breast cancer cells through AMPK

signaling activation [34]. mTOR is a downstream target of AMPK, which acts as an intracellular nutrient sensor to control protein synthesis, cell growth, and metabolism and is regulated by the PI3K/AKT pathway [35]. Additionally, CAP combined with docetaxel hinders the growth of prostate cancer cells through the PI3K/AKT/mTOR signaling pathway [36]. Therefore, we investigated whether CAP combined with DDP exerted anti-metastatic effects through AMPK or PI3K/AKT pathway in TSCC cells.

In this study, we demonstrated that CAP combined with DDP inhibited non-TGF-β1-induced EMT and migration in TSCC through the AMPK/mTOR pathway, whereas suppressed TGF-β1-induced EMT and migration by the Claudin-1/PI3K/AKT/mTOR pathway. Furthermore, the distant metastasis was significantly restrained by the combined utilization of CAP and DDP in lung metastasis model of nude mice, which further confirmed our conclusions from in vitro experiments.

Methods

Cell lines

The human TSCC cell lines CAL27 (CRL-2095) and SCC9 (CRL-2095) were obtained from the American Type Culture Collection (ATCC). HN6 cells were gifted by the Stomatological Hospital Affiliated to Shanxi Medical University. The HN6 and CAL27 cell lines were cultured in DMEM high-glucose medium (Biosharp Life Sciences, CN, BL304A) containing 10% fetal bovine serum (FBS) (Jitai Excellbio Biotechnology, FSP500) and 1% penicillin and streptomycin (Biosharp Life Sciences, BL505A). SCC9 cells were cultured in DMEM-F12 (Biosharp Life Sciences, BL305A) containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, and 400 ng/ml hydrocortisone (Macklin Inc., H917755). Cells were incubated at 37 °C in a humidified incubator supplemented with 5% CO_2 and passaged every 2 days.

Reagents and antibodies

CAP and DDP were purchased from MedChemExpress (USA, 126924, 101436), and TGF-β1 was purchased from PeproTech (100–21 C). The primary antibodies used here included anti-E-cadherin (20874-1-AP, 1:5000), anti-N-cadherin (22018-1-AP, 1:3000), anti-vimentin (10366-1-AP, 1:3000), anti-LKB1 (Ser428, 10746-1-AP, 1:1000), anti-Claudin-1 (28674-1-AP, 1:1000), anti-AMPK (10929-2-AP, 1:1000), and anti-p-AKT (Ser473, 28731-1-AP, 1:1000), which were purchased from Proteintech. Anti-AKT1 (BS2987, 1:1000), anti-p-LKB1 (AP0088, 1:1000), anti-mTOR (BS61794, 1:500), anti-p-AMPKα (Thr172, BS5003, 1:1000), anti-p-mTOR (Ser2481, BS79380, 1:1000), which were purchased from Bioworld. Anti-PI3K (4257 S, 1:1000) was purchased from Cell Signaling Technology. Anti-p-PI3K (AP0854, 1:1000) and anti-β-Actin (AC026, 1:8000) were purchased from ABclonal. Peroxidase-labeled secondary anti-rabbit IgG (BS22357, 1:10000) was purchased from Bioworld.

Cell proliferation assay

Cell proliferation was detected using the Cell Counting Kit-8 (CCK8) (MA0218-5; Meilunbio). HN6, CAL27, or SCC9 cells were inoculated into 96-well plates and treated with CAP (0, 50, 100, 150, 200, 250 μM) or DDP (0, 2, 4, 6, 8 μg/ml) for different periods (24–48 h) post 24 h. Subsequently, 10 μl of CCK8 solution was incorporated into each well, and incubation was continued in a 37 °C incubator for 1 h. The absorbance at 460 nm was measured by a microplate reader (Mindray, MR-96 A).

Wound healing assay

A wound-healing assay was conducted to evaluate the cell migration rate. Tongue cancer cells were inoculated in a 6-well plate at 1×10^6 cells per well density and incubated for 24 h. After the cell density reached 90%, the bottom of the 6-well plates was scratched with a 200 μl pipette tip, after which all detached cells were washed away with phosphate-buffered saline (PBS). TGF-β1 (5 ng/ml), CAP (150 μM), and DDP (4 μg/ml) were incorporated to serum-free medium and then incubated in an incubator for 24 h. Wound healing was imaged using an inverted light microscope (Olympus, Tokyo, Japan) at 0 and 24 h. The migration rate was determined by quantifying the change in the wound area using Image J 1.8.0.

Transwell assay

In the Transwell migration assay, approximately 8×10^4 cells were suspended in 200 μl serum-free medium containing DDP (4 μg/ml) and CAP (150 μM), followed by transfer to the upper chamber (Corning Incorporated). Then, 500 μl of medium containing TGF-β1 (5 ng/ml), DDP (4 μg/ml), and CAP (150 μM) in 10% FBS was incorporated into the lower chamber. After 24 h at 37 °C in 5% CO_2 incubation, the cells were fixed with 4% paraformaldehyde (Biosharp Life Sciences, BL539A) for 20 min and stained with crystal violet for 15 min. Photography and analysis were conducted into 3–5 fields per chamber.

Plasmids and cell transfection

To construct AMPK and Claudin-1 knockdown TSCC cells, we co-transfected 293T cells with the lentiviral vector pLenti-shRNA-GFP-Puro and the packaging plasmids PSPAX-2 and PMD2G (Bioeagle Biotech Company) using Neofect® DNA transfection reagent (Genomtech, TF201201) to generate recombinant lentivirus. The collected viral supernatants were infected into HN6 cells and filtered with 1 μg/ml puromycin (Solarbio, 1013×041). A non-targeting vector was used as a control. The target sequences for AMPK knockdown were sh1 G

CTTGATGCACACATGAATGC, sh2 GGAAGTTCTCA GCTGTCTTTA, and sh3 GGATTTCCGTAGTATTGAT GA. The target sequences for Claudin-1 (CLDN1) knockdown were as follows: sh1 AGGAGAAAATCATGTTGA A, sh2 GCAATAGAATCGTTCAAGA, and sh3 GTCTT TGACTCCTTGCTGA.

Western blotting analysis

Western blotting was used to detect the expression of target proteins. Cells were washed with PBS. Next, the appropriate RIPA lysate (Biosharp Life Sciences, BL504A) was incorporated into the cells, which were mixed with protease and phosphatase inhibitors. Post centrifugation, the protease suspension supernatant was quantified using a BCA (Yeasen Biotechnology, 2020ES76) qualification system. The protein lysates were then incorporated into loading buffer (Biosharp Life Sciences, BL529A) and denatured in a boiling water bath. The protein (20 μg per sample) was loaded onto 7.5 or 10% SDS–PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, IPVH00010), which was then immunoblotted in fresh skim milk for 1.5 h at room temperature. The membrane was incubated with primary antibodies overnight at 4 °C on a horizontal shaker, followed by incubation with peroxidase-conjugated antibodies at room temperature for an hour. ImageJ software (NIH) was used to evaluate and measure the membrane bands post visualization with an ECL system (Bio-Rad).

