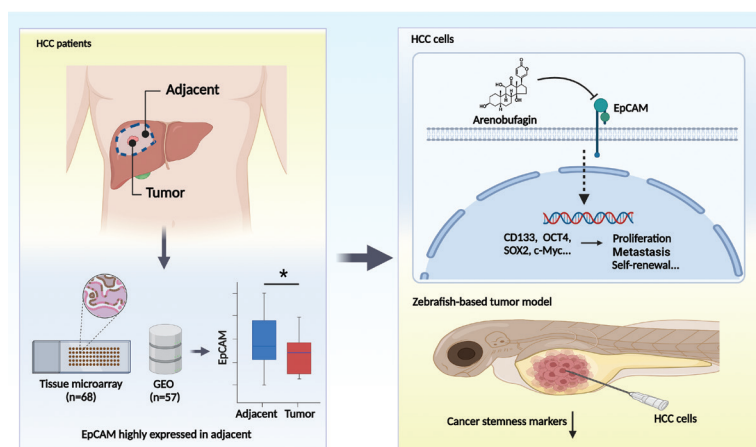


Arenobufagin suppresses the progression of early-stage hepatocellular carcinoma by inhibiting EpCAM-mediated tumor stemness

Graphical abstract



Authors

Lijuan Deng, Lifang Zou, Chunhong Zhou, Fuqin Yang, Fen Ouyang, Yingru Zhu, Huihui Cao, Min Hu, Xiaoshen Zhang and Junshan Liu

Correspondence

liujunshan@smu.edu.cn (J. Liu); xshchang@outlook.com (X. Zhang); humin2019@jnu.edu.cn (M. Hu)

In brief

EpCAM may promote early-stage hepatocellular carcinoma stemness which is counteracted by arenobufagin.

Highlights

- EpCAM is a potential biomarker of early-stage hepatocellular carcinoma.
- Arenobufagin suppresses early-stage hepatocellular carcinoma by inhibiting EpCAM-mediated tumor stemness.

Research Article

Arenobufagin suppresses the progression of early-stage hepatocellular carcinoma by inhibiting EpCAM-mediated tumor stemness

Lijuan Deng^{a,1}, Lifang Zou^{b,1}, Chunhong Zhou^{b,1}, Fuqin Yang^{a,1}, Fen Ouyang^c, Yingru Zhu^a, Huihui Cao^b, Min Hu^{d,*}, Xiaoshen Zhang^{e,*} and Junshan Liu^{b,*}

^aFormula Pattern Research Center, School of Traditional Chinese Medicine, Jinan University, Guangzhou 510630, P. R. China

^bSchool of Traditional Chinese Medicine, Guangdong Provincial Key Laboratory of Chinese Medicine Pharmaceuticals, Guangdong Basic Research Center of Excellence for Integrated Traditional and Western Medicine for Qingzhi Diseases, Southern Medical University, Guangzhou 510515, P. R. China

^cDepartment of Laboratory Medicine, Nanfang Hospital Baiyun Branch, Southern Medical University, Guangzhou 510420, P. R. China

^dDepartment of Hepatobiliary Surgery, First Affiliated Hospital, Jinan University, Guangzhou 510632, P. R. China

^eSchool of Nursing, Department of Cardiovascular Surgery, First Affiliated Hospital, Jinan University, Guangzhou 510630, P. R. China

¹These authors contributed equally to the work.

*Correspondence: liujunshan@smu.edu.cn (J. Liu); xshchang@outlook.com (X. Zhang); humin2019@jnu.edu.cn (M. Hu)

Received: 29 September 2024; Revised: 21 December 2024; Accepted: 26 December 2024

Published online: 17 January 2025

DOI 10.15212/AMM-2024-0064

ABSTRACT

Epithelial cell adhesion molecule (EpCAM) is a biomarker for epithelial cell-derived tumors. However, the specific role of EpCAM itself in early-stage hepatocellular carcinoma progression remains unclear, and small molecules targeting EpCAM have not yet been reported. Here, the protein expression profile of EpCAM in tumor-adjacent regions was found to be higher than that in tumor regions, and to be positively associated with the progression of early-stage liver cancer, as well as high frequency of recurrence, cirrhosis, lymph node metastasis, microvascular invasion and cancer stemness, in 68 patients with hepatocellular carcinoma (HCC). *In vitro*, EpCAM enhanced cancer cell stemness, as reflected by increased abilities of proliferation, self-renewal, migration and invasion, which was counteracted by arenobufagin. Furthermore, arenobufagin inhibited the viability of Hep3B and Huh7 cells with IC₅₀ values of 36.4 nM and 123.4 nM after 72 h of treatment, respectively. Molecular docking data further indicated that arenobufagin binds EpCAM. Moreover, arenobufagin inhibited early progression of HCC through EpCAM in a zebrafish xenograft tumor model mimicking early-stage hepatocellular carcinoma without blood vessels *in vivo*. This study supports a tumor-promoting role of EpCAM in early-stage hepatocellular carcinoma by facilitating cancer stemness and suggests that arenobufagin might be promising candidate for EpCAM inhibition.

Keywords: EpCAM, cancer stemness, arenobufagin, early-stage hepatocellular carcinoma

1. INTRODUCTION

Hepatocellular carcinoma (HCC), the most common type of liver cancer, occurs in nearly 865,000 new cases each year, accounting for 75%–85% of all primary liver cancer cases [1–4]. HCC is the sixth most common malignancy worldwide and the third leading cause of cancer-related death, and its incidence and mortality rates are increasing every year [2–4]. By 2025, at least 1 million people will be affected by liver cancer each year, and approximately half of all primary liver cancers occur in China [2–4].

Although mortality for most cancers is declining, HCC remains one of the fastest growing malignancies in

terms of cancer-related deaths globally [1, 5]. A major reason for the high mortality in patients with HCC is the lack of accurate early detection strategies [5]. Because early-stage liver cancer has not yet developed the distinguishing feature of a rich vascular system, even a combination of blood-based biomarkers (e.g., alpha-Fetoprotein, lens culinaris agglutinin-reactive fraction of AFP and des-γ-carboxy prothrombin), small-molecule contrast agents and traditional imaging techniques, such as ultrasound, computed tomography and magnetic resonance imaging, can detect only small liver cancer lesions with a diameter of approximately 2–3 cm [5, 6]. However, detecting early-stage HCC foci less than

2 cm in diameter remains difficult. Furthermore, owing to difficulties in detecting and diagnosing early-stage HCC, achieving a cure remains challenging [5, 6]. Therefore, a high-sensitivity biomarker for early-stage HCC—comprising either neoplastic or recurrent tumors with diameters less than 2 cm, and without formation of a mature vascular system—would be of great clinical value.

Epithelial cell adhesion molecule (EpCAM), a marker of liver cancer stem cells, plays a key role in maintaining cell self-renewal [7]. EpCAM-positive severe cirrhosis tissues and EpCAM⁺ HCC cells have tumorigenic ability *in vivo*, thus suggesting that EpCAM promotes the progression of advanced cirrhosis to HCC and the self-renewal of cells [8, 9]. By down-regulating the chromatin remodeling enzyme chromodomain helicase DNA binding protein 4 in EpCAM⁺ HCC subsets, the sensitivity of cells to chemotherapy can be improved [10]. In addition, the expression of EpCAM decreases during the self-renewal of EpCAM⁺ HCC cells, thereby further explaining the differentiation characteristics of EpCAM as a stemness marker [9]. In summary, EpCAM expression is strongly associated with the status of cell differentiation and the expression of reprogramming factors, such as octamer-binding transcription factor 4 (OCT4), SRY-Box transcription factor 2 (SOX2) and Nanog Homeobox (NANOG), which play important roles in maintaining cell stemness [11]. Thus, an urgent need exists to better characterize the role of EpCAM in the progression of early-stage HCC, to discover novel therapies that inhibit tumorigenesis.

Venenum Bufonis, the dry secretion of the post-auricular and cutaneous glands of *Bufo gargarizans* Cantor or *Bufo melanostictus* Schneider, is widely used clinically for the treatment of malignant tumors in China [12]. Pharmacological studies have indicated that bufadienolides, the main active ingredients in *Venenum Bufonis*, have multiple antitumor activities, including inhibition of proliferation, invasion and migration; attenuation of angiogenesis; induction of apoptosis; and reversal of multidrug resistance in tumor cells [13]. Arenobufagin (Are), is a representative bufadienolide from *Venenum Bufonis*. In a prior high-throughput screen, this compound had the strongest anti-HCC activity [14]. Arenobufagin increases intracellular levels of reactive oxygen radicals and calcium ions; induces autophagy-dependent ferroptosis through the p62/Keap1/Nrf2 pathway [15]; induces cell apoptosis and cell cycle arrest [16, 17]; and induces tumor-associated macrophage polarization [18]. In addition, arenobufagin induces autophagy in HCC cells by activating the autophagy-related gene LC-3II and inhibiting the PI3K/Akt/mTOR signaling pathway [19]. Despite the broad antitumor activity of arenobufagin, its effects on the biological properties of liver cancer stemness have not been reported.

In this study, EpCAM was found to serve as an indicator of early-stage HCCs—as evidenced by higher EpCAM expression in tumor-adjacent regions than tumor

regions—and to positively correlate with the high frequency of recurrence, cirrhosis, lymph node metastasis and microvascular invasion in patients with HCC. EpCAM also promoted the stemness of HCC cells (Hep3B and Huh7) *in vitro*. Moreover, arenobufagin, a natural bufadienolide isolated from *Venenum Bufonis*, suppressed the stemness of HCC cells by targeting EpCAM. Our study sheds new light on the roles of EpCAM in promoting malignant behavior and phenotype in HCC, and demonstrates that arenobufagin has potential to be developed as a promising agent for treating HCC by targeting EpCAM.

