Research Article

Extract of the Medicinal Plant Pao Pereira Inhibits Pancreatic Cancer Stem-Like Cell In Vitro and In Vivo

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Abstract

Pancreatic cancers are enriched with cancer stem-like cells (CSCs), which are resistant to chemotherapies, and responsible for tumor metastasis and recurrence. Here, we investigated the extract of a medicinal plant Pao Pereira (Pao) for its activity against pancreatic CSCs. Pao inhibited overall proliferation of human pancreatic cancer cell lines with IC_{50} ranging from 125 to 325 μg/mL and had limited cytotoxicity to normal epithelial cells. Pancreatic CSC population, identified using surface markers CD24+ CD44+ EpCam+ or tumor spheroid formation assay, was significantly reduced, with IC_{50}s of ~100 μg/mL for 48 hours treatment, and ~27 μg/mL for long-term treatment. Nuclear β-catenin levels were decreased, suggesting suppression of Wnt/β-catenin signaling pathway. In vivo, Pao at 20 mg/kg, 5 times/week gavage, significantly reduced tumorigenicity of PANC-1 cells in immunocompromised mice, indicating inhibition of CSCs in vivo. Further investigation is warranted in using Pao as a novel treatment targeting pancreatic CSCs.

Keywords

pancreatic cancer, Pao Pereira, cancer stem-like cells, xenograft models

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Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States with a 5-year overall survival rate of only 8%.[1] The American Cancer Society estimated that 55 440 (men = 29 200, women = 26 240) people will be diagnosed with pancreatic cancer in 2018, and 44 330 (men = 23 020, women = 21 310) will die from it.[2] Treatment outcomes are far from satisfactory.[3,4] Because of the lack of efficient early detection methods, only about 10% of patients are diagnosed with local disease, for whom the 5-year survival rate is about 32%. For the majority of patients who are diagnosed at an advanced stage, the 5-year survival rate is less than 5%, which is among the lowest of all types and stages of malignancies.[5] Gemcitabine as the first-line chemotherapy provides very limited benefit on the overall survival of patients with locally advanced or metastatic pancreatic cancers.[6,7] New treatment regimens that have been designed either by adding chemotherapy drugs to gemcitabine, such as adding nab-paclitaxel,[8] or using gemcitabine-free combination, such as FOLFIRINOX,[9,10] show limited improvement in survival and response rates, and significantly increase toxic side effects.[11,12] New treatment options are urgently needed for pancreatic cancer.

The poor treatment outcomes may be partially due to an enriched cancer stem-like cell (CSC) population in pancreatic cancer. Accumulating evidence has shown that pancreatic CSCs are resistant to current treatments, and therefore survive and eventually generate new tumors, either at the primary site or at metastatic site.[13-15] CSCs share characteristics with normal stem cells. An important characteristic is the ability to self-renew. Depending on the microenvironment, a stem cell can divide and generate daughter cells that do not differentiate but keep the full potential of differentiation as the parent stem cell (self-renewal), and/or raise daughter cells which will differentiate.[16] CSCs possess self-renewal ability and are able to give rise to all cell types found in a particular bulk of tumor.[17] CSCs are resistant to current chemo and radiation therapy,[18] are responsible for tumor metastasis[19] and recurrence,[4] which are the main reasons for cancer-related death. Therefore, therapies that

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inhibit CSCs would hold great promises in eliminating the whole cancer cell population.

Natural products have been a rich resource for bioactive anticancer agents. They are used in folk medicines all over the world and have been used by oncologic patients and integrative medicine practitioners for many years. Pao Pereira (*Geissospermum vellosii*) is an Amazonian tree in the Apocynaceae family. This family of plants have been used as a folk medicine in South American to treat a variety of health-related conditions, including cancer.[20] A number of compounds isolated from this family of plants were reported to have antiplasmodial,[21] antiviral,[22] and antiparasitic[23] bioactivities. The extract of the bark of Pao Pereira (Pao) has long been used in complementary and alternative medicine on cancer patients, and has been reported recently to have tumor inhibitory effect toward prostate, ovarian and pancreatic cancers.[20,24-26] We previously reported that Pao induced pancreatic cancer cells apoptosis, and inhibited pancreatic tumor growth in mice.[25] The combination of Pao and gemcitabine showed synergistic antitumor effects.[25] Here, we investigated the activities of Pao in inhibiting pancreatic CSCs both in vitro and in vivo.