KEGG analysis

Key CAP signaling pathways in TSCC were screened using KEGG analysis. Firstly, 100 CAP-related genes were identified from the TCMSP database (https://old.tcmsp-e.com/tcmsp.php), and concurrently 1503 genes related to TSCC with Relevance score ≥ 20 were extracted from the Genecards database (https://www.genecards.org/). Next, 38 genes related to CAP and TSCC were identified using the Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/). These 38 genes were entered into Metascape (https://metascape.org/gp/index.html) for KEGG analysis.

In vivo experiments

All animal experiments were approved by the Ethics Committee of Hubei University of Chinese Medicine (HUCMS02225741) and conducted according to standard ethical regulations. We procured 35 5-week-old male nude mice, which were acclimatized and fed for 1 week, and then randomly divided into seven groups. We constructed a lung metastatic tumor model by injecting the treated HN6 cells (1×10^6) into nude mice via the tail vein. Drugs were administered starting on the 3rd day post-inoculation, and intraperitoneal injections were conducted every 3 days according to the dosage of CAP (10 mg/kg) and DDP (4 mg/kg). Body weight changes were recorded. The mice were sacrificed post 4 weeks, and the number of metastatic nodules was determined by removing the lungs, which were fixed in 4% paraformaldehyde. The lung metastases of the tumors were determined by HE staining and photographed under a microscope.

Statistical analysis

Data analysis was conducted using GraphPad Prism 9.5 (San Diego, CA, USA). Statistical analyses were conducted using one-way ANOVA, two-way ANOVA, and Tukey's multiple comparison test. All results are reported as the mean ± SD of at least three independent experiments. Statistical significance was set at $P<0.05$.

Results

TGF-β1 induces EMT in TSCC cells

To determine whether TGF-β1 could successfully induce EMT in TSCC cells, HN6 cells were cultured in diverse TGF-β1 concentrations (0, 2, 5, and 10 ng/ml) for 72 h, and the results demonstrated that HN6 cells underwent mesenchymal-like changes, which were shuttle or spindle-shaped (Fig. 1A). Meanwhile, the expression of the epithelial marker molecules Claudin-1 and E-cadherin were downregulated, whereas the expression of the mesenchymal marker molecules N-cadherin and Vimentin was upregulated (Fig. 1B), which indicated that TGF-β1 induced EMT in HN6 cells. Based on the above experimental results, HN6, CAL27, and SCC9 cells were treated with 5 ng/ml TGF-β1 for 24 and 48 h, and the results demonstrated that mesenchymal-like alterations occurred in all three groups of cells (Fig. 1C), with mesenchymal markers upregulation and epithelial markers downregulation (Fig. 1D). These results indicated that EMT could be successfully induced post treatment with 5 ng/ml TGF-β1 for 24 and 48 h in TSCC cells.

CAP and DDP respectively inhibited cell viability in TSCC

To select the appropriate CAP and DDP concentrations, we used the CCK8 cell proliferation assay to determine HN6, CAL27, and SCC9 cell viability. The results demonstrated that the viability of treated cells was inhibited in a concentration-dependent manner, and the IC50 of CAP and DDP for HN6 cells were 170.3 μM and 4.0 μg/ml (24 h) and 141.4 μM and 3.2 μg/ml (48 h), respectively. The IC50 of CAP and DDP for CAL27 cells were 169.3 μM and 5.5 μg/ml (24 h) and 126.6 μM and 3.8 μg/ml (48 h), respectively. The IC50 of CAP and DDP for SCC9 cells were 173.1 μM and 5.9 μg/ml (24 h) and 145.2 μM and 4.4 μg/ml (48 h), respectively. Due to the different effects of the drugs on cell viability in the three groups, we chose drug concentrations that were below the IC50

