# Betulinic acid inhibits stemness and EMT of pancreatic cancer cells via activation of AMPK signaling

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**Abstract.** Cancer stem cells (CSCs), which are found in various types of human cancer, including pancreatic cancer, possess elevated metastatic potential, lead to tumor recurrence and cause chemoradiotherapy resistance. Alterations in cellular bioenergetics through the regulation of 5' adenosine monophosphate-activated protein kinase (AMPK) signaling may be a prerequisite to stemness. Betulinic acid (BA) is a well-known bioactive compound with antiretroviral and anti-inflammatory potential, which has been reported to exert anticancer effects on various types of cancer, including pancreatic cancer. The present study aimed to investigate whether BA could inhibit pancreatic CSCs via regulation of AMPK signaling. The proliferation of pancreatic cancer cells was examined by MTT and colony formation assays. The migratory and invasive abilities of pancreatic cancer cells were assessed using wound-scratch and Transwell invasion assays. In addition, the expression levels of candidate genes were measured by reverse transcription-quantitative polymerase chain reaction and western blotting. The results revealed that BA inhibited the proliferation and tumorsphere formation of pancreatic cancer cells, suppressed epithelial-mesenchymal transition (EMT), migration and invasion, and reduced the expression of three pluripotency factors [SRY-box 2 (Sox2), octamer-binding protein 4 (Oct4) and Nanog]. Furthermore, immunohistochemical analysis confirmed that there was a significant inverse association between the expression levels of phosphorylated (P)-AMPK and Sox2 in pancreatic cancer, and it was revealed that BA may activate AMPK signaling. Notably, knockdown of AMPK reversed the suppressive effects of BA on EMT and stemness of pancreatic cancer cells. In addition, BA reversed the effects of gemcitabine on stemness and enhanced the sensitivity of pancreatic cancer cells to gemcitabine. Collectively, these results indicated that BA may effectively inhibit pluripotency factor expression (Sox2, Oct4 and Nanog), EMT and the stem-like phenotype of pancreatic cancer cells via activating AMPK signaling. Therefore, BA may be considered an attractive therapeutic candidate and an effective inhibitor of the stem-like phenotype in pancreatic cancer cells. Further investigation into the development of BA as an anticancer drug is warranted.

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## Introduction

Pancreatic cancer is a highly malignant tumor of the digestive system with a 5-year survival rate of <7%; the incidence rate of pancreatic cancer is almost equal to its mortality rate (1). The majority of patients with pancreatic cancer lack apparent symptoms until it has evolved into an advanced stage. Therefore, only ~20% of patients with pancreatic cancer have the opportunity for radical resection (2). Even among those who undergo potentially curative resection, the majority of patients will eventually relapse, and the 5-year survival rate of patients who undergo a complete resection is <25% (3). Autopsy cases have revealed that ~90% of patients with pancreatic cancer also have distant metastases (4); therefore, it is imperative to

clarify the mechanism underlying its early metastasis and high recurrence rate, in order to improve therapeutic efficacy.

It has previously been reported that there is a subpopulation of cells in pancreatic ductal adenocarcinomaå(PDAC), which is the most common type of pancreatic cancer, that exhibits stem cell-like traits; these cells are termed pancreatic cancer stem cells (PCSCs), and have the capacity of self-renewal and differentiation into heterogeneous cancer cells. PCSCs serve a key role in tumor initiation, invasion, metastasis and therapeutic resistance, as well as in local recurrence following curative resection. Therefore, the elimination of PCSCs from pancreatic cancer is considered an effective strategy for the treatment of this highly refractory malignancy (5-7). Although additional molecular markers of CSCs are still being discovered, three transcription factors; namely, SRY-box 2 (Sox2), octamer-binding protein 4 (Oct4) and Nanog, have been reported as master mediators of pluripotency (8). Epithelial-mesenchymal transition (EMT) is characterized by the loss of intercellular adhesion and decreased expression of epithelial markers, including E-cadherin, and enhanced expression of mesenchymal markers, such as vimentin (9). EMT has an important role in tumor invasion, metastasis and therapeutic resistance. Notably, a direct connection has been reported between EMT and the acquirement of stem cell-like properties (10), as EMT generates neoplastic stem cells. In addition, neoplastic stem cells tend to express higher levels of the molecular markers of EMT during metastasis (10). Furthermore, non-PCSCs can be converted into PCSCs by introducing ectopic expression of zinc finger E-box binding homeobox 1, a key transcriptional factor associated with EMT (11), which facilitates cell invasion and treatment resistance to enhance the malignant phenotype (12). This further highlights the close association between the properties of CSCs and EMT.

5' adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric complex that consists of catalytic α-subunits, and regulatory β- and γ-subunits. It exerts a crucial role in regulating energy metabolism in cancer as an energy sensor (13). AMPK activation inhibits energy consumption under nutrient deprivation, leading to the suppression of protein synthesis and cellular proliferation (14). Previous studies have confirmed that AMPK signaling is also involved in regulating various pathological aspects of fibrosis, and modulates migration and invasion (15-19). Alteration of cellular bioenergetics through the regulation of AMPK signaling may be a prerequisite to stemness (20). Recently, Sengupta *et al* revealed that activation of tumor suppressor-liver kinase B1 by honokiol subsequently enhances AMPK phosphorylation, which in turn restricts the recruitment of signal transducer and activator of transcription 3 (STAT3) to the promoter regions of Sox2, Oct4 and Nanog, leading to inhibition of the stem-like phenotype in breast cancer (8). Similarly, methylisoindigo, which is a natural product of indirubin and a derivative of isoindigo, is able to kill PCSCs by modulating cell metabolism via activation of AMPK in PDAC (21). Metformin is an activator of AMPK, which also serves important roles in targeting PCSCs via regulating metabolism and microRNA expression (22,23). Although AMPK signaling is involved in the stemness of pancreatic cancer, its explicit mechanism has not been completely clarified and there is currently a lack of effective drugs that preferentially kill PCSCs via the modulation of AMPK signaling.

Betulinic acid (BA) is a natural pentacyclic triterpene purified from bark, particularly bark from *Betula sp.*, which exhibits a wide spectrum of pharmacological and biological activities. BA has been reported to exert antidepressive (24), anti-inflammatory (25,26) and anti-acquired immune deficiency syndrome (AIDS) (27,28) effects, and possesses hepatoprotective potential (29) and anticancer efficacy (30-32). It has previously been suggested that the combined use of BA and mithramycin A may effectively suppress angiogenesis, proliferation and invasion of pancreatic cancer through down-regulation of SP1 (33). A previous study further verified that lamin B1 is a novel therapeutic target in BA-treated pancreatic cancer independent of SP1 signaling (34). BA may also effectively ameliorate non-alcoholic fatty liver disease (NAFLD) via activation of AMPK and modulation of calcium/calmodulin-dependent protein kinase kinase-AMPK-mammalian target of rapamycin (mTOR)-sterol regulatory element-binding protein 1 signaling (35). However, whether BA exerts anticancer effects on pancreatic cancer and the underlying mechanism of action remain elusive. Therefore, the present study aimed to demonstrate whether BA could inhibit the EMT and stemness of pancreatic cancer cells through regulating the expression of pluripotency-induced transcription factors (i.e. Sox2, Oct4 and Nanog) via the activation of AMPK signaling. In addition, the study aimed to elucidate the contribution of BA to pancreatic cancer therapy.