Materials and Methods

Cell Lines and Reagents

Human pancreatic cancer cell lines PANC-1, AsPC-1, HPAF-II, BxPC-3, and MIA PaCa-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and have been maintained in the lab. An immortalized human lung epithelial cell line MRC-5 was provided by Dr Sitta Sittampalam at the National Center for Advancing Translational Sciences, National Institutes of Health, and was used as a comparison to the cancer cells. All cells were cultured at 37°C in 5% CO_2/95% air in recommended growth media containing 10% fetal bovine serum (FBS) and 1% antibiotics. The extract of Pao Pereira (Pao) was provided by the Natural Source International Ltd (New York, NY, USA). Samples of the plant were authenticated by the vendor by use of voucher specimens. Aqueous alcoholic extraction from the bark of Pao yielded a proprietary extract, and then on spray drying yielded a free-flowing powder. The powder was the extract used in this study. Quality control was ensured by high-performance liquid chromatography. Pao was prepared in sterile phosphate buffered saline (PBS) in 10 mg/mL stock solutions and stored at −20°C. All the experiments used the extract of Pao from a single lot.

Cell Viability Assay

Cells were assessed for viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 48 hours of treatment. Cells in exponential growth phase were exposed to serial dilutions of Pao for 48 hours. Cells were then changed into fresh media containing MTT and were incubated for 4 hours. The colorimetric MTT assay assesses relative proliferation, based on the ability of living, but not dead cells, to reduce MTT to formazan. Cells did not reach plateau phase during the incubation period. Fifty percent inhibitory concentration (IC_{50}) was defined as the concentration of drug that inhibited cell growth by 50% compare to the untreated control.

Tumor Spheroid Formation Assay

Single-cell suspension of PANC-1 cells was plated into 24 well ultra-low attachment plates (Corning Inc, Corning, NY) at a density of 5000 cells/well in stem cell media and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For MIA PaCa-2 cells, single-cell suspension was plated into 96-well ultra-low attachment plates (Corning Inc, Corning, NY) at a density of 100 cells/well in stem cell media and incubated under the same conditions. The stem cell media consist of Dulbecco's modified Eagle medium (DMEM; Corning Inc, Corning, NY) supplemented with 1X B27 Supplement, 20 ng/mL human basic fibroblast growth factor, 20 ng/mL epidermal growth factor, 100 units/mL penicillin/streptomycin (Invitrogen, Grand Island, NY), and 4 µg/mL heparin calcium salt (Fisher Scientific, Pittsburg, PA). PANC-1 spheroids were counted after 4 weeks and MIA PaCa-2 spheroids were counted after 2 weeks under the microscope. Spheroids diameter was measured by Image J.

Flow Cytometry for Detection of Cancer Stem-Like Cells Surface Markers

Cells were exposed to various concentrations of Pao for 24 or 48 hours. Cells were then washed with PBS 3 times and resuspended in binding buffer (PBS supplemented with 0.1% bovine serum albumin) for 15 minutes. PE conjugated anti-CD24 antibody, PE-Cy7 conjugated anti-CD44 antibody, and APC conjugated anti-EpCam antibody (Biolegend, San Diego, CA) were added into cell suspension and incubated for 15 minutes according to the manufacturer's protocol. Cells were washed with PBS 3 times after staining and then analyzed by BD LSR II Flow Cytometer. The data were normalized to cell death (normalized CSC population = original CSC population detected with flow cytometry × % cell viability detected with MTT assay).