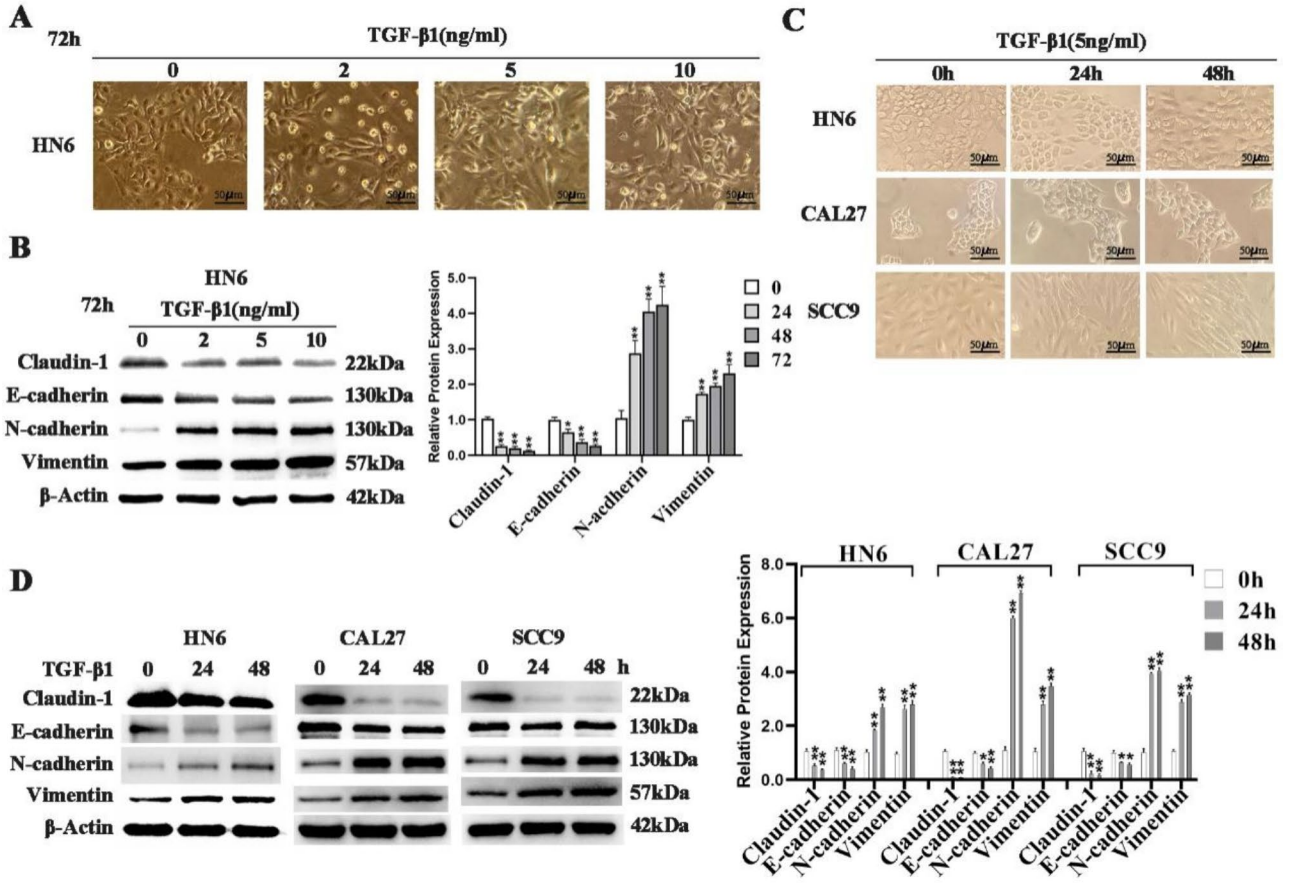

Fig. 1 TGF-β1 induces EMT in TSCC cells. (**A**, **B**) The effect of different doses of TGF-β1 on the morphology and EMT of HN6 cells. Cells are treated with the indicated concentrations of TGF-β1 for 72 h. An inverted microscope (×100) is used to observe the cell morphology, and western blotting is conducted to detect EMT-related protein expression. (**C**, **D**) Effects on TSCC cell morphology and EMT post TGF-β1 treatment. Cells are treated with the indicated concentrations of TGF-β1 for 0, 24, or 48 h. Cell morphology is observed using inverted microscopy, and the expression of EMT-related proteins is detected using western blotting. β-Actin serves as a control. $*p < 0.05$ and $**p < 0.01$ vs. control (0 ng/ml). All data are presented as the mean ± SD of three independent experiments

(CAP:150 μM-24 h; DDP: 4 μg/ml-24 h) but effective for the following experiments (Fig. 2A).

CAP combined with DDP activates the LKB1/AMPK signaling pathway and inhibits TGF-β1-induced EMT and migration of TSCC cells

To determine the effects of CAP and DDP on cell migration induced by TGF-β1, we conducted wound healing and Transwell assays in HN6, CAL27, and SCC9 cells. The results demonstrated that either CAP or DDP could inhibit the migration induced by TGF-β1, and the effect was more pronounced when CAP and DDP were combined (Fig. 2B). After treatment with CAP and/or DDP in HN6, CAL27 and SCC9 cells, the LKB1/AMPK signaling pathway is activated by p-LKB1 and p-AMPK upregulation, Claudin-1 and E-cadherin were upregulated, whereas N-cadherin and vimentin were downregulated (Fig. 2C). These results suggested that CAP combined with DDP activates the LKB1/AMPK signaling pathway and inhibits TGF-β1-induced EMT and migration of TSCC cells.

AMPK knockdown unable to reverse TGF-β1-induced EMT in TSCC

To further verify whether AMPK regulates TGF-β1-induced EMT, we knocked down AMPK expression in HN6 cells with three shRNAs (sh1, sh2, and sh3) and selected the effective shRNA sh1 for subsequent experiments (Fig. 3A). We found that p-AMPK and AMPK levels were significantly reduced in the treated group (shAMPK) compared to those in the control group (shCon). However, the expression of Claudin-1, E-cadherin, and N-cadherin was almost identical to that of the vehicle group (shCon) (Fig. 3B), which indicated that AMPK knockdown was unable to reverse TGF-β1-induced EMT in TSCC. The above results demonstrated that AMPK was not the target kinase for CAP and DDP in combination to reverse TGF-β1-induced EMT.