2. MATERIALS AND METHODS

2.1 Reagents and antibodies

Arenobufagin (≥98% purity) was purchased from Baoji Herbest Bio-Tech Co., Ltd. (Baoji, Shanxi, China). A tissue chip (G6035) was provided by Servicebio® (Wuhan, Hubei, China). In detail, the normal adjacent tissue must be strictly collected 2–4 cm from the tumor margin and must not contain tumor [20–23]. Cell Tracker™ CM-Dil dye (40718ES60) was purchased from Yeasen (Shanghai, China). A cell-Light 5-ethynyl-2'-deoxyuridine (EdU) Apollo 488 In Vitro Kit (100T, C10310-3) was purchased from RiboBio (Guangzhou, Guangdong, China). Hematoxylin and eosin (H&E, G1120-3) were purchased from Solarbio (Shanghai, China), and paraffin (39601095) was provided by Leica (Wetzlar, HE, Germany). Tricaine (T0941) was obtained from Macklin (Shanghai, China). A bicinchoninic acid (BCA, 23225) protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Polyvinylidene fluoride membrane (PVDF, IPVH00010) was purchased from Merck Millipore (Darmstadt, HE, Germany), and an enhanced chemiluminescence kit (WBKLS0500) was purchased from Thermo Fisher Scientific™ (Waltham, MA, USA). Anti-EpCAM (21050-1-AP), anti-CD133 (66666-1-Ig), anti-CD44 (60224-1-Ig), anti-OCT4 (60242-1-Ig), anti-SOX2 (11064-1-AP) and anti-c-Myc (67447-1-Ig) were purchased from Proteintech Group (Wuhan, Hubei, China). Anti-β-actin (4970), anti-rabbit IgG (7074) and anti-Mouse IgG (7076) were purchased from Cell Signaling Technology (Danvers, MA, USA). Dimethyl sulfoxide (DMSO, D2650), agarose (111860) and other reagents were all supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cell lines and cell culture

Huh7 (SCSP-526) and Hep3B (SCSP-5045) cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Huh7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (C11995500BT, Gibco™, Carlsbad, CA, USA), whereas Hep3B cells were cultured in minimum essential medium (PM150410, Procell, Wuhan, Hubei, China) with 10% (v/v) fetal bovine serum (ExCell Bio, Shanghai, China) and 1% (v/v) penicillin/streptomycin (15140122, Gibco™,

Research Article

Carlsbad, CA, USA) at 37°C in a humidified incubator containing 5% CO₂. The cells were passaged when 70%–90% confluency was achieved.

2.3 Cell viability assay

Hep3B and Huh7 cells were seeded in 96-well plates at 1×10^4 cells per well for 24 h at 37°C, then treated with various concentrations of arenobufagin (0–1500 nM) for 24, 48 or 72 h. Cells were then incubated with 10 μ L 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/mL) per well for another 4 h at 37°C. The supernatants were then removed, and 100 μ L dimethyl sulfoxide was added to dissolve the formazan crystals. Finally, the absorbance at 570 nm was measured with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), and the viability of Hep3B and Huh7 cells was calculated.

2.4 Cell proliferation ability assay

5-ethynyl-2'-deoxyuridine (EdU) assays were used to evaluate tumor cell proliferation potential. Hep3B and Huh7 cells were seeded in 96-well plates at 4×10^3 cells per well and incubated overnight, then treated with various concentrations of arenobufagin (20, 100 or 500 nM) for 24 h, and incubated with EdU (50 μ M, 100 μ L/well) for 2 h. Subsequently, the cells were washed with phosphate buffered saline (PBS) to remove the unlabeled EdU and fixed with 4% (v/v) paraformaldehyde at room temperature for 30 min. After addition of glycine solution (100 μ L/well, 2 mg/mL) to each well to remove excess aldehyde, PBS was added to wash the cells. To enhance cell membrane permeability, we added 0.5% (v/v) TritonX-100 (T8787, Sigma, St. Louis, MO, USA) to each well and incubated the cells at room temperature for 10 min. The cells were then incubated in Apollo staining reaction solution for 30 min at room temperature. Subsequently, the cells were washed three times with 0.5% Triton X-100 followed by PBS (100 μ L/well). Finally, the nuclei were stained with Hoechst 33342 reaction solution and observed under a microscope (Axiovert. A1; Carl Zeiss AG, Jena, TH, Germany).

2.5 Colony formation assay

Hep3B and Huh7 cells were seeded in six-well plates at 300 cells per well and incubated overnight. The cells were then exposed to arenobufagin (12.5, 25, 50, 100 or 500 nM) for 24 h, and the medium was replaced every 3 days. After 10 days, the cells were fixed with 4% (v/v) paraformaldehyde for 30 min and stained with 1% crystal violet for 30 min. After the cells were washed twice with PBS, the numbers of colonies were counted manually. The rate of colony formation was determined as (number of clones/number of inoculated cells) $\times$ 100%.

2.6 Wound healing assay

Hep3B and Huh7 cells were seeded in a six-well plate at 5×10^5 cells per well with serum-free MEM and DMEM,

respectively. When cells had grown to approximately 90% confluence, wounds were created across the monolayer with a 10 μ L pipette tip. Subsequently, arenobufagin (6.25 or 12.5 nM) was added for 24 h, and migration of cells into the wound area was observed and photographed under a microscope (IX 53, Olympus, Tokyo, Japan).

2.7 Transwell migration and invasion assays

Transwell migration and invasion assays were performed with 6.5 mm Transwells with 8.0 μ m pore polycarbonate membrane inserts (3422, Corning, Tewksbury, MA, USA), in the presence or absence of Matrigel (356234, BioCoat, Horsham, PA, USA). Cells suspended in serum-free medium containing arenobufagin (20 nM) were added to the upper chamber at 3×10^4 cells per well, whereas the lower chamber was filled with 600 μ L fresh complete medium. After 24 h, the Matrigel and cells remaining in the upper chamber were removed, whereas cells on the lower surface were considered to have invaded and were fixed with paraformaldehyde. After being stained with 0.1% crystal violet, the cells that had migrated or invaded were photographed and analyzed in Image-Pro Plus 6.0.

2.8 Cell transfection

EpCAM small interfering RNA (siRNA, A10001) and short hairpin RNA (shRNA) were provided by GenePharma (Suzhou GenePharma Co., Ltd., suzhou, jiangsu, China); EpCAM overexpression vector (EpCAM^{OE}) pcDNA3.1(+)-mCherry-P2A-hEpCAM receptor and pLKD-U6-hEpCAM-shRNA-CMV-mCherry-2A-Puro receptor were purchased from Umire-Bio (Guangzhou, Guangdong, China); and Lipofectamine 2000 (11668019) was purchased from Invitrogen™ (Grand Island, NY, USA). Hep3B and Huh7 cells were cultured in a six-well plate at 5×10^5 cells per well for 24 h, then transfected with EpCAM siRNA, shRNA or non-targeting siRNA with Lipofectamine™ 2000 according to the manufacturer's protocol. After 24 h of transfection, the cells were treated with arenobufagin (20, 100 or 500 nM), and functional assays were performed.

2.9 3D organoid culture assay

Hep3B and Huh7 cells were collected and resuspended in stem cell culture medium before being seeded into a low adhesion six-well plate at 5×10^3 cells per well. After incubation with arenobufagin (12.5, 25, 50, 100 or 500 nM) for 5 days, the numbers and sizes of the cell spheres were observed under a microscope, and the numbers of suspended tumor spheres including more than 50 cells were recorded.

2.10 Western blotting assay

Hep3B and Huh7 cells were cultured in a six-well plate (5×10^5 cells/well) and treated with arenobufagin (20, 100 or 500 nM). Protein levels of EpCAM, CD133, CD44, c-Myc, OCT4, SOX2 and β -actin were subsequently measured with western blotting. Briefly, after cell lysis,

total cellular proteins were collected, and the concentrations were determined with a BCA protein assay kit. The protein samples were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred to polyvinylidene fluoride membranes (Waltham, MA, USA). After being blocked in Tris buffered saline with Tween-20 (TBST) containing 5% skimmed milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with antibodies to β -actin (1:5000), EpCAM (1:1000), CD133 (1:1000), CD44 (1:1000), OCT4 (1:1000), SOX2 (1:1000) and c-Myc (1:1000). After being washed three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h. Proteins on the membranes were visualized with an enhanced chemiluminescence detection kit and a Chemiluminescence Image Analyzer (ProteinSimple, San Jose, CA, USA).

2.11 Zebrafish feeding and embryo collection

Zebrafish were reared in a circulating water purification system with a temperature of 28.5 °C, pH of 7.4, salinity of 0.03%–0.04% and a photoperiod alternating cycles of 14 h light/10 h dark. The female-to-male ratio of mature zebrafish was maintained at 1:1 or 2:1, and the light was switched on to stimulate the zebrafish to mate and lay eggs. After careful collection in a clean Petri dish, the embryos were sterilized with embryo-culture water that contained 0.002% methylene blue. Zebrafish embryos were subsequently raised in an incubator at 28.5°C. All zebrafish procedures were approved by the Experimental Animal Welfare and Ethics Committee of Southern Medical University.

2.12 Xenotransplanted zebrafish model

To establish the zebrafish xenotransplant model, we labeled Hep3B cells with chloromethylbenzamido-Dil (CM-Dil) and then microinjected them into the yolk sac in 2 days post-fertilization (dpf) zebrafish. Next, the larvae were soaked in the indicated concentrations of arenobufagin (250, 500 or 1000 nM) or the positive control sorafenib (0.25 μ M) for 72 h. At the end of the treatment, zebrafish were observed under a fluorescence microscope (MVX10, Olympus, Tokyo, Japan).

2.13 Histopathological staining

After treatment with arenobufagin for 72 h, the xenotransplanted zebrafish were fixed overnight with 4% paraformaldehyde, dehydrated with graded ethanol, rendered transparent with graded xylene and embedded in paraffin. The paraffin blocks were then cut into 5 μ m thin slices, dewaxed with fractionated xylene and rehydrated with ethanol. Subsequently, Hematoxylin and eosin (H&E) staining was performed according to the manufacturer's instructions, and sections were observed under a microscope (IX53, Olympus, Tokyo, Japan).

Immunohistochemistry (IHC) was performed to examine the protein expression levels of EpCAM, CD133 and CD44. After antigen retrieval with sodium citrate solution, the sections were blocked with 5% (w/v) bovine serum albumin and then incubated with antibodies to EpCAM (1:200), CD133 (1:200) and CD44 (1:200) at 4°C overnight. Subsequently, the sections were washed with PBS and incubated with HRP conjugated secondary antibody, visualized with DAB and counterstained with hematoxylin. Five representative fields per section were observed under an optical microscope (Olympus, Tokyo, Japan).