Flow Cytometry for Sorting of Side Population From Pancreatic Cancer Cells

Dye Cycel Violet (DCV, Invitrogen, Grand Island, NY) was used for staining of the non-CSC population. Cells that efficiently exclude DCV from cytoplasm are considered CSC-like population (DCV negative cells). Pancreatic cancer cells

were suspended at a density of $1x10^6$ cells/mL in DMEM supplemented with 10% FBS and 10 mM HEPES. DCV (10 μM) were added and incubated for 30 minutes at room temperature. Cells were then washed twice with PBS and resuspended in DMEM supplemented with 10% FBS and 10 mM HEPES for 1 hour. Cells were transferred to ice-cold Hanks' balanced salt solution (HBSS)/2% FBS/10 mM HEPES buffer right before flow cytometry sorting. The DCV-negative and -positive cells were separately collected for further analysis. Gate setting was performed by using cells treated with a pump inhibitor verapamil (200 μM) prior to DCV staining.

SDS-PAGE and Western Blot

Cells were lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors (Sigma Aldrich, St Louis, MO), followed by sonication for 10 seconds. Either whole cell lysate or supernatant was used for further experiment, depending on the proteins of interest. Primary and secondary antibodies were from Cell Signaling Technology Inc (Danvers, MA): rabbit anti- β-catenin (Cat# 9582, 1:1,000), rabbit anti-vinculin (Cat# 4650, 1:1,000), rabbit anti-Histone H3 (Cat# 4499, 1:2,000), rabbit anti-Nanog (Cat# 4903, 1:2,000), mouse anti-β-actin (Cat# 3700, 1:2,000), and goat anti-rabbit (Cat# 7074) or anti-mouse (Cat# 7076) IgG (1:5,000). BCA method was used for protein quantification (Pierce BCA protein assay kit, Waltham, MA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed routinely. Blots were established using a chemiluminescence detection kit (Pierce ECL or ECL+ western blotting substrate, Thermo Scientific, Rockford, IL). The intensities of the bands were measured by using Image Studio v5.0.

RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cells or tissue samples by using TRIZOL reagent according to the protocol of the manufacturer (Invitrogen, Grand Island, NY). cDNA synthesis was performed with 1 μg of total RNA using Omniscript RT kit according to manufacturer's protocol (Qiagen, Valencia, CA). cDNA was diluted 1:5 in DEPC-treated nanopure water and used for further analysis. Real-time PCR was performed using Bio-Rad iQ iCycler detection system with iQ SYBR green supermix (Bio-Rad Laboratories Ltd, Hercules, CA). Reactions were performed in a total volume of 10 μL, including 5 μL of 2X iQ SYBR green supermix, 0.4 μL of primers at 20 pmol/μL and 0.4 μL of cDNA template. All reactions were carried out at 4 repeats for every sample and 3 independent experiments for each group. GAPDH was used as a housekeeping gene for normalization. Primers used in Real-time PCR were according to previous studies.[27]

Pancreatic Cancer Mouse Model

All animal studies followed a protocol approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. One-time treatment and repeated treatment were each used for measurement of tumorigenicity. In the one-time treatment model, pancreatic cancer cells PANC-1 at different numbers were used for tumor inoculation: 2×10^4 cells per injection, 2×10^5 cells per injection, or 1×10^6 cells per injection. PANC-1 cells were suspended in PBS as single cell suspension and then mixed with either 200 μg/mL Pao or PBS. At each cell injection number, cells mixed with Pao were injected subcutaneously into the left flank of the mouse, and cells mixed with PBS into the right flank of the same mouse. Ten mice were used for each cell number. Formation of tumors was monitored daily, and longitudinal tumor growth was measured by a caliper.

In the repeated treatment model, single cell suspension of PANC-1 cells were mixed with 200 μg/mL Pao, and then inoculated into 10 mice at 2×10^5 cells per injection, at both left and right flanks. Treatment started the next day with oral gavage of 20 mg/kg Pao, 5 × per week for 3 weeks. Control group (10 mice) was inoculated with the same number of cells in PBS, and then was gavaged with equivalent volume of saline solution. Tumor formation was monitored daily, and longitudinal tumor growth was measured by a caliper.

Data Analysis

IC_{50} estimation was calculated by using linear regression with Pao concentration as X and the % cell viability compared against untreated (MTT assay) / total number of spheroids (spheroid formation assay) /CSC population (flow cytometry) as Y. IC_{50} value was then estimated using the fitted line: $Y = a * X + b$, when $Y = 0.5b$, $IC_{50} = X = -0.5b/a$.