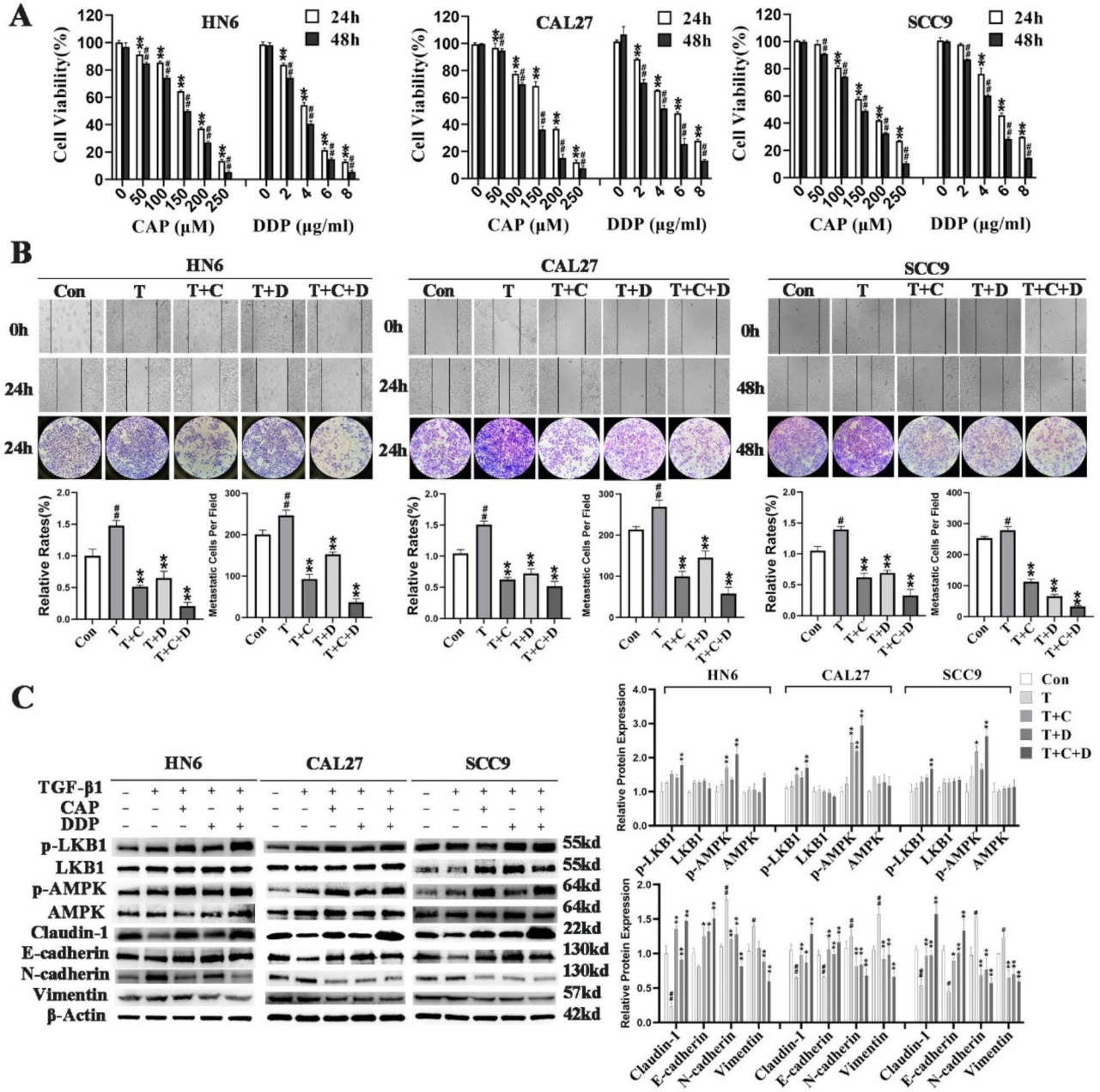

Fig. 2 CAP combined with DDP activates the LKB1/AMPK signaling pathway and inhibits TGF-β1-induced EMT and migration of TSCC cells. (**A**) Effects of the diverse CAP or DDP doses on TSCC cell viability. Cells are treated with CAP or DDP at the indicated concentrations for 24 and 48 h. Cell viability is determined by CCK-8 assay and expressed as percentages of the control. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control. (**B**) Cell migration is assessed by wound healing and Transwell assays. The migration of HN6, CAL27, and SCC9 cells is induced by TGF-β1, and the closure distance and the number of migrated cells are photographed (×20) and measured post CAP and/or DDP treatment for 24–48 h. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. TGF-β1 group, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control. (**C**) Effects of CAP and/or DDP treatments on EMT and the LKB1/AMPK pathways. HN6, CAL27, and SCC9 cells are pretreated with TGF-β1 for 3 h, and then CAP and/or DDP are incorporated to treat the cells for 24 h. The levels of the EMT-associated proteins p-LKB1, LKB1, p-AMPK, and AMPK are determined by Western blot. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. TGF-β1 group, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control. Con: control. All data are presented as the mean ± SD of three independent experiments

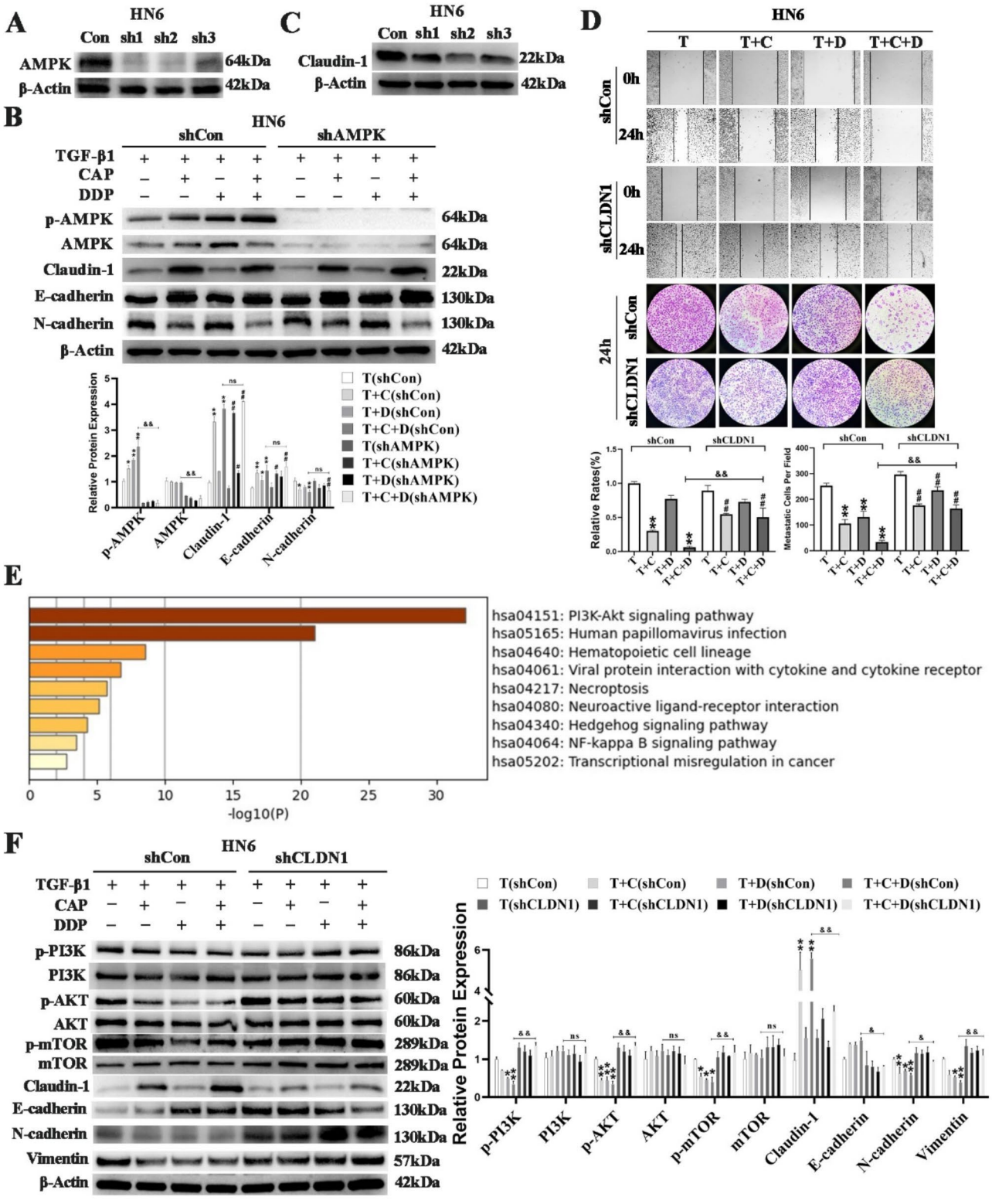

Fig. 3 (See legend on next page.)