2.14 Molecular docking

The three-dimensional structure of arenobufagin was constructed with ChemDraw 3D. AutoDockTools was used to process the protein, including hydrogenation, charge and water removal. Molecular docking of arenobufagin into the structure of EpCAM (UniProt ID: P16422) was performed with AutoDock Vina software.

2.15 Statistical analysis

Data are expressed as mean $\pm$ SD or mean $\pm$ SEM from at least three independent *in vitro* or *in vivo* experiments, respectively. The statistical software GraphPad Prism 8.0 and SPSS V20.0 (IBM Statistics, USA) was used to compare the means among groups through single-factor analysis of variance (ANOVA). A two-tailed unpaired *t*-test and paired *t*-test were used to assess differences between groups, and $p \leq 0.05$ was considered statistically significant. All experiments were independently repeated at least three times.

3. RESULTS

3.1 EpCAM expression in the tumor-adjacent region is an indicator of early-stage HCC

An abnormal expression profile of EpCAM has been demonstrated to be strongly associated with the occurrence and poor prognosis of multiple malignant tumors [11], as confirmed by the GEPIA2 database. The results revealed that *EpCAM* was highly expressed in breast invasive carcinoma (BRCA), stomach adenocarcinoma (STAD), pancreatic adenocarcinoma (PAAD), lung adenocarcinoma (LUAD) and prostate adenocarcinoma (PRAD), and the strongest expression was observed in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) (Supplementary Figure 1A). However, in patients with HCC, *EpCAM* expression in tumors was much lower than that in paired normal tissues (Supplementary Figure 1A & B). Because *EpCAM* expression is strongly associated with the status of tissue-specific stem cell differentiation [11], we aimed to further characterize the role of *EpCAM* in HCC progression.

We examined the expression of *EpCAM* in 68 patients with HCC by using tissue microarray technology in combination with immunohistochemistry. Representative

Research Article

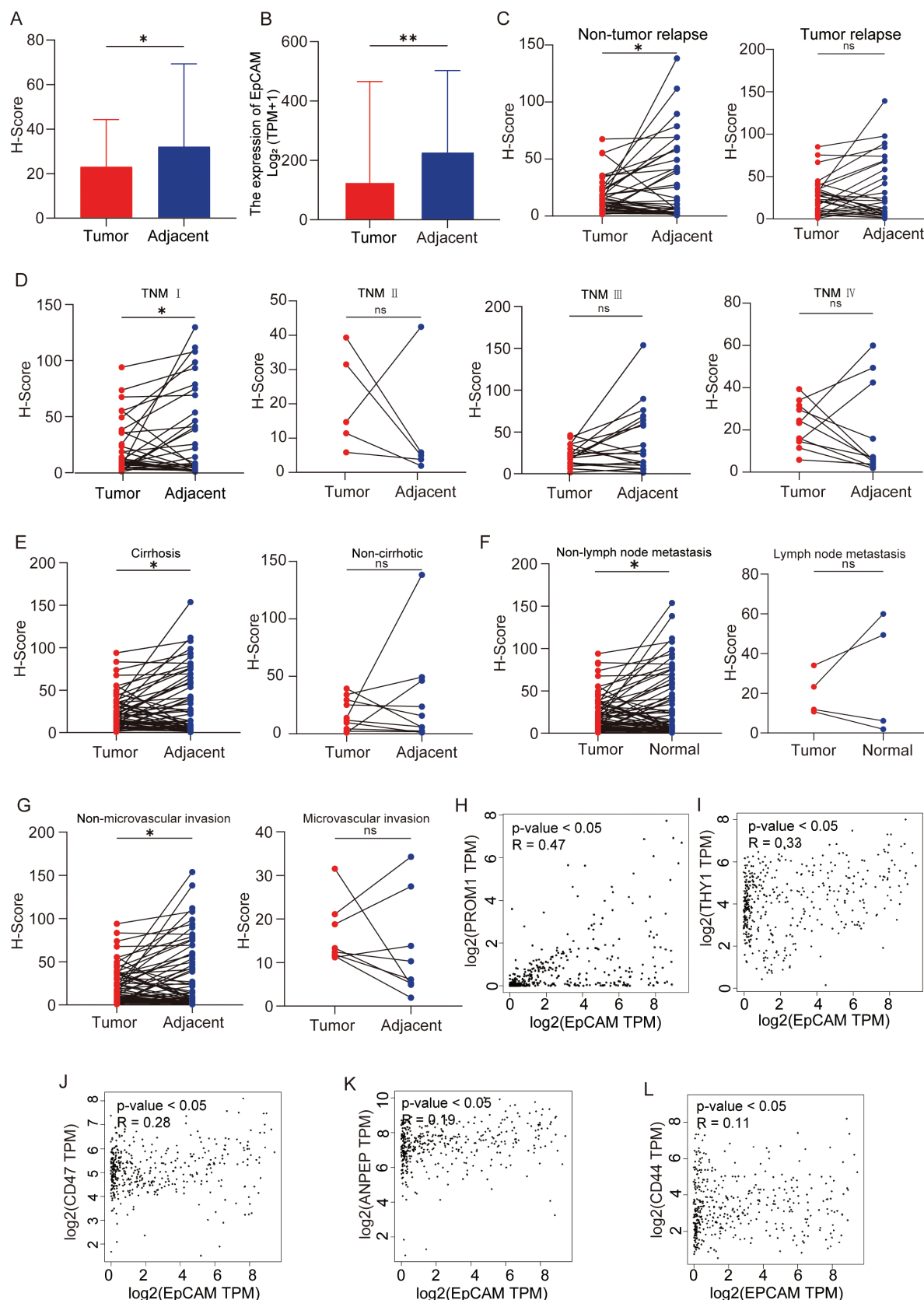


Figure 1 | EpCAM expression in tumor-adjacent regions is an indicator of early-stage HCC.

(A) The H-score of EpCAM in patient tumor tissue is lower than that in adjacent regions, according to a tissue microarray ($n = 68$). (B) The expression of EpCAM in patients with HCC is lower than that in adjacent regions, as analyzed with the GEO database (GSE174570, $n = 57$). (C-G) Clinical characteristics of the enrolled patients, according to a tissue microarray. Correlation between EpCAM expression in tumor-adjacent regions and tumor recurrence (C), stages of HCC (D), cirrhosis (E), lymph node metastasis (F) and microvascular invasion (G). * $p < 0.05$, ** $p < 0.01$ vs. tumor region by paired t -test. (H-L) Correlation between the expression levels of EpCAM and cancer stemness markers, detected according to the GEPIA 2 platform. The expression levels of EpCAM and CD133 (also named PROM1, H), CD90 (also named THY1, I), CD47 (J), ANPEP (K) and CD44 (L) were positively correlated in patients with HCC. The Pearson correlation coefficient analysis method was used. "Plot" was used to obtain the correlation coefficients in HCC.

staining results for EpCAM in tumor-adjacent and tumor regions are presented in online [Supplementary Figure 1C & D](#). Notably, the expression of EpCAM in the tumor-adjacent region was significantly higher than that in the tumor region ([Figure 1A](#)), and this phenomenon was further confirmed by the GEO database ([Figure 1B](#)). Furthermore, we observed a similar trend only in TNM stage I of HCC; however, no significant difference was observed between adjacent and tumor regions in TNM stages II, III and IV of HCC ([Figure 1D](#)). These data indicated that the expression level of EpCAM in the tumor-adjacent region was strongly associated with HCC progression, particularly in early stages of HCC.

Next, we performed prognosis analyses by using patient information. In HCC patients without tumor relapse, the expression of EpCAM in tumor-adjacent regions was significantly higher than that in tumor regions. In contrast, in HCC patients with tumor relapse, no significant difference was observed in EpCAM expression between tumor-adjacent and tumor regions ([Figure 1C](#)). EpCAM levels were higher in tumor-adjacent regions than tumor regions in patients with cirrhotic HCC. However, in patients with HCC without cirrhosis, no significant difference was observed in EpCAM expression between adjacent and tumor tissues ([Figure 1E](#)). In HCC patients without lymph node metastasis and microvascular invasion, the levels of EpCAM were significantly lower in tumors than adjacent regions ([Figure 1F & G](#)). In contrast, in HCC patients with lymph node metastasis and microvascular invasion, the EpCAM expression showed no significant difference between adjacent and tumor tissues. The clinical characteristics of the enrolled patients are presented in [Supplementary Table 1](#). This evidence suggested that patients with advanced HCC with a mature tumor vascular system, no significant difference in EpCAM expression was observed between adjacent and tumor tissues, whereas in the early-stage HCC patients without lymph node metastasis and microvascular invasion, EpCAM expression in adjacent tissues served as an indicator of early-stage HCC that had not yet developed a tumor vascular system.

3.2 EpCAM facilitates liver cancer stemness in HCC cells

The correlation of EpCAM with several cancer stem cell markers, such as CD133/PROM1, CD90/THY1, ANPEP

and CD47, was further investigated with the GEPIA 2 database. The expression level of EpCAM was positively correlated with that of cancer stem cell markers ([Figure 1H-L](#)).

Moreover, we examined EpCAM and CD133 expression in HCC cell lines and observed higher expression in Hep3B cells than Huh7 cells ([Figure 2A](#)), thereby indicating that Hep3B cells showed stronger cancer stemness than Huh7 cells. We next explored the role of EpCAM in cancer stemness by knocking down EpCAM in Hep3B cells with EpCAM siRNA and overexpressing EpCAM in Huh7 cells with an EpCAM-overexpressed plasmid ([Figure 2B](#)). As shown in [Figure 2C-F](#), knockdown of EpCAM in Hep3B cells had inhibitory effects on cell viability, proliferation and colony formation, whereas overexpression of EpCAM in Huh7 cells showed opposite effects. Furthermore, EpCAM was found to play an important role in promoting the tumor cell invasion and migration ability of HCC cells ([Figure 2H](#)). To further investigate whether EpCAM might affect cancer stemness in HCC cells, we conducted 3D organoid experiments, which showed that EpCAM knockdown significantly inhibited the self-renewal ability of Hep3B cells, whereas overexpression of EpCAM enhanced the self-renewal ability of Huh7 cells ([Figure 2G](#)). Moreover, EpCAM siRNA inhibited wound closure in Hep3B cell monolayers, whereas EpCAM overexpression showed opposite effects in Huh7 cells ([Figure 2I](#)). Together, these results revealed that EpCAM promotes tumor stem characteristics, by enhancing cancer cell survival, proliferation, cloning, invasion, migration and self-renewal of HCC cells *in vitro*.