Statistical analysis was performed using SPSS software for Student's t test and log-rank test. A difference was considered significant at the $P < .05$ level.

Results

Pao Inhibited Pancreatic Tumor Spheroids Formation In Vitro

Five different human pancreatic cancer cell lines (PANC-1, MIA PaCa-2, AsPC-1, HPAF-II, and BxPC-3) and an immortalized epithelial cell line (MRC-5) were treated with Pao, and cell viability was detected after 48 hours. Pao inhibited proliferation of all 5 cancer cells (Figure 1A), with IC_{50} values ranging from 125 to 325 μg/mL. The noncancerous epithelial cell MRC-5 was less affected, with a higher IC_{50} value of 547 μg/mL (Figure 1B). These results

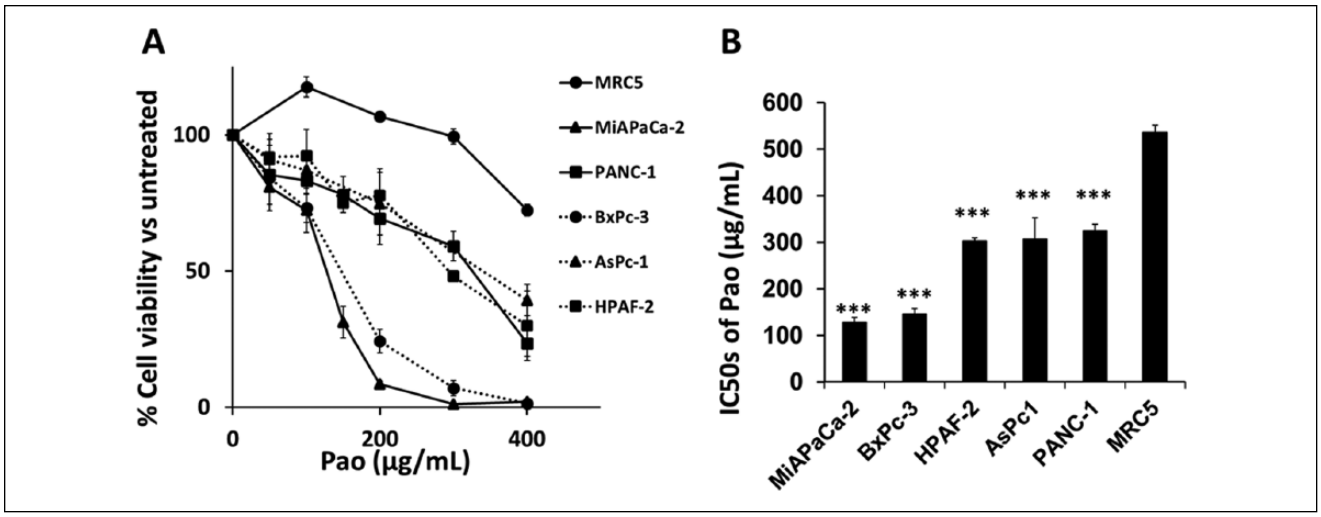

Figure 1. Inhibition of the proliferation of pancreatic cancer cells by Pao. (A) Dose-response curves. Human pancreatic cancer cells PANC-1, AsPC-1, HPAF-II, BxPC-3, and MIA PaCa-2 were exposed to serial concentrations of Pao for 48 hours. Cell viability was detected by MTT assay. An immortalized noncancerous epithelial cell line, MCR-5, was subjected to the same treatment. (B) IC_{50} values of Pao in pancreatic cancer cells and MRC-5 cells. ***$P < .001$ compared with the IC_{50} of MRC5 cells. All values are expressed as means ± SD of 3 independent experiments, each done in triplicates.

are consistent with our previous studies that Pao inhibited the overall proliferation of pancreatic cancer cells.[25]