**Materials and methods**

*Reagents and antibodies.* BA, gemcitabine, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), dimethyl sulfoxide (DMSO) and MTT were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). BA and AICAR were initially dissolved in dimethyl sulfoxide at stock concentrations of 50 mM and 2 M, respectively. Working concentrations for BA and AICAR were diluted immediately in culture medium prior to use. Human epidermal growth factor (EGF) and fibroblast growth factor (FGF) were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA).The antibodies used in this study were as follows: Rabbit anti-Sox2 (1:1,000 dilution; cat. no. ab97959), anti-Oct4 (1:1,000 dilution; cat. no. ab18976) and anti-Nanog (1:1,000 dilution; cat. no. ab80892) (all from Abcam, Cambridge, UK), mouse anti-β-actin (1:10,000 dilution; cat. no. 19526; KangChen Bio-Tech, Inc., Shanghai, China), mouse anti-E-cadherin (1:1,000 dilution; cat. no. sc-71008), rabbit anti-cluster of differentiation (CD)133 (1:1,000 dilution; cat. no. sc-11406), mouse anti-aldehyde dehydrogenase (ALDH)1 (1:1,000 dilution; cat. no. sc-374149) and mouse anti-epithelial cell adhesion molecule (EpCAM) (1:1,000 dilution; cat. no. sc-66020) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and rabbit anti-vimentin (1:800 dilution; cat. no. 12826), anti-AMPK (1:800 dilution; cat. no. 5831) and anti-phosphorylated (P)-AMPK (Thr172) (1:800 dilution; cat. no. 2535) (all from Cell Signaling Technology, Inc., Danvers, MA, USA).

*Cell culture.* The Mia PaCa-2 and Panc-1 human pancreatic cancer cell lines were purchased from the Type Culture

Collection of the Chinese Academy of Sciences (Shanghai, China). Mia PaCa-2 and Panc-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cells were cultured in a humidified atmosphere at 37˚C containing 5% $CO_2$.

*Cell viability assay.* Mia PaCa-2 and Panc-1 cells were seeded into 96-well plates at a density of $5x10^3$ cells/well and treated with various concentrations of BA (0, 12.5, 25, 50, 100 and 200 $\mu$M) for 24, 48 and 72 h at 37˚C. Subsequently, 10 $\mu$l MTT (Sigma-Aldrich; Merck KGaA) was added and incubated for 4 h at 37˚C. The supernatant was then replaced with 100 $\mu$l DMSO (Sigma-Aldrich; Merck KGaA) and absorbance was detected at 490 nm using a multiwell microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

*Colony formation assay.* Mia PaCa-2 and Panc-1 cells were digested, counted and seeded into 6-well plates at a density of 800 cells/well. After adherence overnight, pancreatic cancer cells were treated with BA (50 $\mu$M), gemcitabine (5 $\mu$M), or gemcitabine (5 $\mu$M) combined with BA (50 $\mu$M) for 24 h at 37˚C, after which the medium was replaced with drug-free medium containing 10% FBS. The cells were then allowed to grow for 2 weeks to form colonies. After 2 weeks, the colonies were washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature and stained for 10 min at room temperature with 0.1% crystal violet solution, followed by rinsing and imaging. The number of colonies with a diameter >0.5 mm was counted under a microscope (Nikon Eclipse Ti-S; Nikon Corporation, Tokyo, Japan).

*Apoptosis assay.* The apoptosis of pancreatic cancer cells was assessed using flow cytometry with an Annexin V-fluorescein isothiocyanate (FITC)/7-aminoactinomycin D (7-AAD) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. The preprocessing of pancreatic cancer cells was performed as previously described (36). Cells treated with gemcitabine (5 $\mu$M), BA (50 $\mu$M), or gemcitabine (5 $\mu$M) combined with BA (50 $\mu$M) for 24 h at 37˚C were washed twice in cold PBS and resuspended in 1X binding buffer (BD Biosciences) at a density of $1x10^6$ cells/ml. Cell suspensions ($2.5x10^5$ cells) were added to 1.5 ml Eppendorf tubes, to which 5 $\mu$l allophycocyanin Annexin V and 7-AAD were added, and were gently vortexed. Subsequently, cells were incubated at room temperature for 15 min in the dark, and the percentage of apoptotic cells was quantified by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using Winmdi2.9 software (The Scripps Research Institute, San Diego, CA, USA). The total apoptotic rate was assessed by adding the rate of Annexin V-FITC+/7-AAD- cells (early apoptotic cells) and Annexin V-FITC+/7-AAD+ cells (late apoptotic cells) together.

*Tumorsphere formation assay.* Following treatment with BA (50 $\mu$M) or AICAR (2 mM), or with BA (50 $\mu$M) combined

with siRNA to knockdown AMPK or siControl for 24 h at 37˚C, pancreatic cancer cells were digested, counted and seeded in 6-well ultra-low attachment plates (Corning Incorporated, Corning, NY, USA) at a density of 5,000 cells/well in serum-free DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 20 ng/ml human FGF, 20 ng/ml human EGF and 1% B27 (Gibco; Thermo Fisher Scientific, Inc.). Cells were subsequently cultured at 37˚C in an atmosphere containing 5% $CO_2$ for 1 week to form tumorspheres. After 1 week, tumorsphere formation was counted and recorded under a light microscope (Nikon Corporation) at a magnification of x200.

*Wound-scratch assay.* The wound-scratch assay was conducted to examine the migratory capacity of pancreatic cancer cells. Briefly, once Mia PaCa-2 and Panc-1 cells were cultured to 90-100% confluence, a 10-$\mu$l pipette tip was used to generate a wound in the surface of the cells in a 6-well plate, and then the cells were treated with or without BA (50 $\mu$M) for 48 h at 37˚C. Images of the same fields at the indicated time-points (0 and 48 h) were captured under a light microscope (Nikon Corporation) at a magnification of x100.

*Transwell invasion assay.* Transwell chamber (EMD Millipore, Billerica, MA, USA) assays were performed to assess the invasive ability of pancreatic cancer cells, in accordance with a previously described protocol (37). Briefly, Mia PaCa-2 and Panc-1 cells were serum-starved for 6-8 h and were pretreated with BA (50 $\mu$M) or AICAR (2 mM) for 24 h at 37˚C. In addition, the upper chamber was coated with Matrigel (Sigma-Aldrich; Merck KGaA) and was incubated at 37˚C in an atmosphere containing 5% $CO_2$ for 5 h. Subsequently, Mia PaCa-2 and Panc-1 cells ($1x10^5$) were digested, resuspended in serum-free medium and seeded into the upper chamber. The pancreatic cancer cells were allowed to invade into the lower chamber, which contained medium supplemented with 10% FBS, for 24 h at 37˚C. The non-invading cells on the upper side were scraped off using a cotton swab, and the membrane was then fixed with 4% paraformaldehyde for 30 min at room temperature and stained for 10 min at room temperature with 0.1% crystal violet. Subsequently, the number of cells on each membrane was counted in 10 random fields and images were captured under a light microscope (Nikon Corporation) at x200 magnification. The values reported are the mean of triplicate experiments.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).* Following treatment with or without BA (50 $\mu$M), with various concentrations of gemcitabine (0, 1 and 5 $\mu$M), or with gemcitabine (5 $\mu$M) combined with BA (50 $\mu$M) for 24 h at 37˚C, total RNA was extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The isolated total RNA was then reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. An iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a SYBR Green PCR kit (Takara Biotechnology Co., Ltd.) were used to conduct RT-qPCR, according to the manufacturers' protocols. The RT-qPCR

experimental steps were conducted as previously described (38). The specificity of the amplified PCR products was evaluated by melting curve analysis, and the comparative Cq method was used to determine the expression levels of each target gene, with GAPDH as a normalization control, as previously described (39). The primer sequences used for RT-qPCR were as follows: Sox-2, forward 5'-GCCGAGTGGAAACTT TTGTCG-3',reverse 5'-GGCAGCGTGTACTTATCCTTCT-3'; Nanog, forward 5'-TTTGTGGGCCTGAATAAGCAG-3', reverse 5'-AGGGCTGTCCTGAATAAGCAG-3'; Oct4, forward 5'-CTGGGTTGATCCTCGGACCT-3', reverse 5'-CCATCGG AGTTGCTCTCCA-3'; CD133, forward 5'-TACAACGCCAA ACCACGACTGT-3', reverse 5'-TCTGAACCAATGGAATTC AAGACCCTTT-3'; ALDH1, forward 5'-TGTTAGCTGATGC CGACTTG-3', reverse 5'-CTTCTTAGCCCGCTCAACAC-3'; EpCAM, forward 5'-ATGTTTGGT GATGAAGGCAGAA-3', reverse 5'-ATCGCAGT CAGGATCATAAAGC-3'; and β-actin, forward 5'-ATCGTGCGTGACATTAAGGAGAAG-3' and reverse 5'-AGGAAGAAGGCTGGAAGAGTG-3'.