3.3 Arenobufagin shows significant anti-stemness effects in vitro

To identify effective agents for treating HCC, we tested more than 40 natural bufadienolides and evaluated cancer cell viability. Arenobufagin potentially decreased the viability of HCC cells and was selected for subsequent studies [14]. Our results revealed that arenobufagin markedly decreased the viability of HCC (Hep3B and Huh7) cells in a concentration- and time-dependent manner, with IC_{50} values of 36.4 nM in Hep3B cells and 123.4 nM in Huh7 cells after 72 h treatment ([Figure 3A](#)). Moreover, arenobufagin treatment significantly decreased the clonogenicity of HCC cells, as evaluated with colony formation assays ([Figure 3B](#)). Arenobufagin

Research Article

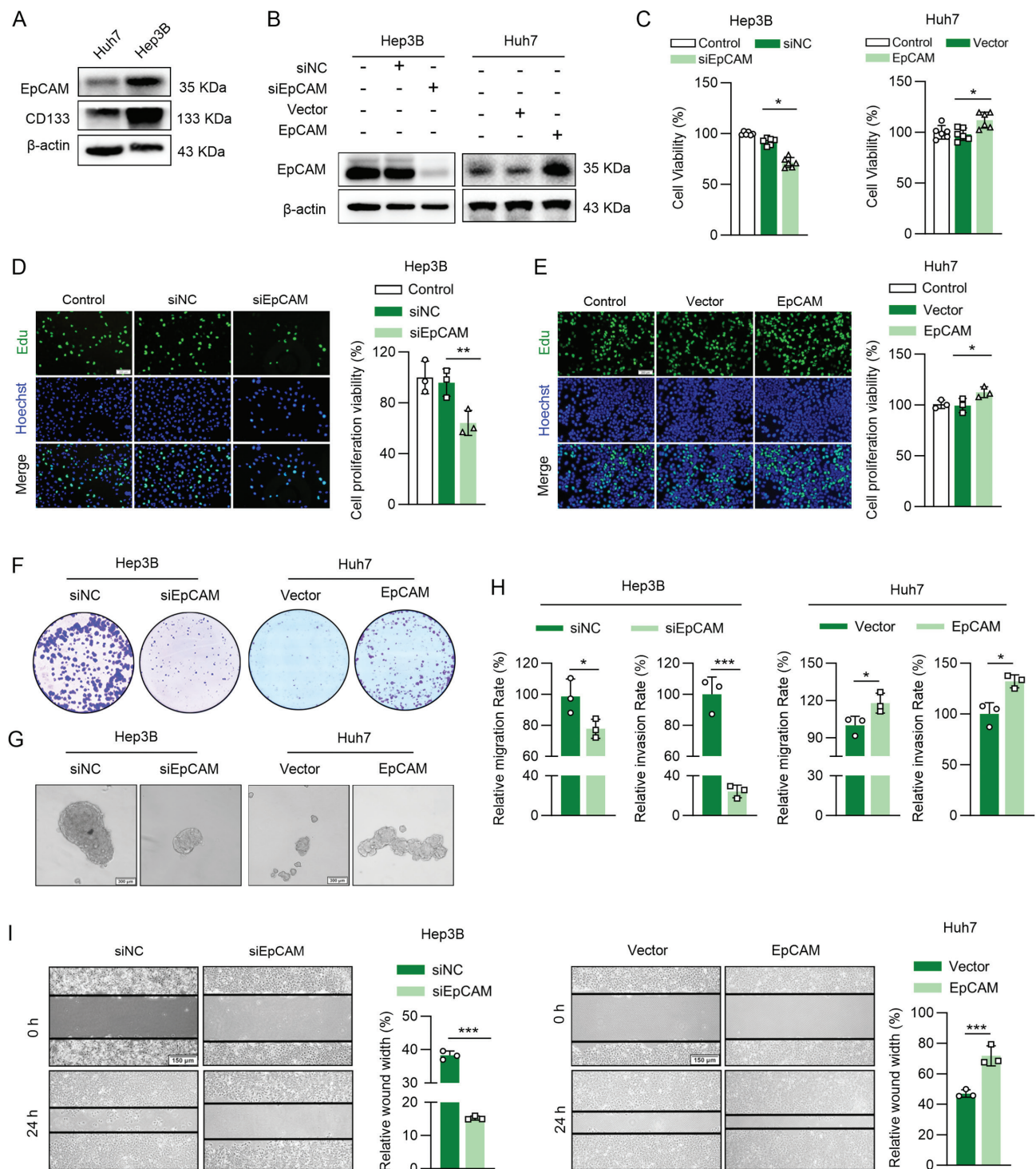


Figure 2 | EpCAM facilitates liver cancer stemness in HCC cells.

(A) The expression of EpCAM and CD133 is high in Hep3B and is low in Huh7 cells. (B) HCC cells were transfected with EpCAM siRNA or EpCAM overexpression plasmid for 24 h, and the levels of EpCAM in HCC cells were evaluated by western blotting. (C) EpCAM knockdown decreases viability in Hep3B cells, whereas EpCAM overexpression increases that in Huh7 cells, as determined with MTT assays. (D-F) EpCAM knockdown decreases proliferation in Hep3B cells, whereas EpCAM overexpression increases that in Huh7 cells, as detected with EdU assays (C & D, scale bar = 300 μm) and colony formation assays (F). (G) EpCAM knockdown inhibits the self-renewal ability of Hep3B cells, whereas EpCAM overexpression enhances that of Huh7 cells. Sphere-forming assays were performed by culturing Hep3B and Huh7 cells in sphere suspension medium containing EpCAM shRNA or EpCAM overexpression plasmid. The images were taken at a magnification of 200×

Scale bar = 300 μm . (H) EpCAM knockdown inhibits the migration and invasion ability of Hep3B cells, whereas EpCAM overexpression enhances that of Huh7 cells, as determined with Transwell migration and invasion assays. (I) EpCAM knockdown inhibits the migration ability of Hep3B cells, whereas EpCAM overexpression increases that of Huh7 cells, as evaluated with wound healing assays. Scale bar = 150 μm . Data are expressed as mean $\pm$ SD. $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Tukey's test. A two-tailed unpaired t -test was used to assess differences between groups, * $p < 0.05$, *** $p < 0.001$.

also inhibited the proliferation of HCC cells (Figure 3C & D). Furthermore, arenobufagin inhibited wound closure in HCC cell monolayers in a concentration-dependent manner (Figure 3E & F), and markedly decreased the migration and invasion ability of Hep3B and Huh7 cells (Figure 3G & H). Together, these results revealed that arenobufagin has antiproliferative and antimetastatic effects on HCC cells *in vitro*.

Furthermore, the 3D organoid experimental results showed that arenobufagin significantly inhibited the self-renewal ability of HCC cells (Figure 4A), and arenobufagin treatment potentially decreased the expression of cancer stemness markers, such as EpCAM, CD44, c-Myc, OCT4 and SOX2 in a dose-dependent manner, as assessed with western blotting (Figure 4B & C). Together, these results revealed that arenobufagin inhibited tumor stemness characteristics *in vitro*.

3.4 Arenobufagin inhibits cancer stemness by down-regulating EpCAM *in vitro*

Considering the importance of EpCAM in maintaining the biological properties of liver cancer stem cells, we investigated whether arenobufagin might inhibit liver cancer stemness *via* blocking EpCAM signaling. Primarily, we conducted molecular docking of arenobufagin with the EpCAM protein to investigate potential direct binding interactions. As shown in Figure 5A, arenobufagin binds the extracellular domain of EpCAM (EpEX). The hydroxyl group of arenobufagin on ^{24}C forms hydrogen bonds with ASP280 and ASP211, whereas the oxygen atom on ^{6}C forms a hydrogen bond with GLN204, thereby cooperatively stabilizing the interaction between arenobufagin and EpCAM. The EpEX domain participates in the adhesion process. We propose that arenobufagin inhibits tumor stemness *via* decreasing the activation of signaling pathways by binding the EpEX domain of EpCAM. Moreover, the arenobufagin-mediated decrease in cell viability in Hep3B cells was synergistically enhanced by EpCAM shRNA treatment, and the cytotoxicity of arenobufagin in Huh7 cells was reversed by overexpression of EpCAM (Figure 5B & C). EpCAM shRNA knockdown or overexpression of EpCAM produced similar effects on the proliferation of HCC cells (Figure 5D & E). Next, the effects of EpCAM shRNA or the overexpression of EpCAM on arenobufagin-induced HCC cell migration and invasion were investigated. EpCAM shRNA significantly decreased the migration and invasion ability of Hep3B cells treated with arenobufagin (Figure 5F). In contrast, arenobufagin

treatment also markedly suppressed EpCAM-induced Huh7 cell migration, and similar results were obtained in Transwell invasion assays (Figure 5G). Furthermore, EpCAM shRNA weakened wound closure in Hep3B cell monolayers, whereas wound closure was significantly facilitated by overexpression of EpCAM in Huh7 cells (Figure 5H). Additionally, EpCAM shRNA enhanced the inhibitory effect of arenobufagin on the self-renewal ability of Hep3B cells, and the overexpression of EpCAM reversed the self-renewal inhibitory effect of arenobufagin in Huh7 cells (Figure 5I & J). Moreover, the protein expression of cancer stemness markers inhibited by arenobufagin increased after EpCAM knockdown in Hep3B cells and was partially restored after EpCAM overexpression in Huh7 cells, as assessed with western blotting (Figure 5K & L). Together, these results suggested that EpCAM contributes to the inhibitory effects of arenobufagin on HCC cell stemness.