To investigate inhibition in CSCs, tumor spheroid formation was detected. The ability to form tumor spheroids is an indication of CSCs' self-renewal and tumorigenic capacity in vitro. When cancer cells are cultured in serum-free, nonadherent conditions, the non-CSC population dies by anoikis, whereas CSCs overcome anoikis and go through division leading to formation of tumor spheroids.[28,29] At the concentration of 50 μg/mL, Pao significantly reduced the number of the PANC-1 tumor spheroids (Figure 2A and B). At the concentration of 100 μg/mL and above, Pao completely eliminated the PANC-1 tumor spheroids (Figure 2A and B). The estimated IC_{50} value for PANC-1 spheroids inhibition is 27 μg/mL. In comparison, the IC_{50} value of Pao to the bulk of PANC-1 cells is about 300 μg/mL (Figure 1A). In the bulk PANC-1 cell population, 100 μg/mL of Pao inhibited the overall proliferation by 20%, whereas 100% tumor spheroids were inhibited at this concentration (Figure 2A). MIA PaCa-2 pancreatic cancer cells were also subjected to Pao treatment for detection of tumor spheroids. Similar results were obtained. Pao reduced the number of the MIA PaCa-2 spheroids at 50 μg/mL, and completely inhibited spheroid formation at 100 μg/mL and above (Figure 2C and D). The estimated IC_{50} value is 35 μg/mL (Figure 2D), which is much lower than the IC_{50} value to the bulk MIA PaCa-2 cells (Figure 1A).

The side population of cells that exclude dyes is indicative of CSCs.[30,31] MIA PaCa-2 cells were sorted by flow cytometry to separate CSC-like side populations by DCV staining. Both DCV− cells (CSC-like) and DCV+ (non-CSC-like) cells were collected and treated with Pao. Cell viability was examined by MTT assay. Pao inhibited viability in all unsorted, DCV+ and DCV− cells, with preference in inhibiting DCV− cells (Figure 2E). The estimated IC_{50}s were 147 μg/mL in unsorted cells, 145 μg/mL in DCV+ cells, and 84 μg/mL in DCV− cells. This suggests that Pao preferentially inhibits CSC-like cells.

DCV− cells formed large spheroids as expected. While some cell spheroids were also formed in DCV+ cell culture, they were significantly smaller (Figure 2F). The spheroid formation in DCV+ cells may be due to the DCV staining, and the sorting method is not an exclusive method to pinpoint CSCs, as to date there is no efficient way to pinpoint pancreatic CSCs. The DCV staining and sorting, rather, provided us a side population enriched with "stemness." Pao at 50 μg/mL inhibited spheroids from both DCV− and DCV+ populations (Figure 2F), a result consistent with those in unsorted cells.

Pao Reduces Number of Pancreatic Cancer Stem-Like Cells In Vitro

The CSC population can be identified by specific cell surface markers. In pancreatic cancer, a subpopulation of cells with high expression of surface markers CD44, CD24, and EpCAM (CD44+ CD24+ EpCAM+ cells) were reported to possess strong self-renewal ability and the ability to produce differentiated progeny and to generate new tumors in mice that were histologically identical to parent tumors.[32] Here, we use these markers as indicative markers for pancreatic CSCs and detected changes in these markers with Pao treatment. PANC-1 cells were treated with Pao for 24 or 48 hours at 50, 100 or 200 μg/mL. CD44, CD24, and