*Immunofluorescence staining.* Following treatment with or without BA (50 $\mu$M) for 24 h at 37˚C, pancreatic cancer cells (1x10$^6$) were washed with PBS and fixed in 4% paraformalde-hyde for 30 min at room temperature. Fixed cells were washed again with PBS and permeabilized in 0.5% Triton X-100 diluted in PBS for 10 min; cells on the slides were subse-quently blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology, Guangzhou, China) for 1 h at room temperature and were then incubated with a Sox2 antibody (1:150 dilution) at 4˚C overnight. Following incubation with a secondary antibody (1:150 dilution; cat. no. 913921; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature, the nuclei of the cells were stained with DAPI and the cells were sealed on glass slides. A confocal microscope (Zeiss AG, Oberkochen, Germany) was used to capture images and record the cells at a wavelength of 488 nm and a magnification of x400.

*Western blotting.* Following treatment with various concentra-tions of BA (0, 25 and 50 $\mu$M), with BA (50 $\mu$M) for various durations (0, 5, 15, 30, 60 and 120 min), various concentra-tions of AICAR (0 and 2 mM), various concentrations of gemcitabine (0, 1 and 5 $\mu$M), or with BA (50 $\mu$M) combined with siRNA to knockdown AMPK or siControl, or with gemcitabine (5 $\mu$M) combined with BA (50 $\mu$M) for 24 h at 37˚C, total proteins were extracted from Mia PaCa-2 and Panc-1 cells (1x10$^6$) grown under experimental conditions using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine protein concentrations, according to the manufac-turer's protocol. Western blotting was conducted as previously described (40). Briefly, proteins (150 $\mu$g) were separated by 10% SDS-PAGE and were transferred onto polyvinylidene difluoride membranes. Subsequently, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween (TBST) and were incubated with primary antibodies overnight at 4˚C. After three washes in TBST (10 min/wash), the membranes were incubated with goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase

(HRP) (1:10,000 dilution; cat. no. ab6721; Abcam) or goat anti-mouse IgG-HRP (1:10,000 dilution; cat. no. ab6789; Abcam) secondary antibodies for 1 h at room temperature, and were washed again. The immunoreactive bands were visualized using a chemiluminescence detection system (EMD Millipore) through the peroxidase reaction, and images of the bands were recorded using the ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc.). β-actin was used as an internal loading control.

*Immunohistochemical analysis.* The present study was approved by the Ethical Committee of The First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China), and written informed consent was obtained from the patients. A total of 72 pancreatic cancer tissues and eight normal pancreatic samples were collected from the Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University. The details of these samples were provided in our previous study (41). The tissue samples were fixed in 10% paraformaldehyde and were embedded in paraffin for further immunohistochemical analysis, as previ-ously described (42). Briefly, pancreatic tissues were cut into 5-$\mu$m sections on glass slides, and the sections were deparaf-finized and rehydrated, after which, the sections underwent antigen retrieval and endogenous enzyme blocking. The sections were then incubated with primary antibodies (anti-P-AMPK and anti-Sox2; 1:150 dilution) overnight at 4˚C, followed by incubation with streptavidin peroxidase (Dako LSAB + HRP kit; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 min at room temperature. The sections were incubated with DAB for 5 min followed by counterstaining with hematoxylin for 2 min at room temperature. Finally, the sections were observed under a light microscope (Nikon Corporation).

*Gene silencing by small interfering (si)RNA.* Loss-of-function analysis was conducted using siRNA to knockdown AMPK, which was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of siRNA and corre-sponding siControl are provided in our previous study (41). Each siRNA (100 nM) was mixed with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as a carrier and were transfected into pancreatic cancer cells (1x10$^6$) for 10 h at 37˚C, according to the manufacturer's protocol. The efficacy of AMPK knockdown was validated by western blot analysis. A total of 48 h post-transfection, these cells underwent further experimentation (Transwell invasion assay and tumorsphere formation assay).

*Statistical analysis.* Data shown are representative of three independent experiments, and for each experiment, at least three samples were analyzed for each treatment group. Data are presented as the means ± standard deviation. Differences were evaluated using Student's t-test, or one-way analysis of variance for multiple comparisons with the Student-Newman-Keuls method as a post hoc test. Statistical analysis of the human tissue data was performed using Pearson's $\chi^2$ test. All statistical analyses were performed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.
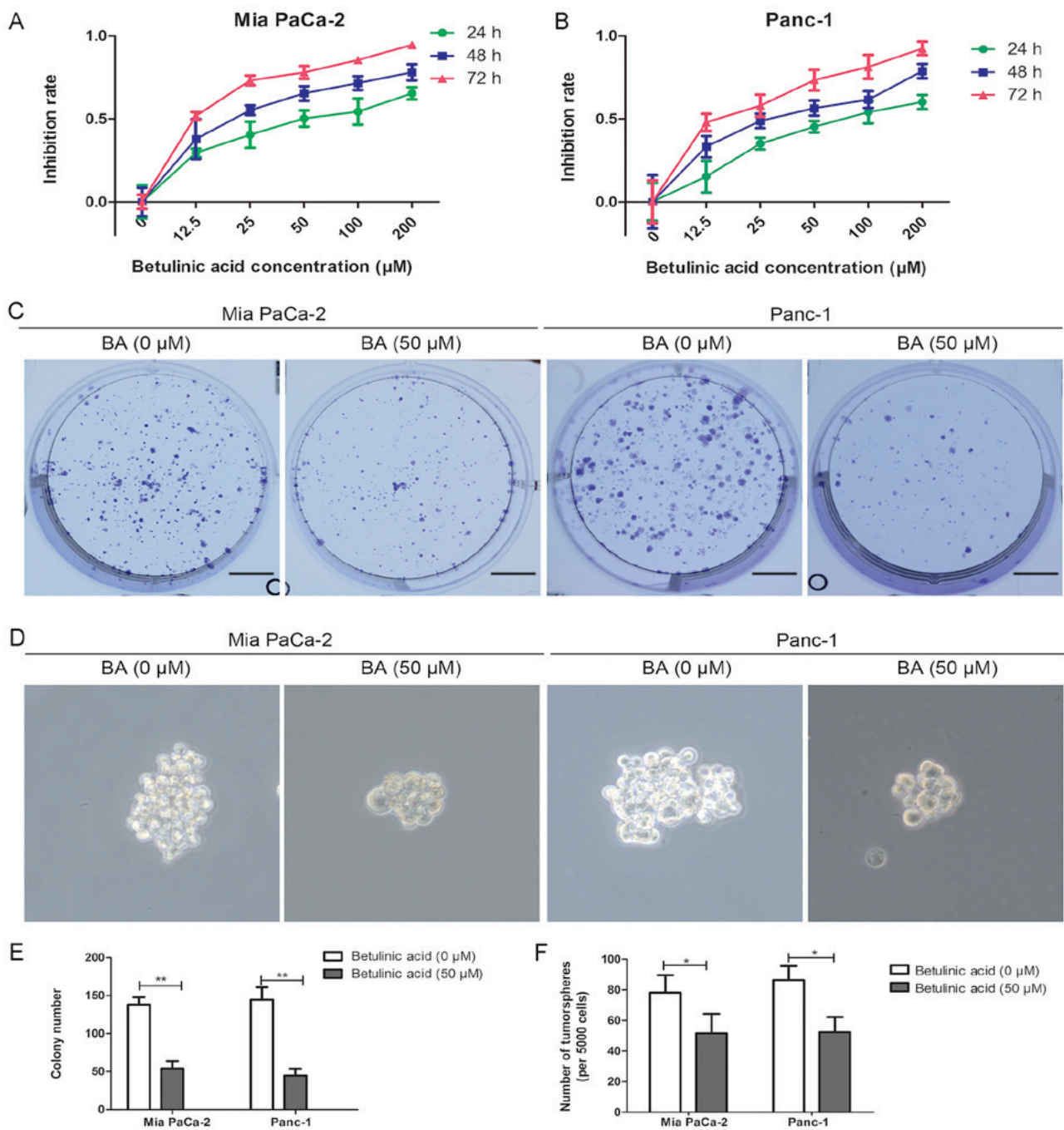
Figure 1. Effects of BA on the proliferation and tumorsphere formation of pancreatic cancer cells. (A and B) Mia PaCa-2 and Panc-1 cells were incubated with a series of BA concentrations (0, 12.5, 25, 50, 100 and 200 $\mu$M) for 24, 48 and 72 h, and cell viability was evaluated by MTT assay. (C and E) Effects of BA on the colony-forming ability of Mia PaCa-2 and Panc-1 cells were assessed by colony formation assay. Images are representative of three independent experiments, and the colony number was counted and plotted. Scale bar, 1 cm. (D and F) Tumorsphere formation assay of Mia PaCa-2 and Panc-1 cells treated with or without 50 $\mu$M BA. The number of tumorspheres was counted and plotted. Magnification, x200. $^{*}$P<0.05, $^{**}$P<0.01. BA, betulinic acid.