3.5 Arenobufagin exerts anti-HCC effects through inhibiting EpCAM *in vivo*

We first evaluated the toxicity of arenobufagin in zebrafish. Arenobufagin concentrations in the range of 250–1000 nM did not show clear toxicity (data not shown). Therefore, 250, 500 and 1000 nM were selected as the concentration gradient for subsequent experiments. To evaluate the *in vivo* anti-HCC effect of arenobufagin, we fluorescently labeled Hep3B cells with CM-Dil and transplanted them into 2 dpf zebrafish yolk sacs through microinjection to establish a xenografted zebrafish tumor model. The area of red fluorescence in the yolk sac in zebrafish indicated tumor size. Sorafenib, an FDA-approved anti-HCC drug, was used as a positive control. Red fluorescence in the zebrafish yolk sac decreased after treatment with various concentrations of arenobufagin or sorafenib, in contrast to findings in the model group, thus indicating that arenobufagin significantly inhibited the growth of tumors in xenotransplanted zebrafish (Figure 6A). Histopathological analysis of zebrafish also revealed many Hep3B cells in the yolk sac in zebrafish in the model group (Figure 6B). After 72 h of arenobufagin treatment, the numbers of Hep3B cells, and the expression of EpCAM, CD133 and CD44 in Hep3B cells in the yolk sac in zebrafish decreased in a dose-dependent manner (Figure 6C).

Hep3B cells transfected with EpCAM shRNA or EpCAM^{OE} were subcutaneously microinjected into 2 dpf zebrafish. Inhibition of EpCAM markedly decreased the fluorescence in the zebrafish yolk sac; therefore, the

Research Article

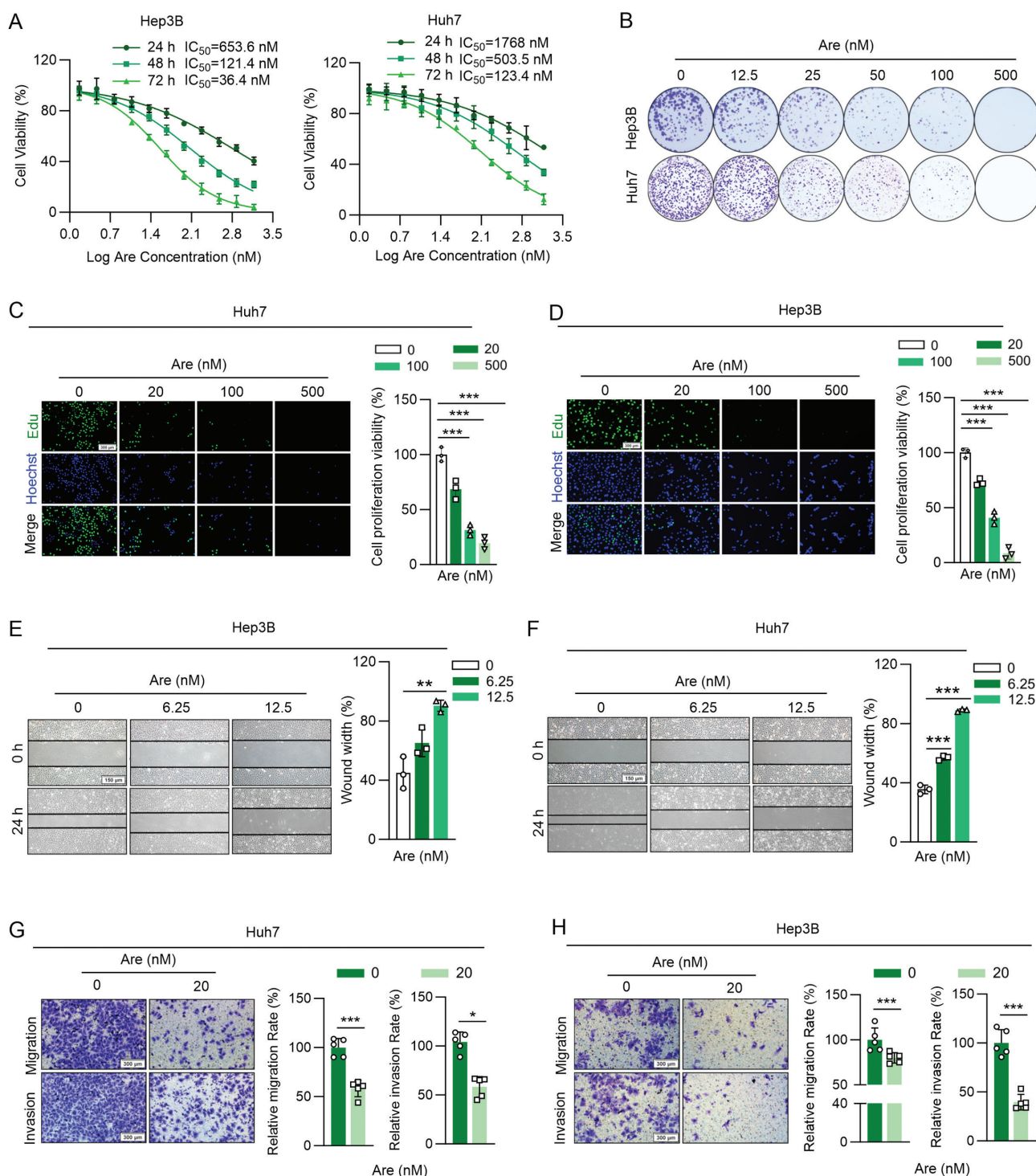


Figure 3 | Arenobufagin shows antiproliferative and antimetastatic effects on HCC cells in vitro.

(A) Arenobufagin inhibits the viability of HCC cells (Hep3B and Huh7). (B) Arenobufagin inhibits colony formation in Hep3B and Huh7 cells. Hep3B and Huh7 cells were treated with arenobufagin (0–500 nM) for 24 h, and cultured 10 days before assessment via colony formation assays. Representative images of cell colonies are shown. (C & D) Arenobufagin inhibits proliferation of HCC cells. Hep3B and Huh7 cells were treated with arenobufagin (20, 100 or 500 nM) for 24 h and evaluated with EdU assays. Scale bar = 300 μ m. (E & F) Arenobufagin decreases the migration of Hep3B and Huh7 cells, as detected with wound healing assays. Representative images and quantification of the numbers of HCC cells that migrated and invaded are shown. Scale bar = 150 μ m. (G & H) Arenobufagin suppresses the migration and invasion of HCC cells. Hep3B and Huh7 cells were treated with arenobufagin (20 nM) for 24 h, then evaluated with Transwell migration and invasion assays.

Representative images and quantification of the numbers of HCC cells that migrated and invaded are shown. Scale bar = 300 μ m. Data are presented as mean $\pm$ SD. $n = 3$. ** $p < 0.01$, *** $p < 0.001$ vs. the control group by one-way ANOVA with Tukey's test. A two-tailed unpaired t -test was used to assess differences between groups, * $p < 0.05$, *** $p < 0.001$.

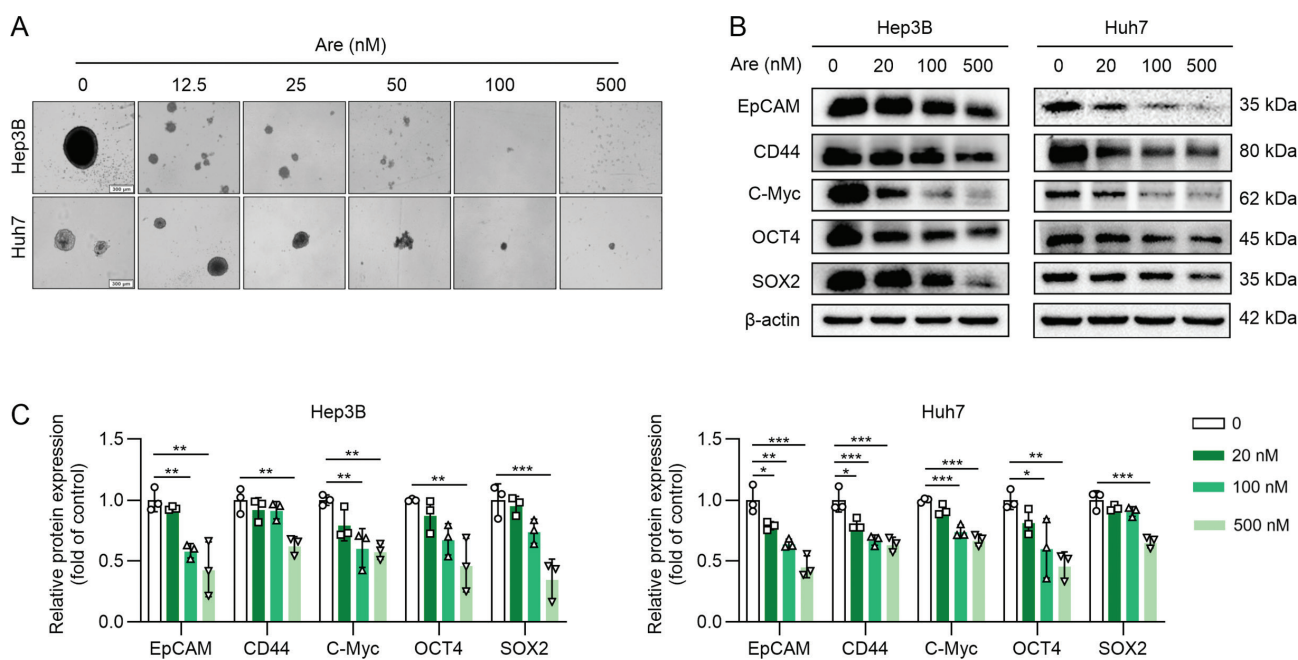


Figure 4 | Arenobufagin inhibits liver cancer stemness in vitro.