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Fig. 3 CAP combined with DDP inhibits TGF-β1-induced EMT and TSCC cells migration through the Claudin-1/PI3K/AKT/mTOR signaling pathway. (**A**) The efficiency of AMPK knockdown is assessed by Western blot in HN6 cells (**B**) The levels of the EMT-related proteins p-AMPK and AMPK are detected by Western blot post 24 h of treatment with CAP and/or DDP in HN6 cells upon AMPK knockdown. $^{*}p<0.05$, $^{**}p<0.01$ vs. T (shCon) group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. T (shAMPK) group, $^{\&}p<0.05$, $^{\&\&}p<0.01$, T + C + D (shAMPK) group vs. T + C + D (shCon) group. (**C**) The efficiency of Claudin-1 (CLDN1) knockdown is assessed by Western blot in HN6 cells (**D**) The migration of cells is assessed by wound healing and Transwell assays. The migration of HN6 cells is induced by TGF-β1, and the closure distance and the number of migrated cells are photographed (×20) and measured post CAP and/or DDP treatment for 24 h. $^{*}p<0.05$, $^{**}p<0.01$ vs. T (shCon) group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. T (shCLDN1) group, $^{\&}p<0.05$, $^{\&\&}p<0.01$, T + C + D (shCLDN1) group vs. T + C + D (shCon) group (**E**) The PI3K/AKT signaling pathway is enriched by KEGG bioanalysis using the GOBP database (**F**) The levels of EMT-related proteins and the PI3K/AKT/mTOR signaling pathway are detected by Western blot post 24 h of treatment with CAP and DDP. $^{*}p<0.05$, $^{**}p<0.01$ vs. the T(shCon) group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. the T (shCLDN1) group; $^{\&}p<0.05$, $^{\&\&}p<0.01$, the T + C + D (shCLDN1) group vs. the T + C + D (shCon) group. T: TGF-β1, C: CAP, D: DDP. All data are presented as the mean ± SD of three independent experiments

CAP combined with DDP inhibits TGF-β1-induced EMT and TSCC cells migration through Claudin-1/PI3K/AKT/mTOR signaling pathway

To identify the key molecules affecting TGF-β1-induced EMT inhibition by CAP combined with DDP, we reviewed previous studies and found that CAP affects cell junctions and adhesion [32]. Claudin-1 is a tight junction protein that is regulated by CAP in TSCC cells (Fig. 2C). Therefore, we knocked down Claudin-1 expression in HN6 cells and observed that the inhibitory effects of CAP and DDP on cell migration were abrogated (Fig. 3C, D). Furthermore, the changing trend for EMT-related proteins in the CAP+DDP treatment group was reversed post Claudin-1 knockdown (Fig. 3F), suggesting that Claudin-1 knockdown counteracts the inhibitory effects of CAP and DDP. We identified the PI3K/AKT signaling pathway as the most relevant signaling pathway between CAP and TSCC using KEGG analysis (Fig. 3E). Hence, the levels of critical proteins in the PI3K/AKT signaling pathway were detected using western blot analysis. The results demonstrated that Claudin-1 knockdown abolished the inhibitory effects of CAP and DDP alone or in combination on p-PI3K, p-AKT and p-mTOR compared to the vehicle group (Fig. 3F). The above results suggested that CAP combined with DDP could inhibit TGF-β1-induced EMT and migration of TSCC through the Claudin-1/PI3K/AKT/mTOR signaling pathway, and the conclusion was further confirmed in CAL27 cells (Fig. S1A, B).

CAP combined with DDP inhibits non-TGF-β1-induced EMT and TSCC migration through AMPK/mTOR signaling pathway

Previously, we demonstrated that AMPK was not the target kinase for CAP and DDP to reverse TGF-β1-induced EMT (Fig. 3B). However, a study found that CAP could directly inhibit EMT and migration of renal cancer cells via the AMPK/mTOR pathway in the absence of TGF-β1 involvement [35]. Therefore, we investigated the inhibitory effects of CAP combined with DDP on EMT and migration in TSCC cells without TGF-β1 treatment and explored the possible AMPK/mTOR pathway. The results demonstrated that CAP combined with DDP significantly inhibited EMT and HN6 cells migration, even without TGF-β1 induction, with AMPK activation (Fig. 4A, B). Furthermore, AMPK knockdown attenuated the inhibitory effect of CAP combined with DDP on EMT and migration and restored the level of p-mTOR (Fig. 4C, D). The above results revealed that mTOR was a downstream target of AMPK and CAP combined with DDP affected non-TGF-β1-induced EMT and TSCC cells migration by regulating the AMPK/mTOR signaling pathway, and the conclusion was further confirmed in CAL27 cells (Fig. S2A, B).

CAP combined with DDP inhibits TSCC metastasis in vivo

We constructed a lung metastatic tumor model to verify the anti-metastatic effect of CAP combined with DDP in vivo. The nude mice started to demonstrate obvious leanness when they were inoculated with tumor cells for approximately 20 days (Fig. 5A). Additionally, CAP combined with DDP failed to reduce weight loss post Claudin-1 knockdown compared to the normal combination group (Fig. 5A). The experiment ended past four weeks, and the lungs, livers, and kidneys of the nude mice were detached. The morphology of lungs and HE staining demonstrated more nodules and a larger area of tumor metastasis post TGF-β1 treatment compared with the control group, while the number of nodules and the area of tumor metastasis were both reduced following treatment with CAP, DDP, or their combination, which was more pronounced in the combination group. Furthermore, the inhibitory effect of CAP combined with DDP on metastasis was attenuated post Claudin-1 knockdown compared to that in the normal combination group (Fig. 5B). No considerable histological changes, including swelling, inflammatory cell infiltration, or necrosis, were observed in the liver or kidneys (Fig. 5C). These results indicated that CAP combined with DDP could dramatically inhibit TSCC metastasis in vivo without obvious injury to the liver and kidneys.

Discussion

Cancer is a global health concern that needs to be solved urgently as it seriously affects the quality of life of human beings and places a heavy economic burden on families