## Results

*BA inhibits the proliferation and tumorsphere formation of pancreatic cancer cells*. To determine whether BA could suppress the viability of pancreatic cancer cells, the MTT assay was used. Briefly, Mia PaCa-2 and Panc-1 cells were treated with a series of gradually increasing concentrations of BA [0 (control), 12.5, 25, 50, 100 and 200 $\mu$M] for 24, 48 and 72 h, and absorbance was measured at 490 nm at the designated time-points to analyze cell viability. The results indicated that

BA impaired the growth of Mia PaCa-2 and Panc-1 cells in time- and dose-dependent manners (Fig. 1A and B). The half maximal inhibitory concentration of BA was ~50 $\mu$M for both Panc-1 and Mia PaCa-2 cells, and it exerted no cytotoxic effects on a pancreatic duct epithelial cell line (data not shown). This concentration of BA was in accordance with previously used dosages (34); therefore, 50 $\mu$M BA was used for subsequent experiments. The present study subsequently examined the effects of BA on colony formation and tumorsphere formation in Mia PaCa-2 and Panc-1 cells (Fig. 1C-F). As shown in Fig. 1C and E, compared
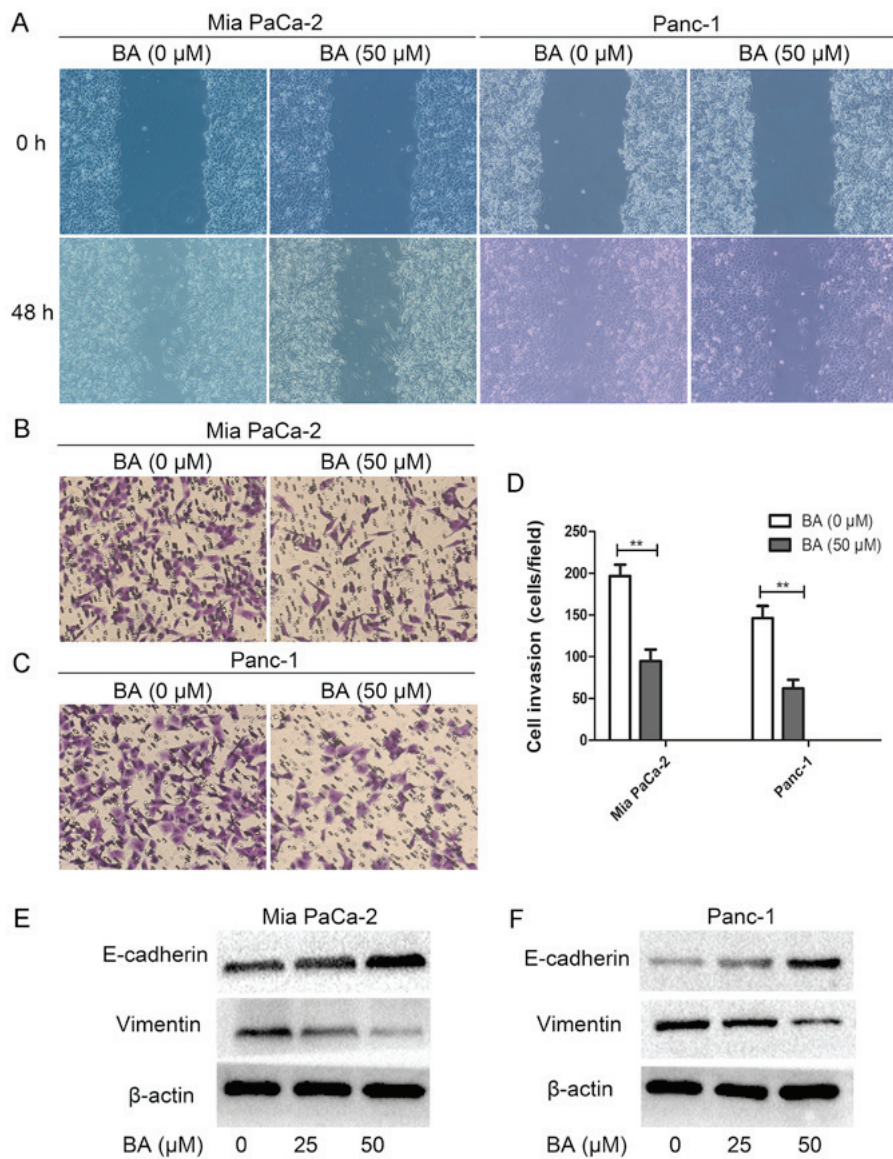
Figure 2. Effects of BA on the migration and invasion of pancreatic cancer cells. (A) Wound-scratch assays were conducted on Mia PaCa-2 and Panc-1 cells pretreated with or without 50 $\mu$M BA. Images were visualized at 0 and 48 h. Magnification, x100. (B-D) Effects of BA on the invasive ability of Mia PaCa-2 and Panc-1 cells were evaluated by Matrigel invasion assay. Images are representative of three independent experiments, and the invasive cells were counted and plotted. Magnification, x200. **P<0.01. (E and F) Mia PaCa-2 and Panc-1 cells were pretreated with BA (0, 25 and 50 $\mu$M) for 48 h, and western blot analysis was performed to assess the expression of epithelial-mesenchymal transition markers (E-cadherin and vimentin). BA, betulinic acid.

with in untreated control cells, the number of colonies in cells treated with 50 $\mu$M BA was markedly decreased. Since cancer stemness is associated with mechanisms underlying cancer metastasis and occurrence, the present study further explored the role of BA in the stemness of pancreatic cancer. As shown in Fig. 1D and F, tumorsphere formation was decreased in Mia PaCa-2 and Panc-1 cells upon BA treatment compared with in untreated cancer cells.

*BA suppresses the migration and invasion of pancreatic cancer cells via inhibiting EMT.* It has previously been reported that the EMT process endows cancer cells with an increased self-renewal capability and mesenchymal phenotype, which is necessary for tumor metastasis (43). Therefore the present study examined the effects of BA on EMT, migration and invasion of pancreatic cancer cells. Notably, BA-treated Mia PaCa-2 and Panc-1 cells exhibited reduced

migration and invasion in vitro compared with untreated cells (Fig. 2A-D). In addition, the results of western blotting suggested that the epithelial marker E-cadherin was elevated, whereas the mesenchymal marker vimentin was decreased in BA-treated Mia PaCa-2 and Panc-1 cells compared with the control (Fig. 2E and F). Collectively, these data indicated that BA may inhibit the migration and invasion of pancreatic cancer cells through targeting EMT.