(A) Arenobufagin inhibits the self-renewal ability of Hep3B and Huh7 cells. Sphere-forming assays were performed by culturing of Hep3B and Huh7 cells in sphere suspension medium containing arenobufagin (0–500 nM) for 5 days. The images were taken at a magnification of 20 $\times$. Scale bar = 300 μ m. (B) Arenobufagin inhibits the expression levels of cancer stemness markers, such as EpCAM, CD44, C-Myc, OCT4 and SOX2. (C) Histogram of the relative protein expression in panel B. Data represent mean $\pm$ SD. $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group by one-way ANOVA with Tukey's test.

decrease in EpCAM exhibited anti-HCC effects, which were further enhanced by arenobufagin treatment (Figure 6D). A similar phenomenon was observed in subsequent H&E staining (Figure 6E). Together, our results suggested that arenobufagin inhibited the progression of early-stage HCC without a mature vascular system by targeting EpCAM *in vivo*.

4. DISCUSSION

Liver cancer is the sixth most common malignant tumor and the third leading cause of cancer-related death worldwide, and has a 5-year survival rate of 18%. HCC accounts for 90% of liver cancers [1–4]. Hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol use and obesity/diabetes are the major risk factors for HCC [24]. Unfortunately, only a subset of patients with HCC can derive specific benefits from systemic therapies based on tyrosine kinase inhibitors (e.g., sorafenib or lenvatinib), immune-checkpoint inhibitors (e.g., pembrolizumab and nivolumab), or a combination of

atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF) [24]. However, despite these unprecedented results, less than 30% of patients respond to existing therapies. To date, biomarkers for early stages of HCC—comprising either neoplastic or recurrent tumors with diameters less than 2 cm, which have not formed mature vascular systems—cannot clearly elucidate the indicators for diagnosis or for evaluating treatment response. Herein, EpCAM expression in the adjacent region was found to serve as an indicator of early-stage HCC in patients with HCC, and EpCAM was found to promote tumorigenesis *in vitro*.

EpCAM is a typical epithelial cancer-associated antigen molecule first identified on the surfaces of colorectal cancer cells in 1979 [25]. The GEPIA 2 database revealed that EpCAM is highly expressed in several types of malignancies, including BRCA, STAD, PAAD, LUAD and PRAD, and the strongest expression was observed in COAD and READ. However, EpCAM was found to be weakly expressed in HCC, renal carcinoma, glioblastoma multiforme (GBM) and other malignant

Research Article

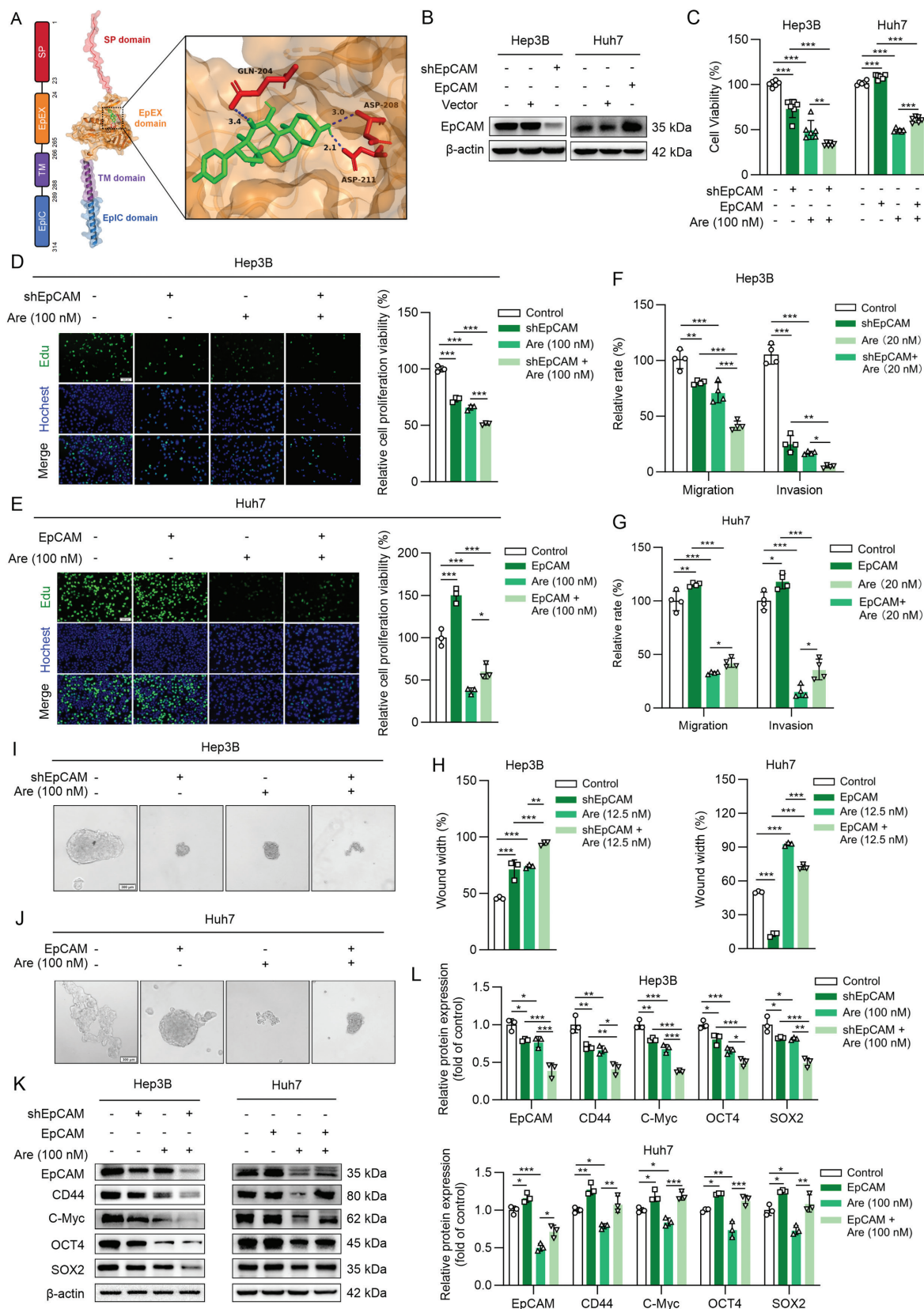


Figure 5 | Arenobufagin inhibits liver cancer stemness by down-regulating EpCAM.

(A) Molecular docking results of arenobufagin binding the extracellular domain (EpEX) of EpCAM. (B) EpCAM expression decreases in Hep3B cells and increases in Huh7 cells after transfection with EpCAM shRNA or EpCAM overexpression plasmid, as detected with western blot analysis. (C) EpCAM knockdown increases the arenobufagin-mediated inhibition of viability in Hep3B cells, whereas EpCAM overexpression decreases that in Huh7 cells, as determined with MTT assays. (D & E) EpCAM knockdown increases the arenobufagin-mediated inhibition of the proliferation in Hep3B cells, whereas EpCAM overexpression has opposite effects in Huh7 cells, as determined with EdU assays. (F-H) EpCAM knockdown increases arenobufagin-mediated inhibition of migration and invasion in Hep3B cells, whereas EpCAM overexpression reverses the inhibitory effect of arenobufagin in Huh7 cells, as assessed with Transwell migration and invasion assays (F & G), and wound healing assays (H). (I & J) EpCAM knockdown increases the arenobufagin-mediated inhibition of self-renewal ability in Hep3B cells, whereas EpCAM overexpression decreases the inhibitory effect of arenobufagin in Huh7 cells. After transfection of EpCAM shRNA or EpCAM overexpression plasmid, Hep3B and Huh7 cells were treated with DMSO or arenobufagin (100 nM) and subjected to sphere-forming assays. Scale bar = 300 μ m. (K & L) EpCAM knockdown increases the arenobufagin-mediated inhibition of cancer stemness marker expression in Hep3B cells, whereas EpCAM overexpression reverses the inhibitory effect of arenobufagin in Huh7 cells, as determined with western blot analysis. Data are expressed as mean $\pm$ SD. $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Tukey's test.

tumors, in agreement with previously reported evidence [25]. Therefore, the multiple functions of EpCAM may vary depending on tumor type and localization, and play decisive roles in the fate of individual tumor cells. As a phenotype of stem cancer cells, high expression of EpCAM promotes tumor cell proliferation and metastasis, and might therefore be associated with poor prognosis. As a marker of epithelial cell differentiation, EpCAM is associated with characteristics of high differentiation, low invasion and migration, and hence better survival. Therefore, the conflicting roles of EpCAM in differential clinical outcomes might be associated with the origin of the cancer or even the stage of cancer progression. Further focused investigation is needed to determine how to use EpCAM expression to determine prognosis.

In HCC, EpCAM is currently one of the most commonly used markers for liver cancer stem cells [26], and its abnormal high expression in tumor tissues is closely associated with intercellular junctions, proliferation, the cell cycle, metastasis and tumor recurrence, and it is considered an important biomarker of poor prognosis [27, 28]. Here, the expression of EpCAM in tumor regions was lower than that in adjacent regions in patients with HCC in the tissue microarray ($n = 68$) results, and the GEO ($n = 57$) and GEPIA2 databases. These results indicate that in HCC patients with relapse tumor, lymph node metastasis or microvascular invasion, the levels of EpCAM in tumors were significantly lower than those in adjacent regions. These results suggested that the expression of EpCAM in adjacent regions might serve as a biomarker for early stages of HCC and have potential roles in patient management of either neoplastic or recurrent tumors that have not formed a mature vascular system. Park *et al.* have also demonstrated that higher EpCAM expression in the peritumoral region is associated with poorer prognosis in patients with HCC [29], thus further supporting the use of EpCAM in tumor-adjacent regions as a biomarker for early stages of HCC.

The EpCAM signaling pathway is activated through the regulation of intramembrane protein hydrolysis. The free short C-terminal cytoplasmic domain (EpICD)

fragment is hydrolyzed by RIP from EpCAM and then released into the cytoplasm, where it forms a nuclear complex with transcriptional regulators β -catenin, lymphoid enhancer-binding factor 1 (LEF1), and four and a half LIM domains 2 (FHL2) [30–32]. This multiprotein complex binds downstream target gene promoters and activates OCT4, KIF4, SOX2, c-Myc and other genes [30]. All these genes are central in the transformation of somatic cells into pluripotent stem cells, and also are closely associated with cell cycle regulation and cell stemness, which are considered direct targets of EpCAM in human embryonic stem cells [33]. Here, we found that EpCAM promoted tumorigenesis in Hep3B and Huh7 cells *in vitro*, by supporting survival, proliferation, cloning, invasion, migration and self-renewal in HCC cells; these findings further confirmed that EpCAM might serve as a cancer stemness marker and therapeutic drug target.