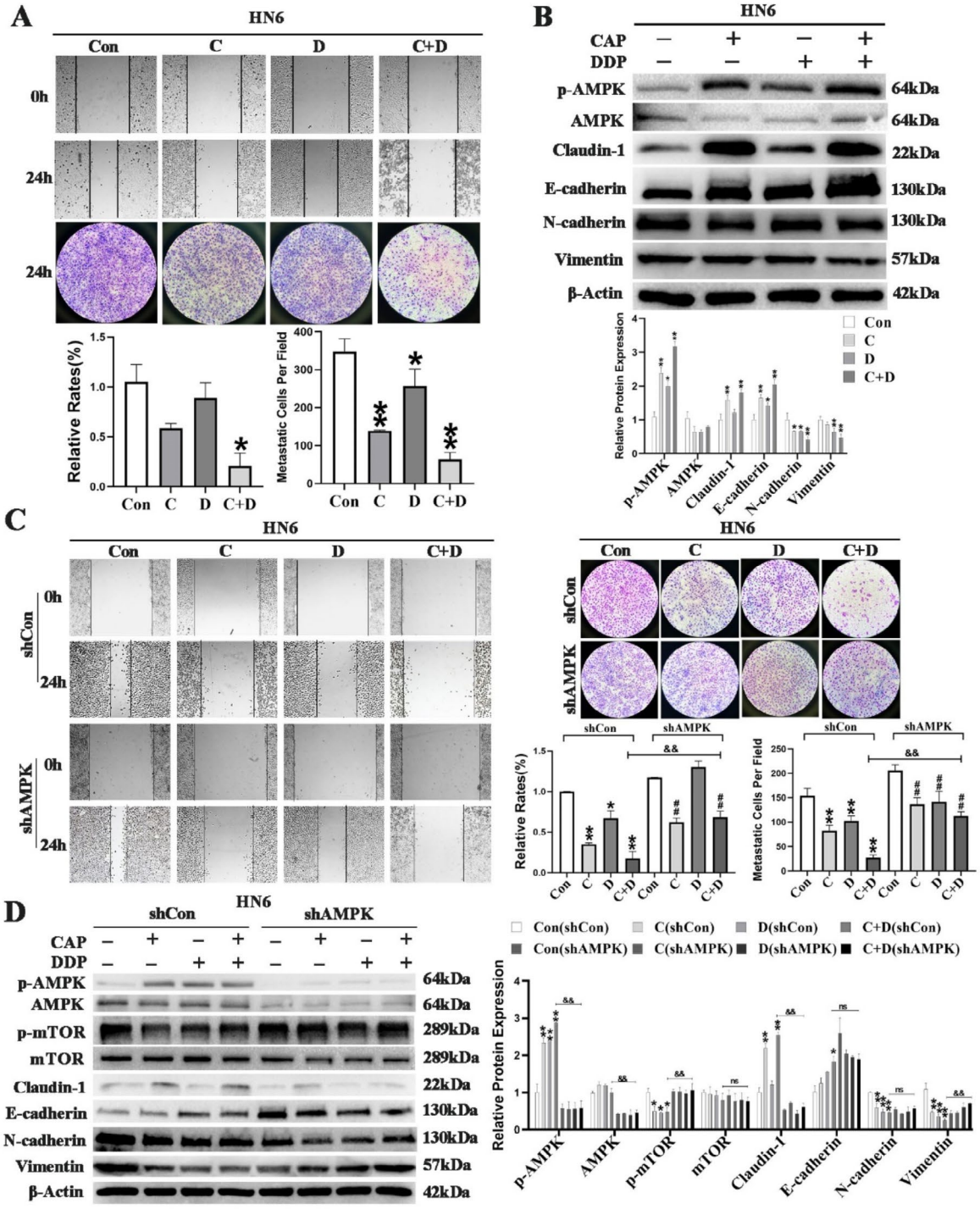

Fig. 4 CAP combined with DDP inhibits non-TGF-β1-induced EMT and migration of TSCC through AMPK/mTOR signaling pathway. (**A**) The migration of cells is assessed by wound healing and Transwell assays. Cells are cultured with CAP and/or DDP for 24 h, and the closure distance and the number of migrated HN6 cells are photographed (×20) and measured. *$p < 0.05$, **$p < 0.01$ vs. control. (**B**) The levels of the EMT-related proteins p-AMPK and AMPK are detected by Western blot. Cells are cultured with CAP and/or DDP for 24 h. *$p < 0.05$, **$p < 0.01$ vs. control. (**C**) The migration of cells is assessed by wound healing and Transwell assays. HN6 cells with AMPK knockdown are cultured with CAP and/or DDP for 24 h, and the closure distance and the number of migrated HN6 cells are photographed (×20) and measured. *$p < 0.05$, **$p < 0.01$ vs. control (shCon) group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control (shAMPK) group, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, C + D (shAMPK) group vs. C + D (shCon) group. (**D**) The levels of EMT-related proteins and the AMPK/mTOR signaling pathway are detected by Western blot. Cells are cultured with CAP and/or DDP for 24 h. *$p < 0.05$, **$p < 0.01$ vs. control (shCon), and $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, C + D (shAMPK) group vs. C + D (shCon) group. C: CAP, D: DDP. All data are presented as the mean ± SD of three independent experiments