*BA inhibits the stemness of pancreatic cancer cells and activates AMPK signaling.* Although the number of known molecular markers of the cancer stem-like phenotype is still increasing, three transcription factors; namely Sox2, Oct4 and Nanog, have been strongly validated as master regulators in the maintenance of the cancer stem-like phenotype (8). The present study examined whether BA treatment affects the expression of these master mediators of pluripotency
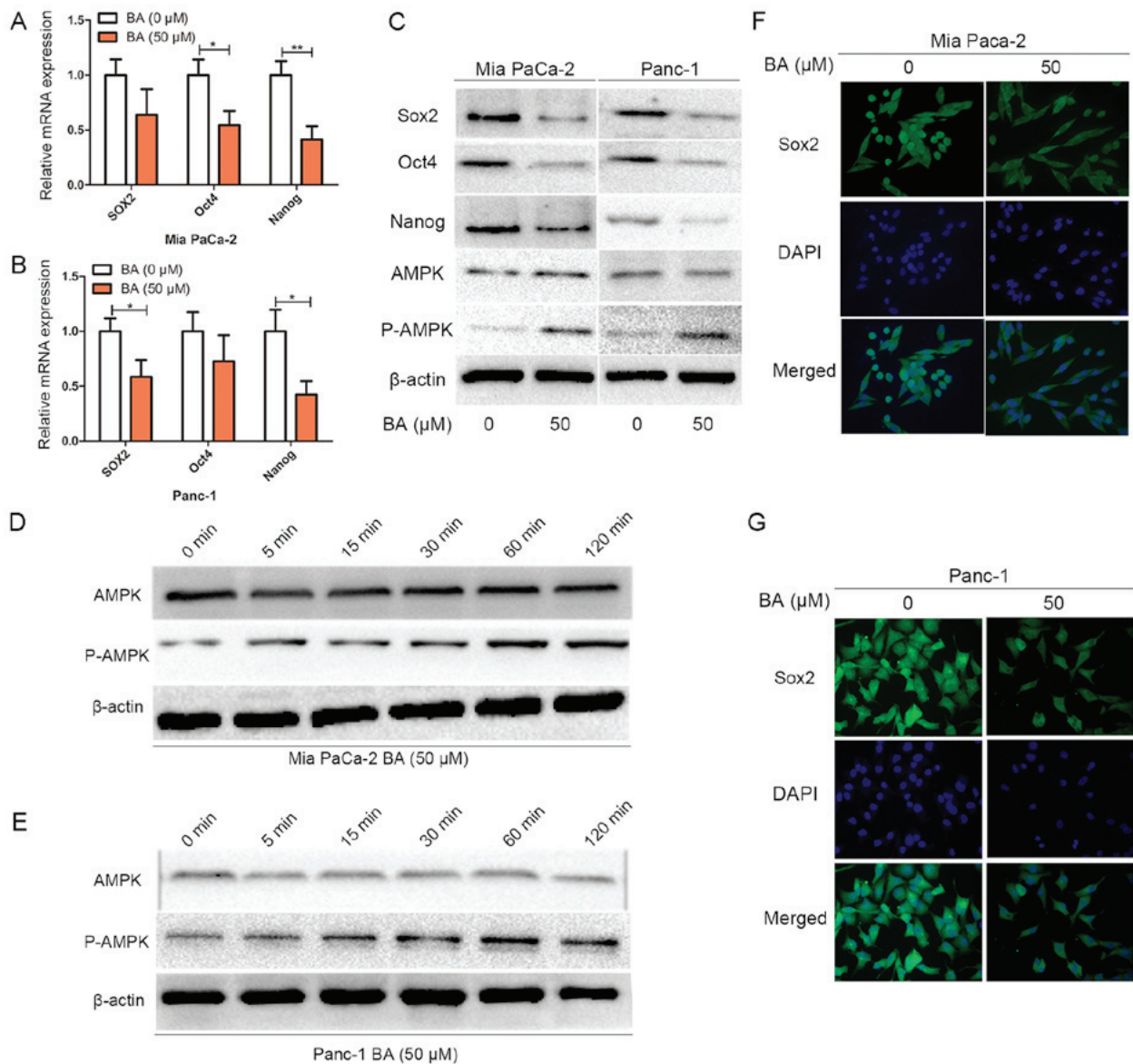
Figure 3. BA inhibits the stemness of pancreatic cancer cells and activates AMPK signaling. (A and B) Mia PaCa-2 and Panc-1 cells were pretreated with BA for 24 h, total RNA was extracted and reverse transcription-quantitative polymerase chain reaction was conducted to detect the expression levels of Sox2, Oct4 and Nanog. *P<0.05 and **P<0.01. (C) Mia PaCa-2 and Panc-1 cells were pretreated with or without 50 µM BA for 48 h, and western blot analysis was performed to assess the protein expression levels of master pluripotency regulators (Sox2, Oct4 and Nanog), total AMPK and P-AMPK. (D and E) Mia PaCa-2 and Panc-1 cells were treated with 50 µM BA at various time-points (0, 5, 15, 30, 60 and 120 min), and total protein was extracted to detect the expression levels of P-AMPK by western blotting. (F and G) Mia PaCa-2 and Panc-1 cells were pretreated with BA for 24 h, and immunofluorescence analysis was conducted to assess the expression and nuclear localization of Sox2 in Mia PaCa-2 and Panc-1 cells. Magnification, x400. AMPK, 5' adenosine monophosphate-activated protein kinase; BA, betulinic acid; Oct4, octamer-binding protein 4; P, phosphorylated; Sox2, SRY-box 2.

(Fig. 3). RT-qPCR analysis revealed that BA treatment suppressed the mRNA expression levels of Sox2, Oct4 and Nanog (Fig. 3A and B). In addition, as shown in Fig. 3C, western blotting indicated that compared with the group without BA treatment, treatment of Mia PaCa-2 and Panc-1 cells with 50 µM BA downregulated the expression levels of Sox2, Oct4 and Nanog, and upregulated P-AMPK expression. The suppressive effects of BA on other stemness markers (CD133, ALDH1 and EpCAM) were similar to those on the aforementioned three master regulators (data not shown). The findings of immunofluorescence analysis also confirmed that BA could suppress the expression and nuclear localization of Sox2 in both pancreatic cancer cell lines (Fig. 3F and G). Notably, AMPK signaling has an important influence on the

cancer stem-like phenotype (20,44). Therefore, to investigate whether BA could activate AMPK signaling, Mia PaCa-2 and Panc-1 cells were treated with BA at various time-points (0, 15, 30, 60, 90 and 120 min), and total protein was extracted a to detect the expression levels of P-AMPK by western blotting. The results revealed that BA treatment enhanced the expression of P-AMPK in a time-dependent manner (Fig. 3D and E). Collectively, these data indicated that BA treatment downregulated the expression of Sox2, Oct4 and Nanog, and activated AMPK signaling in pancreatic cancer cells.