Given that EpCAM is a potential target for cancer therapies, EpCAM-specific antibodies that destroy cancer cells through various mechanisms have been exploited, such as edrecolomab (Panorex[®], mAb 17-1A), ING-1 and 3622W94, MT201 (adecatumumab), catumaxomab and MT110 (solitomab) [25, 34, 35]. Several therapeutic options targeting EpCAM are currently in clinical trials, including anti-EpCAM-based immunotoxin (e.g., MOC31PE) [25] and chimeric antigen receptor T cells (CAR T cells) targeting EpCAM (<https://clinicaltrials.gov/ct2/show/NCT04151186>). However, these strategies have not been successfully developed for various reasons, such as binding affinity, tolerability, toxicity and safety. In addition, Niessing's group has performed a high-content screening of a small-molecule compound library and identified 128 potential inhibitors, among which one compound showed robust inhibitory effects on RIP of EpCAM *in vitro*. However, the inhibitors have not been further validated in animal models for their specific targeting of EpCAM, and their antitumor activity and safety *in vivo*.

Although extensive literature has revealed the anti-cancer activities of arenobufagin and its underlying mechanisms, such as the induction of autophagy and apoptosis, cell cycle arrest, inhibition of angiogenesis,

Research Article

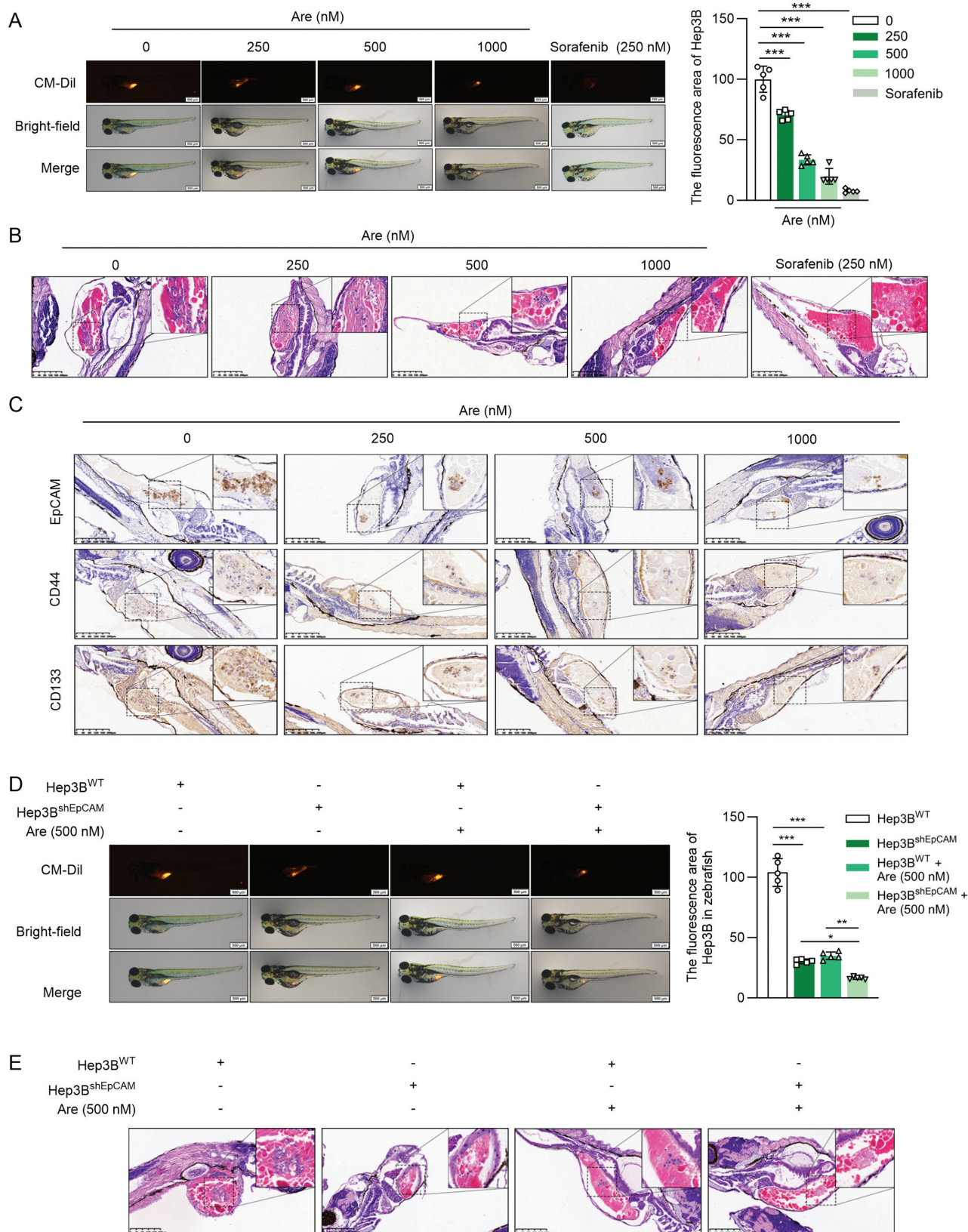


Figure 6 | Arenobufagin exerts anti-HCC effects in vivo through inhibiting EpCAM signaling.

(A & B) Arenobufagin inhibits the growth of Hep3B cells in zebrafish yolk sacs. Hep3B cells were stained with CM-Dil dye and microinjected into the yolk sacs. After arenobufagin (0, 250, 500 or 1000 nM) or sorafenib (250 nM) treatment for 72 h, fluorescence was photographed under a stereoscopic fluorescence microscope (A, left panel, bar = 500 μ m), and the fluorescence intensity was calculated (A, right panel). Data are expressed as mean $\pm$ SD. *** p < 0.001 vs. control group by one-way ANOVA with Tukey's test. Zebrafish tissue slices were stained with H&E and observed under a microscope (B, bar = 200 μ m). (C) Arenobufagin decreases the expression of stemness-related markers in Hep3B cells in zebrafish. Immunohistochemical analysis was used to detect the expression of stemness-related markers (EpCAM, CD44 and CD133) in Hep3B cells in the zebrafish yolk sac. (D & E) The anti-HCC effects of arenobufagin in the zebrafish xenograft tumor model increase after EpCAM inhibition. Hep3B cells were transfected with EpCAM shRNA or scrambled shRNA, and microinjected into 2 dpf zebrafish (D, bar = 500 μ m). Data are expressed as mean $\pm$ SD. * p < 0.05, ** p < 0.01, *** p < 0.001 by one-way ANOVA with Tukey's test. Histopathological analysis of zebrafish (E, bar = 200 μ m).

epithelial-mesenchymal transition (EMT) and tumor-associated macrophage polarization [25], the effects of arenobufagin on the biological properties of liver cancer stemness have not been reported. In this study, we demonstrated that arenobufagin inhibited cancer cell survival, proliferation, cloning, invasion, migration and self-renewal of HCC cells. Because of the critical role of EpCAM in cancer stem cells and its potential as a drug target of cancer therapies, we further explored the relationship between arenobufagin and EpCAM, and found that the inhibitory effects of arenobufagin on HCC cell proliferation and stemness were closely associated with the suppression of EpCAM. The inhibition of EpCAM through siRNA interference promoted the effects of arenobufagin on cell proliferation and stemness, whereas the overexpression of EpCAM through transfection of an overexpression plasmid counteracted these effects. Moreover, we constructed a zebrafish xenograft tumor model to mimic the features of early-stage HCC without a mature vascular system. The data suggested that arenobufagin inhibited early progression of HCC, but also demonstrated that this efficacy was enhanced by EpCAM blockade, thus further confirming the importance of EpCAM in the suppression of tumor stemness mediated by arenobufagin. EpCAM, as a transmembrane protein, is composed of EpEX, a single-spanning transmembrane domain (TM) and EpICD. Our molecular docking data showed that arenobufagin binds the EpEX domain. The function of EpCAM has been demonstrated to be closely associated with the proteolytic process: proteolytic cleavage induces EpEX shedding, which in turn promotes the release of EpICD. After proteasome degradation, EpICD forms a nuclear complex with β -catenin, FHL2 and LEF-1, which regulates stemness-associated genes, such as OCT4, KIF4, SOX2 and c-Myc [30–32]. Thus, we speculated that arenobufagin might inhibit tumor stemness via suppressing EpCAM, particularly through the EpEX domain, thus providing a rationale for the clinical use of *Venenum Bufonis*. However, whether EpCAM is the target of arenobufagin and how arenobufagin regulates EpCAM remain to be determined, and the effects of this agent on the EpCAM signaling pathway should

be further investigated *in vivo*. EpCAM, a critical cancer target, is closely associated with the TGF- β /SMAD, Wnt/ β -catenin, PI3K/AKT/mTOR, p53 and EpEX/EGFR pathways [36]; therefore, these pathways might be involved in the anti-HCC effects of arenobufagin. In addition, arenobufagin is a member of the bufadienolide Na⁺/K⁺-ATPase inhibitors, which exhibit substantial cardiotoxicity [37]. In the future, we will optimize the safety of arenobufagin through structural modifications, by using a prodrug strategy with design of tumor tissue targeting properties [37], or by developing arenobufagin-loaded nanomaterials [38].

5. CONCLUSIONS

In summary, EpCAM was found to serve as an indicator for patients with early-stage HCC, and to promote the proliferation and stemness of HCC cells (Hep3B and Huh7) *in vitro*. Moreover, we found that arenobufagin suppressed the stemness of HCC cells by targeting EpCAM. Overall, our results provide valuable novel insights into EpCAM function in HCC and support arenobufagin as a promising agent for HCC treatment by targeting EpCAM.