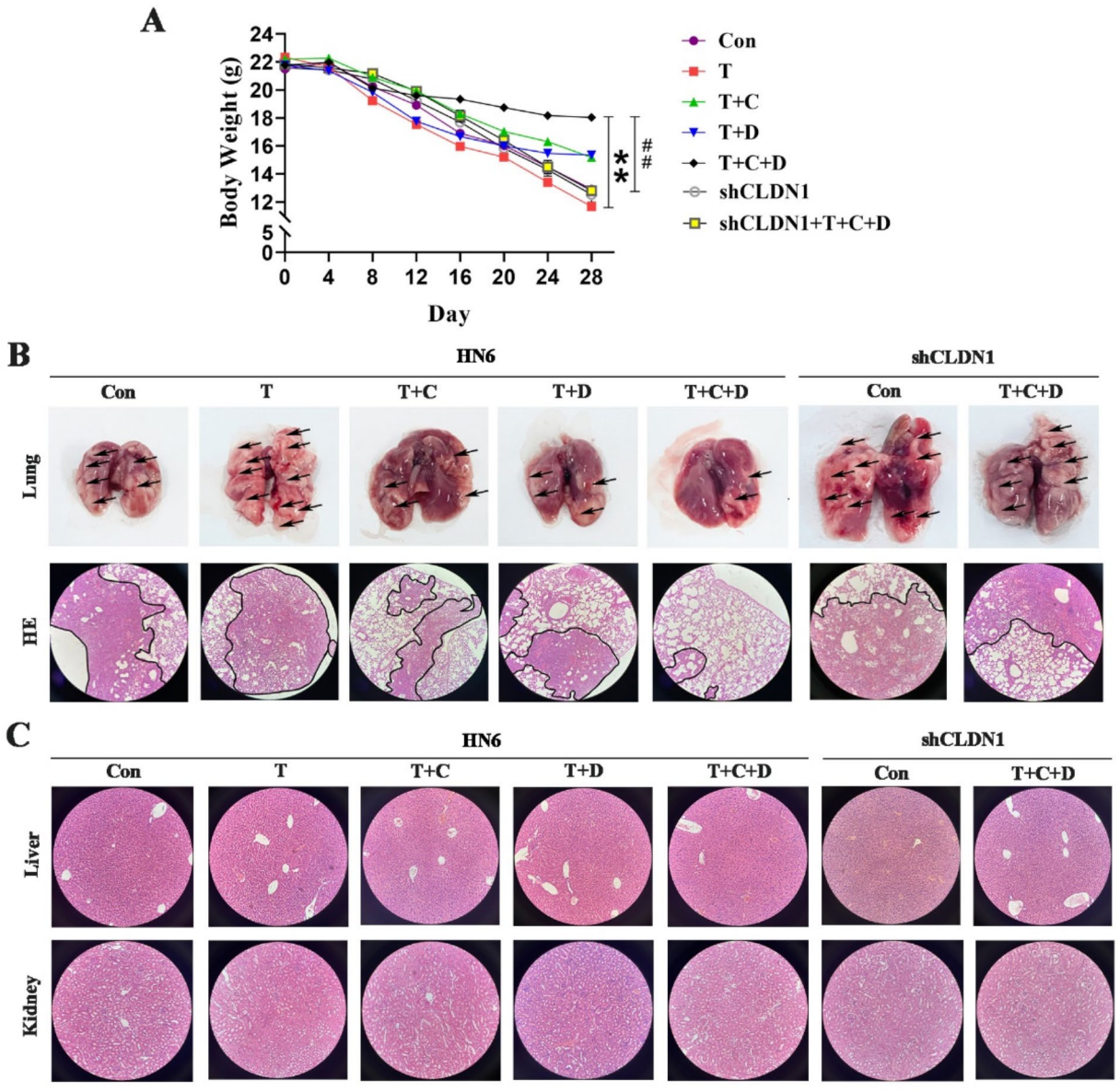

Fig. 5 CAP combined with DDP inhibits TSCC metastasis in vivo. (**A**) Changes in the body weight of mice over 28 days. * $p<0.05$, ** $p<0.01$, T + C + D group vs. T group. $^{\#}p<0.05$, $^{\#\#}p<0.01$, control (shCLDN1) group vs. T + C + D (shCLDN1) group. (**B**) At the end of the experiment, the lungs of the mice are harvested, photographed, (upper line) and examined for tumor metastasis by HE staining (×100) (lower line). (**C**) The tissue sections of mouse livers (upper line) and kidneys (lower line) are stained with HE to detect tumor metastasis and drug toxicity (×100). T: TGF-β1, C: CAP, D: DDP

of patients. Clinical cancer treatments are also limited, with surgery used in the early stages and surgery combined with radiochemotherapy in the middle and late stages. Radiochemotherapy also causes severe side effects, including inflammation, immunosuppression, and chemotherapy resistance [37, 38]. Therefore, development of natural drugs that enhance chemotherapeutic drug efficacy and reduce drug resistance has become a research hotspot. In recent years, several studies have been conducted on natural plant components; for example, one study demonstrated that albumin and paclitaxel combined with DDP could better treat metastatic triple-negative breast cancer, and the efficacy of the combination was more potent than that of DDP combined with gemcitabine, with fewer side effects [39]. Herein, CAP was the active ingredient extracted from Capsicum annuum, a member of the family Solanaceae, which has been demonstrated to inhibit the growth and metastasis of

a vast range of tumor cells in vitro and in vivo [40–42]. Although CAP has been extensively studied in diverse cancers, its role in TSCC metastasis remains obscure.

We investigated the effects of CAP and DDP on the viability of the three TSCC cell lines and found that TSCC cells viability significantly decreased with increasing drug concentrations or extended treatment times. Furthermore, we investigated the effect of CAP combined with DDP on the migration ability of TSCC cells and found that TGF-β1 promoted cell migration, while CAP combined with DDP inhibited TSCC cells migration induced by TGF-β1. Previous studies have described similar effects of CAP on melanoma [43], colon [44], bladder [45], and nasopharyngeal cancers [46]. Our findings indicate that TSCC should be incorporated into this list. Due to the close relationship between EMT and tumor cell migration and the occurrence of EMT accompanied by the epithelial markers downregulation and mesenchymal markers upregulation [47], we next detected changes in EMT-related proteins, and the results suggested that CAP combined with DDP could inhibit TGF-β1-induced EMT. Additionally, a metastatic tumor model in nude mice was constructed by tail vein injection, and the in vivo results verified the in vitro findings.

Based on the above findings, we conducted a deeper exploration to reveal the critical signaling pathways by which CAP and/or DDP inhibit TSCC cells metastasis. The LKB1/AMPK signaling pathway was activated post CAP and/or DDP treatment in TSCC cells. Consistent with our results, CAP activates AMPK in prostate [48] and kidney [35] cancers. However, it was unexpected for us to find no rescue effects on the changes in EMT-related proteins post AMPK knockdown, suggesting that AMPK did not regulate TGF-β1-induced EMT inhibition, and some other molecular mechanisms might exist and need to be explored further.

Previous studies have reported that CAP affects cell junctions and adhesion [35]. Claudin-1, a member of the claudin family of proteins, is the most studied claudin protein in cancer; however, its role as a tumor-promoting or inhibitory factor (or both) has not been determined to date [26]. Claudin-1 tightens paracellular clefts in epithelial cells and regulates EMT via AMPK/TGF-β signaling in head and neck squamous cell carcinoma, thereby mediating its progression [49]. Similarly, Claudin-1 is abnormally elevated in esophageal squamous cell carcinoma tissues and cell lines and promotes esophageal squamous cell carcinoma proliferation and metastasis by triggering autophagy both in vitro and in vivo [50]. Furthermore, Claudin-1 is a major component of epithelial tight junctions and plays an important role in maintaining cell polarity, intercellular communication, and epithelial cell homeostasis [49, 51]. These functions maintain intercellular adhesion, thereby preventing cancer cell metastasis [51]. The PI3K/AKT signaling pathway transmits stimulatory signals from the extracellular to the intracellular, and in the process, exerts an important influence on pathophysiological processes such as cell proliferation, migration, and drug resistance [15]. Meanwhile, it is found that CAP inhibits the viability of breast cancer [52] and nasopharyngeal carcinoma [53] cells by regulating the PI3K/AKT/mTOR signaling pathway, combined with sorafenib exerts a synergistic antihepatocellular carcinoma effect by inhibiting the PI3K/AKT/mTOR signaling pathway [15]. Herein, the antimetastatic function was verified. Claudin-1 was upregulated upon treatment with CAP and DDP, and Claudin-1 knockdown restored the suppressed EMT and migration and rescued the expression of suppressed p-PI3K, p-AKT and p-mTOR, which indicated that CAP combined with DDP inhibited TGF-β1-induced EMT and migration of TSCC cells via the Claudin-1/PI3K/AKT/mTOR signaling pathway.