*AMPK activation by AICAR exhibits similar effects to BA on EMT and stemness of pancreatic cancer cells.* To uncover the potential association between AMPK signaling
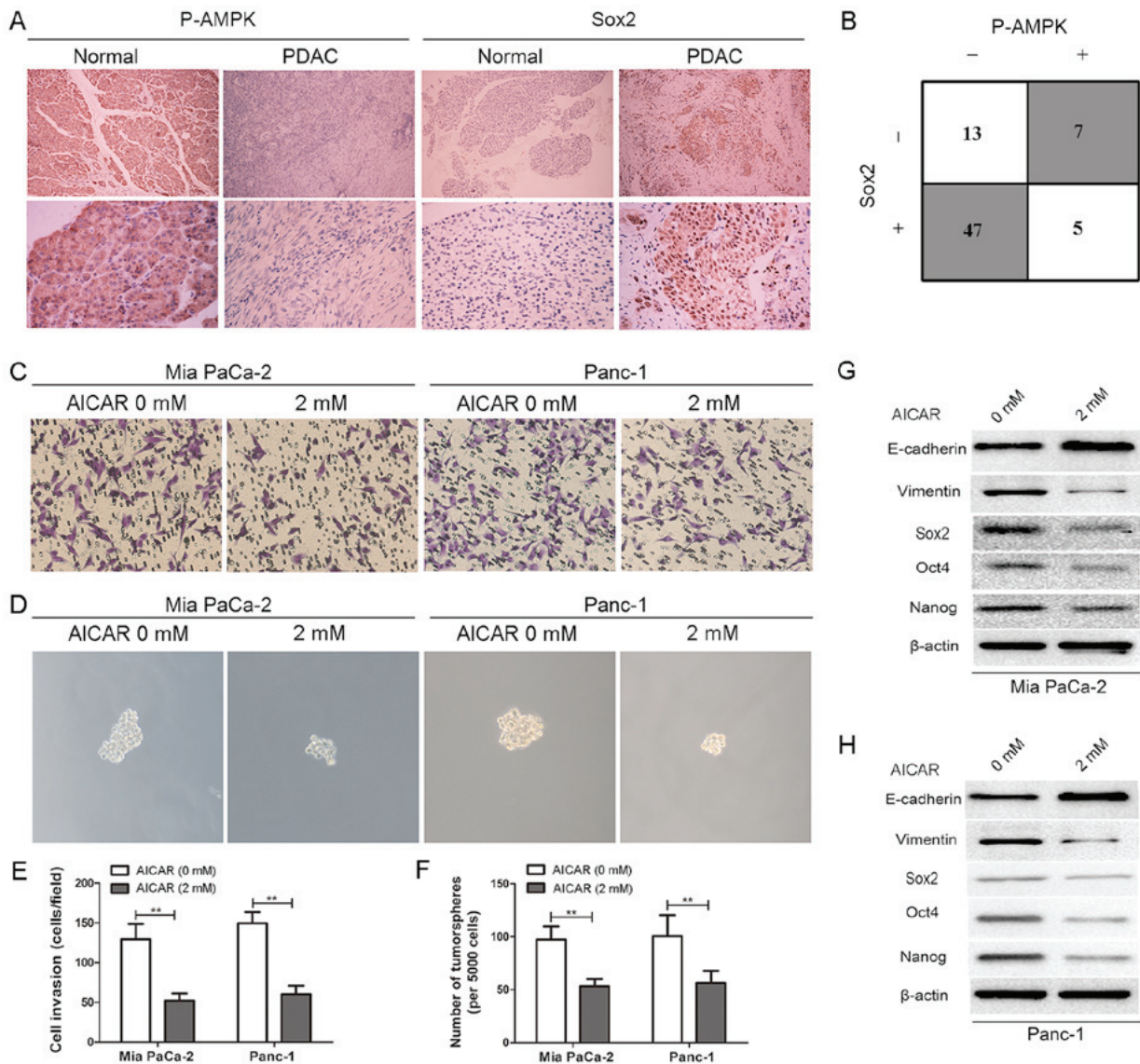
Figure 4. AMPK activation by AICAR exerts similar effects to betulinic acid on EMT and stemness of pancreatic cancer cells. (A) Representative images of immunohistochemical staining of P-AMPK and SOX2 in normal pancreatic tissues and pancreatic cancer tissues. Magnification, x100 for the upper images and x400 for the lower images. (B) An inverse association between P-AMPK and SOX2 expression was detected in pancreatic cancer tissues. P<0.05 by two-tailed $\chi^2$ test. (C and E) Effects of AMPK activation by AICAR (2 mM) on the invasive ability of Mia PaCa-2 and Panc-1 cells were evaluated by Matrigel invasion assay. Images are representative of three independent experiments, and the invasive cells were counted and plotted. Magnification, x200. **P<0.01. (D and F) Tumorsphere formation assay of Mia PaCa-2 and Panc-1 cells treated with or without 2 mM AICAR. The number of tumorspheres was counted and plotted. Magnification, x200. **P<0.01. (G and H) Mia PaCa-2 and Panc-1 cells were pretreated with or without 2 mM AICAR for 48 h, and western blot analysis was performed to assess the expression levels of master pluripotency regulators (Sox2, Oct4, and Nanog) and EMT markers (E-cadherin and vimentin). AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; AMPK, 5' adenosine monophosphate-activated protein kinase; EMT, epithelial-mesenchymal transition; Oct4, octamer-binding protein 4; P, phosphorylated; PDAC, pancreatic ductal adenocarcinoma; Sox2, SRY-box 2.

and cancer stemness, immunohistochemical analysis was conducted to detect the expression levels of P-AMPK and Sox2 in normal pancreatic tissues and pancreatic cancer tissues. Immunohistochemical analysis revealed that, compared with in normal pancreatic tissues, the expression levels of P-AMPK were decreased in pancreatic cancer tissues. Conversely, the expression levels of Sox2 were markedly elevated (Fig. 4A). Furthermore, Sox2 expression was detected in 78.3% (47/60) of pancreatic cancer tissues with negative P-AMPK expression (Fig. 4B), thus suggesting an inverse relationship between P-AMPK signaling and the cancer stem-like phenotype, and indicating that downregulation of P-AMPK may lead to increased cancer stemness.

In order to further clarify the effects of AMPK signaling on the maintenance of pancreatic cancer cell stemness and to determine its role in EMT, an AMPK activator, AICAR (2 mM), was used to treat Mia PaCa-2 and Panc-1 cells for 24 h. The results suggested that AMPK activation by AICAR effectively inhibited the invasion and tumorsphere formation of Mia PaCa-2 and Panc-1 cells compared with the vehicle control (Fig. 4C-F). Furthermore, the results of western blotting indicated that the expression levels of the epithelial marker E-cadherin were elevated, whereas those of the mesenchymal marker vimentin were decreased in AICAR-treated Mia PaCa-2 and Panc-1 cells compared with the control. In addition, the expression levels of the
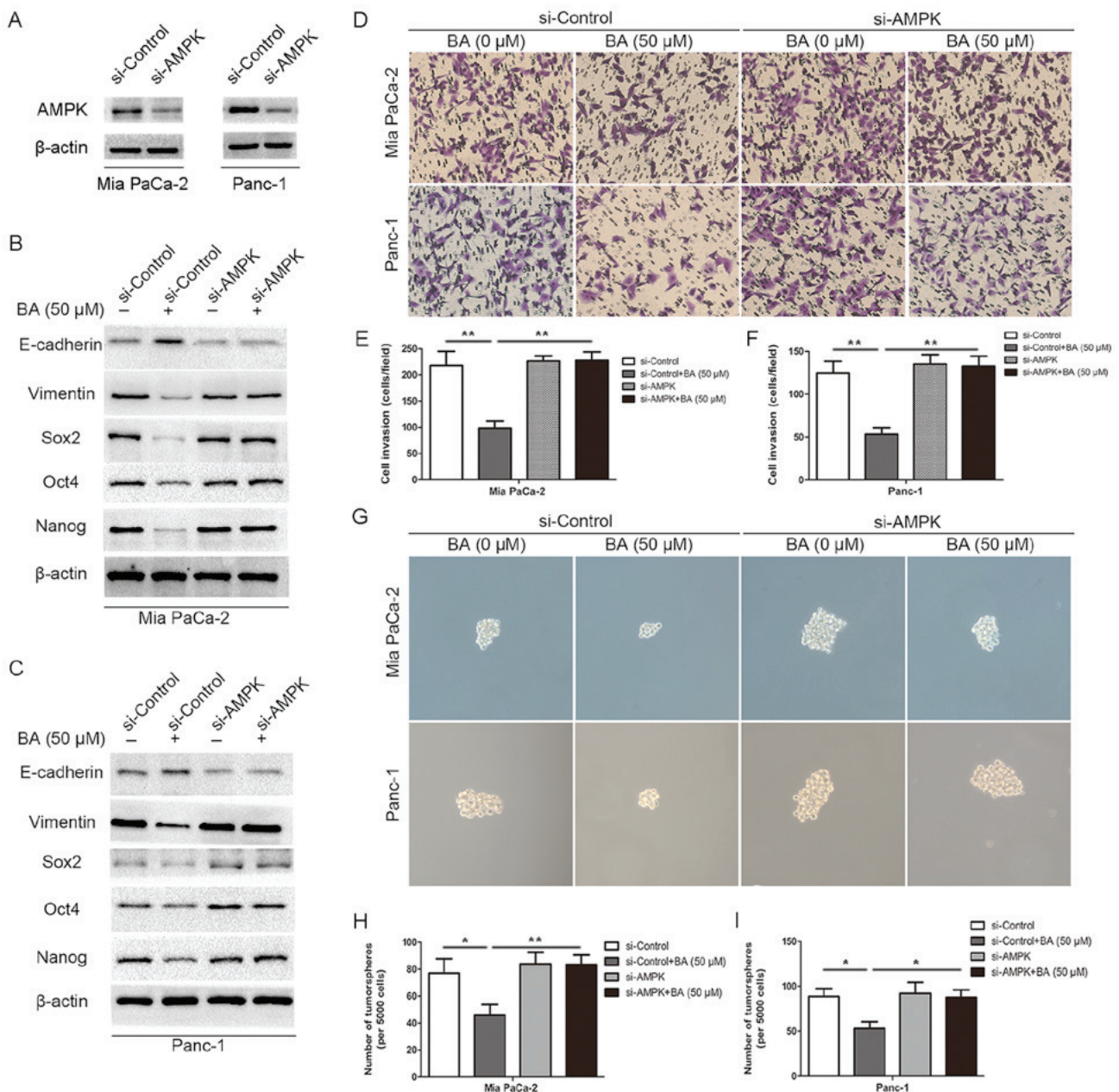
Figure 5. Knockdown of AMPK rescues BA-induced suppression of EMT and stemness in pancreatic cancer cells. (A) Western blotting confirmed the successful silencing of AMPK in Mia PaCa-2 and Panc-1 cells. β-actin was used as an internal loading control. (B and C) Western blot analysis suggested that silencing AMPK by siRNA reversed BA-induced inhibition of the expression of master pluripotency regulators (Sox2, Oct4 and Nanog) and EMT markers (E-cadherin and vimentin) in Mia PaCa-2 and Panc-1 cells. β-actin was used as an internal loading control. (D-F) Matrigel invasion assay revealed that silencing AMPK by siRNA reversed BA-induced suppression of the invasion of Mia PaCa-2 and Panc-1 cells. Images are representative of three independent experiments, and the invasive cells were counted and plotted. Magnification, x200. **P<0.01. (G-I) Tumorsphere formation assay exhibited that knocking down AMPK by siRNA reversed BA-induced inhibition of tumorsphere formation in Mia PaCa-2 and Panc-1 cells. The number of tumorspheres was counted and plotted. Magnification, x200. *P<0.05, **P<0.01. AMPK, 5' adenosine monophosphate-activated protein kinase; BA, betulinic acid; EMT, epithelial-mesenchymal transition; Oct4, octamer-binding protein 4; si/siRNA, small interfering RNA; Sox2, SRY-box 2.