ACKNOWLEDGMENTS

This work was funded by the National Natural Science Foundation of China (No. 81803790), Guangdong Basic and Applied Basic Research Foundation (No. 2024A1515012678), Guangdong National Science Funds for Distinguished Young Scholar (No. 2017A030306006), Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (GDHVP52018) and Young Elite Scientists Sponsorship Program by CACM (2019-QNRC2-C14). The graphical abstract was created with BioRender.com.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary Material can be downloaded from https://amm-journal.org/wp-content/uploads/2025/01/amm20240064_Suppl.zip.

Research Article

REFERENCES

- [1] Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al.: Global Cancer Statistics 2022: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians* 2024, 74:229–263.
- [2] Zhao L, Zhang X, Coday M, Garcia DO, Li X, Mossavar-Rahmani Y, et al.: Sugar-Sweetened and Artificially Sweetened Beverages and Risk of Liver Cancer and Chronic Liver Disease Mortality. *JAMA* 2023, 330:537–546.
- [3] Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, et al.: Hepatocellular Carcinoma. *Nature Reviews Disease Primers* 2021, 7:6.
- [4] Rumgay H, Arnold M, Ferlay J, Lesi O, Cabaasag CJ, Vignat J, et al.: Global Burden of Primary Liver Cancer in 2020 and Predictions to 2040. *Journal of Hepatology* 2022, 77:1598–1606.
- [5] Parikh ND, Tayob N, Singal AG: Blood-based Biomarkers for Hepatocellular Carcinoma Screening: Approaching the End of the Ultrasound Era? *Journal of Hepatology* 2023, 78:207–216.
- [6] Majumdar A, Roccarina D, Thorburn D, Davidson BR, Tsochatzis E, Gurusamy KS: Management of People with Early- or Very Early-stage Hepatocellular Carcinoma: An Attempted Network Meta-analysis. *Cochrane Database of Systematic Reviews* 2017, 3:CD011650.
- [7] Terris B, Cavard C, Perret C: EpCAM, A New Marker for Cancer Stem Cells in Hepatocellular Carcinoma. *Journal of Hepatology* 2010, 52:280–281.
- [8] Khosla R, Rastogi A, Ramakrishna G, Pamecha V, Mukhopadhyay A, Vasudevan M, et al.: EpCAM⁺ Liver Cancer Stem-Like Cells Exhibiting Autocrine Wnt Signaling Potentially Originate in Cirrhotic Patients. *Stem Cells Translational Medicine* 2017, 6:807–818.
- [9] Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, et al.: EpCAM-positive Hepatocellular Carcinoma Cells are Tumor-initiating Cells with Stem/Progenitor Cell Features. *Gastroenterology* 2009, 136:1012–1024.
- [10] Nio K, Yamashita T, Okada H, Kondo M, Hayashi T, Hara Y, et al.: Defeating EpCAM(+) Liver Cancer Stem Cells by Targeting Chromatin Remodeling Enzyme CHD₄ in Human Hepatocellular Carcinoma. *Journal of Hepatology* 2015, 63:1164–1172.
- [11] Xiao D, Xiong M, Wang X, Lyu M, Sun H, Cui Y, et al.: Regulation of the Function and Expression of EpCAM. *Biomedicines* 2024, 12:1129.
- [12] Wei WL, Hou JJ, Wang X, Yu Y, Li HJ, Li ZW, et al.: Venenum Bufonis: An Overview of Its Traditional Use, Natural Product Chemistry, Pharmacology, Pharmacokinetics and Toxicology. *Journal of Ethnopharmacology* 2019, 237:215–235.
- [13] Deng LJ, Li Y, Qi M, Liu JS, Wang S, Hu LJ, et al.: Molecular Mechanisms of Bufadienolides and their Novel Strategies for Cancer Treatment. *European Journal of Pharmacology* 2020, 887:173379.
- [14] Liu JS, Deng LJ, Tian HY, Ruan ZX, Cao HH, Ye WC, et al.: Anti-tumor Effects and 3D-Quantitative Structure-activity Relationship Analysis of Bufadienolides from Toad Venom. *Fitoterapia* 2019, 134:362–371.
- [15] Yang Y, Liu C, Wang M, Cheng H, Wu H, Luo S, et al.: Arenobufagin Regulates the p62-Keap1-Nrf2 Pathway to Induce Autophagy-dependent Ferroptosis in HepG2 Cells. *Naunyn-Schmiedeberg's Archives of Pharmacology* 2024, 397:4895–4909.
- [16] Deng LJ, Peng QL, Wang LH, Xu J, Liu JS, Li YJ, et al.: Arenobufagin Intercalates with DNA Leading to G2 Cell Cycle Arrest via ATM/ATR Pathway. *Oncotarget* 2015, 6:34258–34275.
- [17] Ho HY, Chen MK, Lin CC, Lo YS, Chuang YC, Hsieh MJ: Arenobufagin Induces Cell Apoptosis by Modulating the Cell Cycle Regulator Claspin and the JNK Pathway in Nasopharyngeal Carcinoma Cells. *Expert Opinion on Therapeutic Targets* 2024, 28:461–471.
- [18] Li Y, Chen Y, Zhao C, Yang Y, Zhang M, Cheng H, et al.: Arenobufagin Modulation of PCSK9-Mediated Cholesterol Metabolism Induces Tumor-associated Macrophages Polarisation to Inhibit Hepatocellular Carcinoma Progression. *Phytomedicine* 2024, 128:155532.
- [19] Zhang DM, Liu JS, Deng LJ, Chen MF, Yiu A, Cao HH, et al.: Arenobufagin, a Natural Bufadienolide from Toad Venom, Induces Apoptosis and Autophagy in Human Hepatocellular Carcinoma Cells through Inhibition of PI3K/Akt/mTOR Pathway. *Carcinogenesis* 2013, 34:1331–1342.
- [20] Aran D, Camarda R, Odegaard J, Paik H, Oskotsky B, Krings G, et al.: Comprehensive Analysis of Normal Adjacent to Tumor Transcriptomes. *Nature Communications* 2017, 8:1077.
- [21] Cancer Genome Atlas Research Network. Comprehensive Molecular Characterization of Urothelial Bladder Carcinoma. *Nature* 2014, 507:315–322.
- [22] Cancer Genome Atlas Research Network. Comprehensive Molecular Portraits of Human Breast Tumours. *Nature* 2012, 490:61–70.
- [23] Gu J, Yao J, Song L, Huang D, Gao Z, Gao Q, et al.: The Mutational Landscape of the Adjacent Paracancerous Tissues Confirmed the Safe Margin of 2–5cm in Colorectal Cancer Resection. *Journal of Clinical Oncology* 2020, 38:e16060.
- [24] Donne R, Lujambio A: The Liver Cancer Immune Microenvironment: Therapeutic Implications for Hepatocellular Carcinoma. *Hepatology* 2023, 77:1773–1796.
- [25] Gires O, Pan M, Schinke H, Canis M, Baeuerle PA: Expression and Function of Epithelial Cell Adhesion Molecule EpCAM: Where are We After 40 Years? *Cancer Metastasis Reviews* 2020, 39:969–987.
- [26] Cheng Q, Ning S, Zhu L, Zhang C, Jiang S, Hao Y, et al.: NDRG1 Facilitates Self-renewal of Liver Cancer Stem Cells by Preventing EpCAM Ubiquitination. *British Journal of Cancer* 2023, 129:237–248.
- [27] Trzpis M, McLaughlin PM, de Leij LM, Harmsen MC: Epithelial Cell Adhesion Molecule: More than a Carcinoma Marker and Adhesion Molecule. *American Journal of Pathology* 2007, 171:386–395.
- [28] Schmelzer E, Reid LM: EpCAM Expression in Normal, Non-pathological Tissues. *Frontiers in Bioscience-Landmark* 2008, 13:3096–3100.
- [29] Park DJ, Sung PS, Kim JH, Lee GW, Jang JW, Jung ES, et al.: EpCAM-high Liver Cancer Stem Cells Resist Natural Killer Cell-mediated Cytotoxicity by Upregulating CEACAM1. *Journal for ImmunoTherapy of Cancer* 2020, 8:e301.
- [30] Oishi N, Yamashita T, Kaneko S: Molecular Biology of Liver Cancer Stem Cells. *Liver Cancer* 2014, 3:71–84.
- [31] Munz M, Baeuerle PA, Gires O: The Emerging Role of EpCAM in Cancer and Stem Cell Signaling. *Cancer Research* 2009, 69:5627–5629.

- [32] Maetzel D, Denzel S, Mack B, Canis M, Went P, Benk M, et al.: Nuclear Signalling by Tumour-Associated Antigen EpCAM. *Nature Cell Biology* 2009, 11:162–171.
- [33] Lu TY, Lu RM, Liao MY, Yu J, Chung CH, Kao CF, et al.: Epithelial Cell Adhesion Molecule Regulation is Associated with the Maintenance of the Undifferentiated Phenotype of Human Embryonic Stem Cells. *Journal of Biological Chemistry* 2010, 285:8719–8732.
- [34] Eyvazi S, Farajnia S, Dastmalchi S, Kanipour F, Zarredar H, Bandehpour M: Antibody Based EpCAM Targeted Therapy of Cancer, Review and Update. *Current Cancer Drug Targets* 2018, 18:857–868.
- [35] Dollé L, Theise ND, Schmelzer E, Boulter L, Gires O, van Grunsven LA: EpCAM and the Biology of Hepatic Stem/Progenitor Cells. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 2015, 308:G233–G250.
- [36] Liu Y, Wang Y, Sun S, Chen Z, Xiang S, Ding Z, et al.: Understanding the Versatile Roles and Applications of EpCAM in Cancers: from Bench to Bedside. *Experimental Hematology & Oncology* 2022, 11:97.
- [37] Deng LJ, Wang LH, Peng CK, Li YB, Huang MH, Chen MF, et al.: Fibroblast Activation Protein α Activated Tripeptide Bufadienolide Antitumor Prodrug with Reduced Cardiotoxicity. *Journal of Medicinal Chemistry* 2017, 60:5320–5333.
- [38] Yang JY, Sun B, Wei XL, Zhou YY, Wang HJ, Si N, et al.: Arenobufagin-loaded PEG-PLA Nanoparticles for Reducing Toxicity and Enhancing Cancer Therapy. *Drug Delivery* 2023, 30:2177362.