Previously we have demonstrated that AMPK was not the mediator by which CAP and DDP inhibit TGF-β1-induced EMT. Some studies have found that AMPK activation inhibits tumor metastasis. For example, metformin activates AMPK to release H3K9me2 to reverse E-cadherin downregulation during EMT and inhibit lung cancer metastasis [54]. VSP-17 inhibits EMT through PPARγ/AMPK signaling pathway activation, thereby inhibiting migration and invasion of triple-negative breast cancer cells [55]. Hence, we removed TGF-β1 from the medium and found that the inhibitory effect of CAP combined with DDP on EMT was significantly weakened when AMPK was knocked down, which indicated that AMPK mediated the inhibitory effects of CAP and/or DDP on non-TGF-β1-induced EMT. Furthermore, our KEGG bioanalysis of sensitive genes for both CAP and TSCC using online GOBP databases elucidated the enriched PI3K/AKT signaling pathway. Therefore, we examined the expression of p-mTOR, a downstream signal of PI3K/AKT and found that the combination of CAP and DDP significantly inhibited mTOR activity. However, AMPK knockdown considerably weakened this inhibitory effect. These results collectively demonstrated that CAP combined with DDP inhibited non-TGF-β1-induced EMT and migration of TSCC cells through the AMPK/mTOR signaling pathway.

However, our study has some shortcomings. We did not include tumor samples from TSCC patients, if we could isolate tumor cells from patients to construct organ-like models and transplant them into mice and use CAP combined with DDP to treat mice and observe tumor metastasis, our results would be more convincing and clinically relevant.

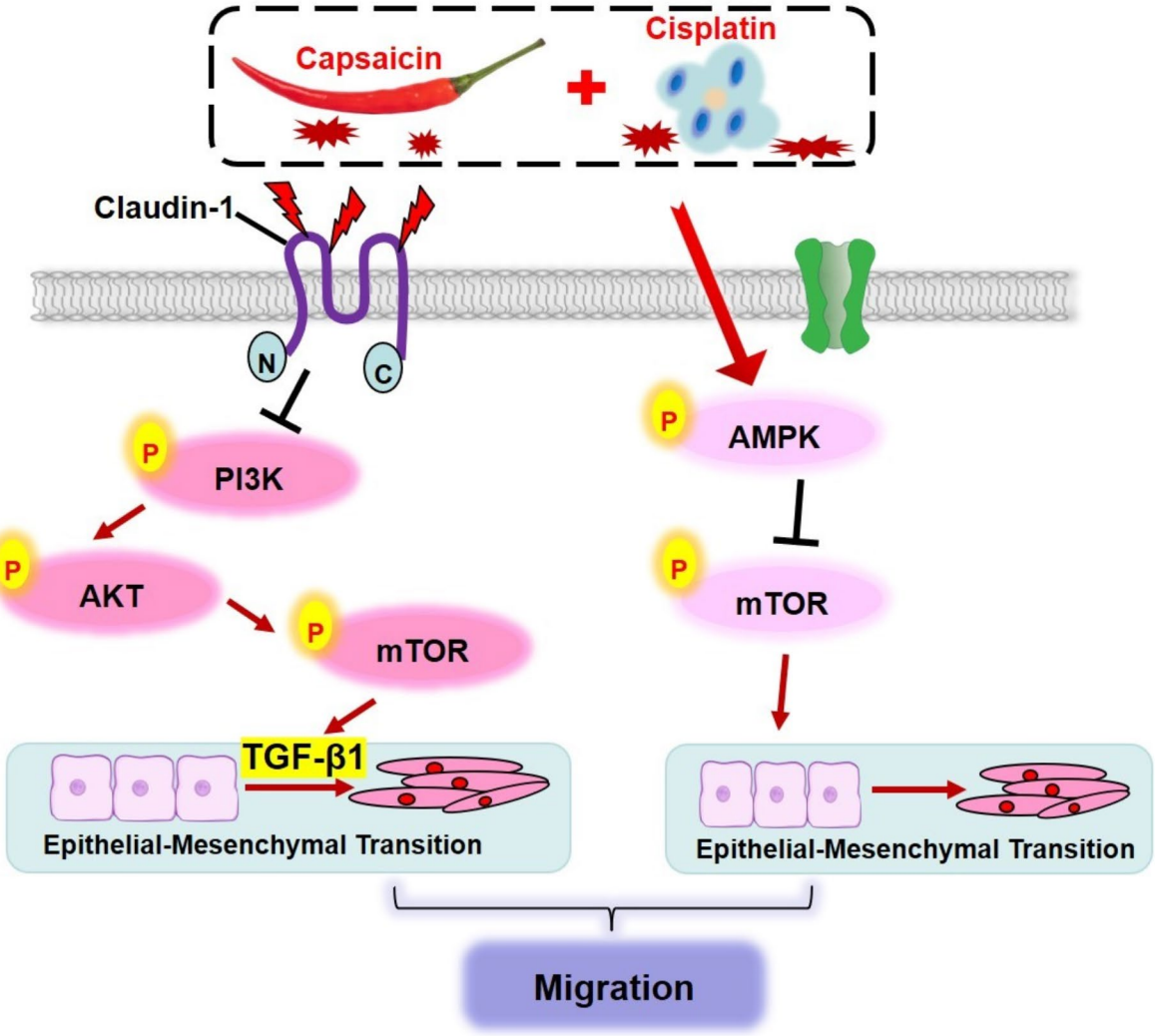

Fig. 6 Diagram of our conclusions in this article. CAP combined with DDP activated Claudin-1 to inhibit PI3K/AKT/mTOR phosphorylation and block TGF-β1-induced EMT and migration, while activating AMPK to reduce p-mTOR expression and inhibit non-TGF-β1-induced EMT and migration.

Conclusions

In conclusion, the current research conducted in vitro and in vivo collectively verified that CAP combined with DDP could inhibit the cell migration in TSCC, in a more pronounced way than monotherapy with either CAP or DDP. Mechanistically, after combined treatment of CAP and DDP, the inhibition of non-TGF-β1-induced EMT and migration was mediated by the AMPK/mTOR pathway, whereas the suppression of TGF-β1-induced EMT and migration were regulated by the Claudin-1/PI3K/AKT/mTOR pathway (Fig. 6). Therefore, our findings verified that CAP could be used as an effective adjuvant of DDP in metastatic tumors treatment with low toxicity and might be a promising strategy for developing clinical chemotherapies for TSCC.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

Zhuang Li: Conceptualization, Study design, Methodology, Resources, Investigation, Writing – original draft and Validation. Qiwei Zhao: Data curation, Methodology and Formal analysis. Xiayang Liu: Formal analysis, Resources and Methodology. Xinyue Zhou: Software and Methodology. Yu Wang: Resources and Software. Min Zhao and Fenghua Wu: Visualization and Supervision. Gang Zhao: Resources and Supervision. Xiaohong Guo:

Writing – review & editing, Funding acquisition, Resources, Visualization and Supervision.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The animal study protocol was approved by the Institutional Review Board of the Ethics Committee of Hubei University of Chinese Medicine.

Consent for publication
All the authors have read and agreed to submit the manuscript to Cancer Cell International with equal responsibility.

Competing interests
The authors declare no competing interests.

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