master pluripotency inducers (Sox2, Oct4 and Nanog) were decreased (Fig. 4G and H). Similar effects were observed on other stemness markers (CD133, ALDH1 and EpCAM; data not shown). These data indicated that AMPK activation by AICAR may exert similar effects to BA on EMT and stemness of pancreatic cancer cells.

*Knockdown of AMPK rescues BA-induced suppression of EMT and stemness in pancreatic cancer cells.* In order to test the hypothesis that BA inhibits stemness and EMT of pancreatic

cancer through activating AMPK signaling, AMPK-specific siRNA (si-AMPK) was used to silence AMPK expression in pancreatic cancer cells in conjunction with BA treatment. Successful silencing of AMPK expression was confirmed in Mia PaCa-2 and Panc-1 cells by western blotting (Fig. 5A). Subsequently, AMPK-depleted and control pancreatic cancer cells (Mia PaCa-2-siAMPK, Mia PaCa-2-siControl, Panc-1-siAMPK and Panc-1-siControl) were treated with BA and were subjected to Transwell invasion assay, tumorsphere formation assay and western blot analysis (Fig. 5). BA could
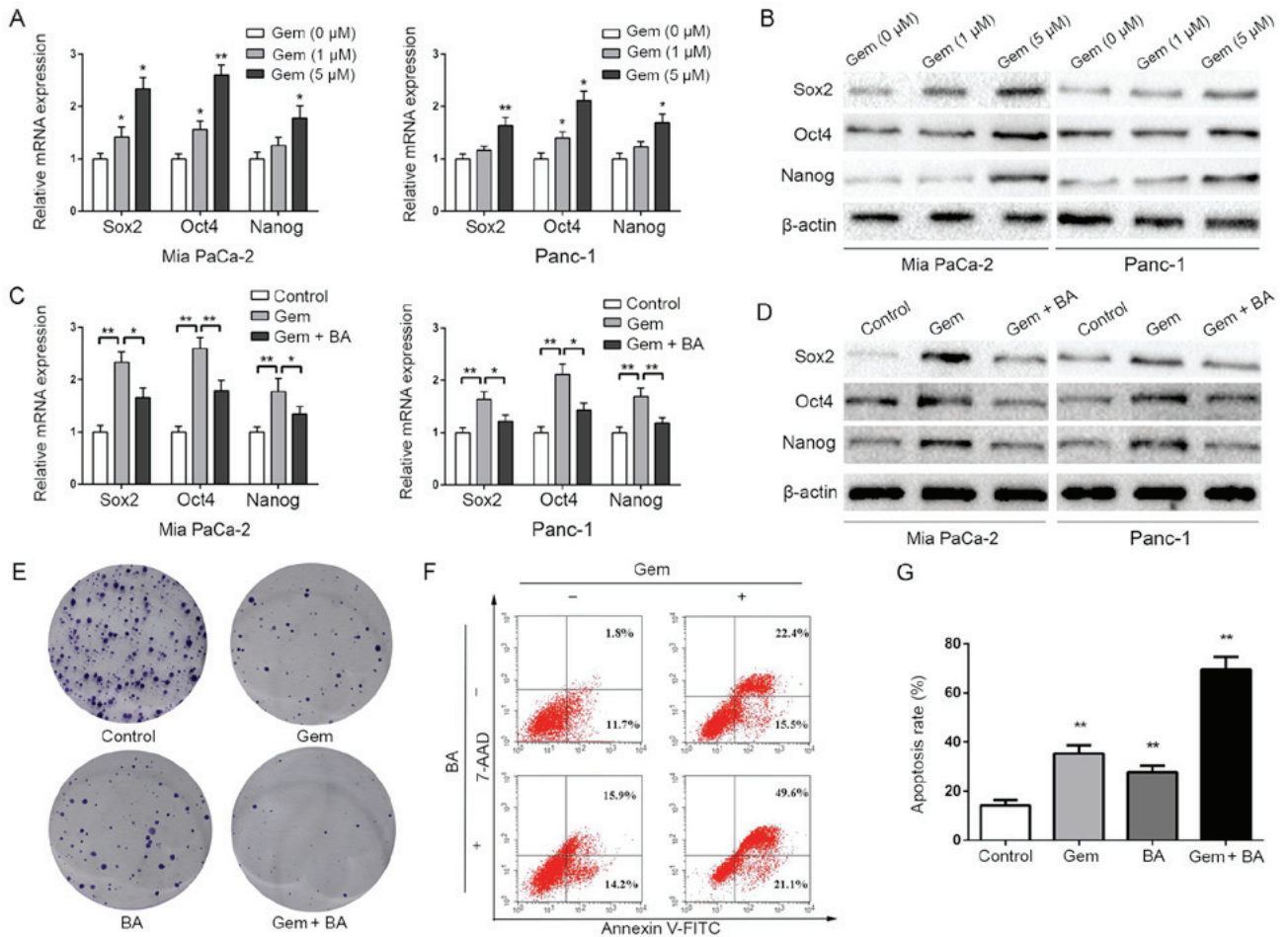
Figure 6. BA reverses gemcitabine-induced stemness and enhances the sensitivity of pancreatic cancer cells to gemcitabine. (A) Mia PaCa-2 and Panc-1 cells were pretreated with various concentrations of gemcitabine (0, 1 and 5 μM) for 24 h, total RNA was extracted and RT-qPCR was conducted to detect the expression levels of Sox2, Oct4 and Nanog. *P<0.05 and **P<0.01, compared with the control group. (B) Mia PaCa-2 and Panc-1 cells were pretreated with various concentrations of gemcitabine (0, 1 and 5 μM) for 24 h, and western blot analysis was performed to assess the expression levels of master pluripotency regulators (Sox2, Oct4 and Nanog). (C) Effects of BA (50 μM) on gemcitabine (5 μM) treatment-induced stemness were measured by RT-qPCR analysis. *P<0.05 and **P<0.01. (D) Effects of BA (50 μM) on gemcitabine (5 μM) treatment-induced stemness were measured by western blot analysis. (E) Effects of BA (50 μM) combined with gemcitabine (5 μM) on the colony-forming ability of Panc-1 cells. (F and G) Effects of BA (50 μM) combined with gemcitabine (5 μM) on the apoptosis of Panc-1 cells. **P<0.01, compared with the control group. 7-AAD, 7-aminoactinomycin D; BA, betulinic acid; FITC, fluorescein isothiocyanate; Oct4, octamer-binding protein 4; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Sox2, SRY-box 2.

effectively inhibit invasion (Fig. 5D-F) and tumorsphere formation (Fig. 5G-I) in Mia PaCa-2-siControl and Panc-1-siControl cells. Conversely, Mia PaCa-2-siAMPK and Panc-1-siAMPK cells exhibited a slightly higher capacity for invasion and tumorsphere formation, which was not suppressed by BA treatment. Furthermore, this study analyzed whether BA could abrogate the expression of master pluripotency inducers (Sox2, Oct4 and Nanog) and EMT markers (E-cadherin and vimentin) in the absence of AMPK. As shown in Fig. 5B and C, BA treatment not only suppressed the expression of Sox2, Oct4, Nanog and vimentin, but also increased the expression of E-cadherin in Mia PaCa-2-siControl and Panc-1-siControl cells; however, no alterations were detected in Mia PaCa-2-siAMPK and Panc-1-siAMPK cells. Similar results were also determined with regards to other stemness markers (CD133, ALDH1 and EpCAM; data not shown). Collectively, these results indicated that BA may inhibit invasion and tumorsphere formation of pancreatic cancer cells, and regulate the expression levels of Sox2, Oct4, Nanog and EMT markers in an AMPK-dependent manner.

*BA reverses stemness induced by gemcitabine and enhances the sensitivity of pancreatic cancer cells to gemcitabine.* Gemcitabine is the major chemotherapeutic agent currently used in the treatment of pancreatic cancer; however, chemoresistance is a serious issue that markedly affects the prognosis of patients. A previous study demonstrated that gemcitabine treatment induces stemness in pancreatic cancer cells (45), which may serve a critical role in resistance to gemcitabine. The present results revealed that gemcitabine treatment dose-dependently increased the expression levels of CSC markers, including Sox2, Oct4 and Nanog (Fig. 6A and B). Similar effects were observed on other stemness markers (CD133, ALDH1 and EpCAM; data not shown). Since BA markedly inhibited the stemness of cancer cells, this study further investigated whether it could attenuate gemcitabine-induced stemness and facilitate the antitumor effects of gemcitabine. Notably, BA reversed gemcitabine-induced stemness, as revealed by decreased Sox2, Oct4 and Nanog expression (Fig. 6C and D). Since Panc-1 cells are resistant to gemcitabine (46), Panc-1 cells were selected for further experiments. Notably, BA

combined with gemcitabine was more effective at suppressing proliferation (Fig. 6E) and inducing apoptosis (Fig. 6F and G) of Panc-1 cells. Taken together, these data suggested that BA may be considered a sensitizer in gemcitabine treatment.

## Discussion

The tumor biology of PDAC is conducive to early metastasis and recurrence, and contributes to chemoradiotherapy resistance (47). It has previously been reported that several tumor types, including pancreatic cancer, exhibit a minority of cells that display a stem-like phenotype; these cells have an elevated metastatic potential, thus contributing to tumor recurrence and chemoresistance (48). The present study indicated a vital role for PCSCs in metastasis and recurrence of pancreatic cancer, and suggested that targeting PCSCs may be a promising strategy for the treatment of this refractory malignancy. In addition, EMT triggers cancer metastasis, which enhances the invasion of cancer cells and impels them to disseminate into secondary tissue sites, forming micrometastatic lesions. Furthermore, the EMT process confers on these cancer cells acquired stem-like traits for self-renewal, with an enhanced proliferative capacity and an ability to form macroscopic metastases from micrometastatic lesions (43,49). Therefore, there is a direct link between the EMT process and CSCs. Notably, the present results revealed that BA effectively inhibited EMT and stemness of pancreatic cancer cells, thus suggesting that BA may be an effective compound to suppress metastasis of pancreatic cancer via targeting PCSCs and EMT.

Although known molecular markers of the cancer stem-like phenotype are still being discovered, three transcription factors, namely Sox2, Oct4 and Nanog, have been strongly validated as master regulators in the maintenance of the cancer stem-like phenotype (8). Overexpression of Sox2, Oct4 and Nanog frequently occurs in poorly differentiated malignancies and overlap with the signatures of embryonic stem cells (50). It has previously been reported that inhibition of AMPK signaling results in activation of the Warburg effect, which further induces stemness during the reprogramming of somatic cells (51). Furthermore, activation of AMPK signaling, via a pharmacological strategy, is a metabolic barrier during the process of somatic cells transforming into stem cells, which cannot be bypassed even under a p53 deficiency (52), thus indicating the critical role of AMPK signaling in stemness. It has also been reported that Sox2-overexpressed breast cancer cells downregulate AMPK signaling and activate mTOR to maintain their cancer stem-like phenotypes (44). In the present study, to explore the relationship between Sox2 and AMPK in pancreatic cancer, immunohistochemistry was conducted; the results indicated that there was an inverse association between P-AMPK and the key stemness regulator, Sox2. Immunohistochemistry also revealed that, compared with in normal pancreatic tissues, the expression levels of P-AMPK were significantly decreased in pancreatic cancer tissues. Conversely, the expression levels of Sox2 were markedly elevated. Investigating the modulation of AMPK signaling and suppression of pancreatic cancer stemness, it was revealed that administration of BA not only enhanced the levels of P-AMPK, but also inhibited the expression and nuclear localization of Sox2 in Mia PaCa-2 and Panc-1 cells, in order to restrain the stem-like phenotype. This study confirmed the finding whereby AMPK signaling exerts an essential role in modulating EMT and stemness of pancreatic cancer.

Gemcitabine has been used as a major therapy for the treatment of advanced pancreatic cancer; however, the majority of patients develop resistance during the initial period of treatment. The definite mechanism underlying chemoresistance remains to be determined. Recent reports have demonstrated that treatment with gemcitabine fortifies the stemness of cancer cells via the NAPDH oxidase/reactive oxygen species/nuclear factor-κB/STAT3 signaling cascade and the long non-coding RNA HOX transcript antisense RNA (45,53). Similarly, the present data indicated that gemcitabine treatment promoted upregulation of stemness markers in MiaPaCa-2 and Panc-1 pancreatic cancer cell lines. Notably, BA reversed gemcitabine-induced stemness and facilitated the antitumor effects of gemcitabine in pancreatic cancer cells; therefore, it may be considered a potential sensitizer for patients with pancreatic cancer.

BA is known to exert antidepressive (24), anti-inflammatory (25,26) and anti-AIDs (27,28) effects; in addition, it has hepatoprotective potential (29) and can alleviate NAFLD (35). Furthermore, its potential as a cancer preventive and therapeutic compound has been verified (30-34). Of particular interest is its direct and relatively selective cytotoxic effect on various tumor cells compared with normal or non-neoplastic cells (54). The present results revealed that BA may inhibit the proliferation, migration, invasion and tumorsphere formation of pancreatic cancer cells, regulate EMT, and alter the expression levels of Sox2, Oct4 and Nanog via activation of AMPK signaling. Furthermore, BA combined with gemcitabine exerted antitumor effects on pancreatic cancer cells. However, there are some limitations to the present study. These findings were based on in vitro experiments, which may not accurately reflect the physiological conditions in vivo. Therefore, BA warrants further functional studies and in vivo investigations, to determine whether it is an effective inhibitor of the stem-like phenotype in pancreatic cancer cells.

## Availability of data and materials

The data and materials used during the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

LS, JC, QM and QX designed the experiments. LS and JC conducted the majority of the experiments. KC analyzed the data. LC, CZ and BY organized the figures and contributed to data analysis. LS and JC wrote the manuscript, and QM

and QX reviewed it. WQ, JL, WD, JM, DQ, EW, ZW and QL contributed to the collection of human specimens and revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All experimental protocols were approved by the Ethical Committee of The First Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was obtained from the patients.

## Patient consent for publication

The authors declare that the patients provided written informed consent for the publication of any associated data in the present study.

## Competing interests

The authors declare that they have no competing interests.

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