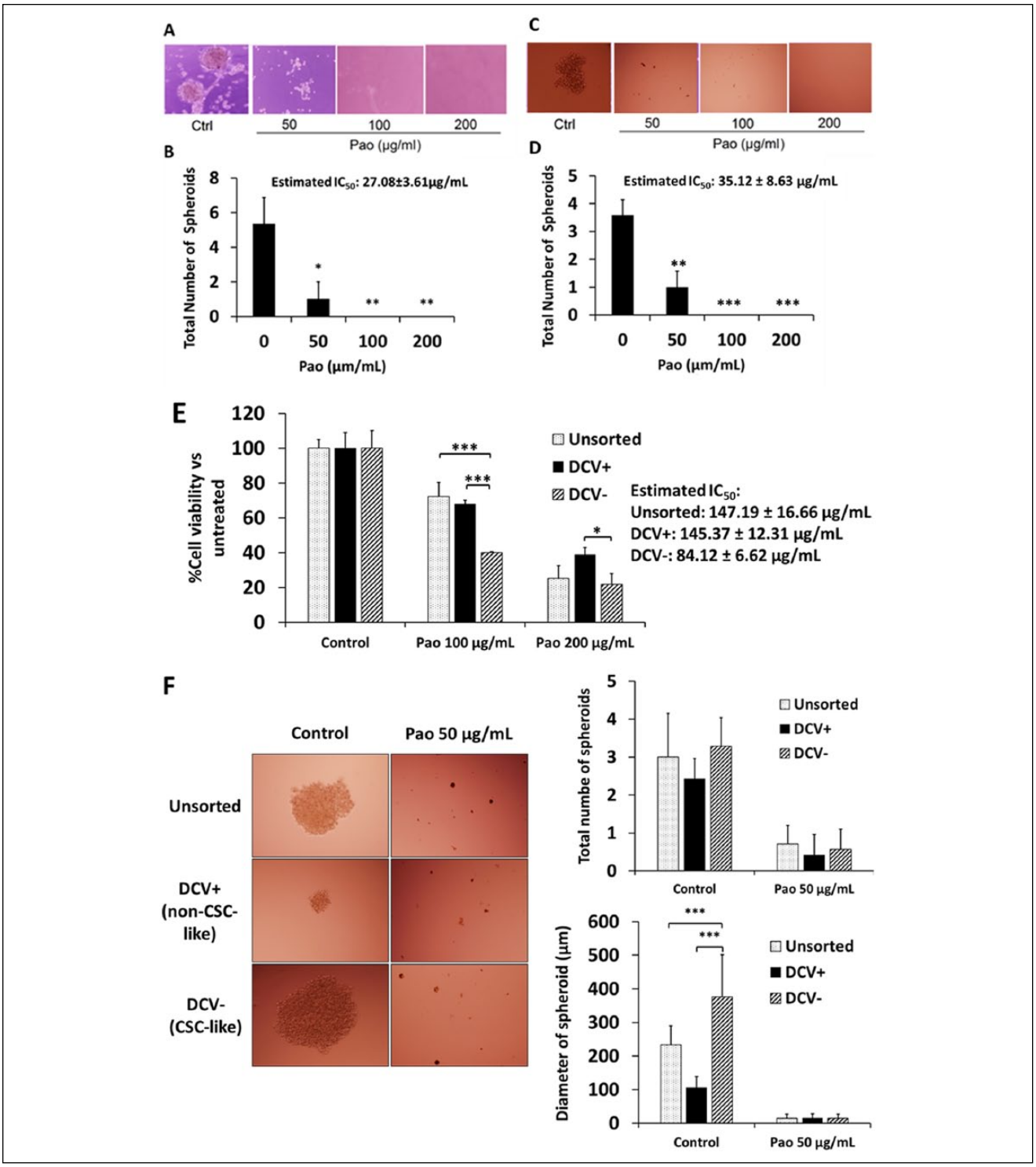

Figure 2. Inhibition of pancreatic tumor spheroids by Pao. (A) Representative images of the PANC-1 spheroids with and without Pao treatment. PANC-1 single-cell suspension was plated into 24-well ultra-low attachment plates at a density of 5000 cells/well in stem cell media. Tumor spheroids were counted after 4 weeks. (B) Number of PANC-1 spheroids (means ± SD of 3 independent experiments). (C) Representative images of the MIA PaCa-2 spheroids with and without Pao treatment. MIA PaCa-2 single-cell suspension was plated into 96-well ultra-low attachment plates at a density of 100 cells/well in stem cell media. Tumor spheroids were counted after 2 weeks. (D) Number of MIA PaCa-2 spheroids (means ± SD of 3 independent experiments). (E) Cell proliferation of unsorted cells, DCV+ cells (non-CSCs-like) and DCV− cells (CSC-like) with Pao treatment for 48 hours (means ± SD of 3 independent experiments). (F) Representative images of the MIA PaCa-2 spheroids from unsorted cells, DCV+ cells and DCV− cells with and without Pao treatment. Number and size of MIA PaCa-2 spheroids are shown in bar graph. *$P < .05$; **$P < .01$; ***$P < .001$, compared with untreated control.

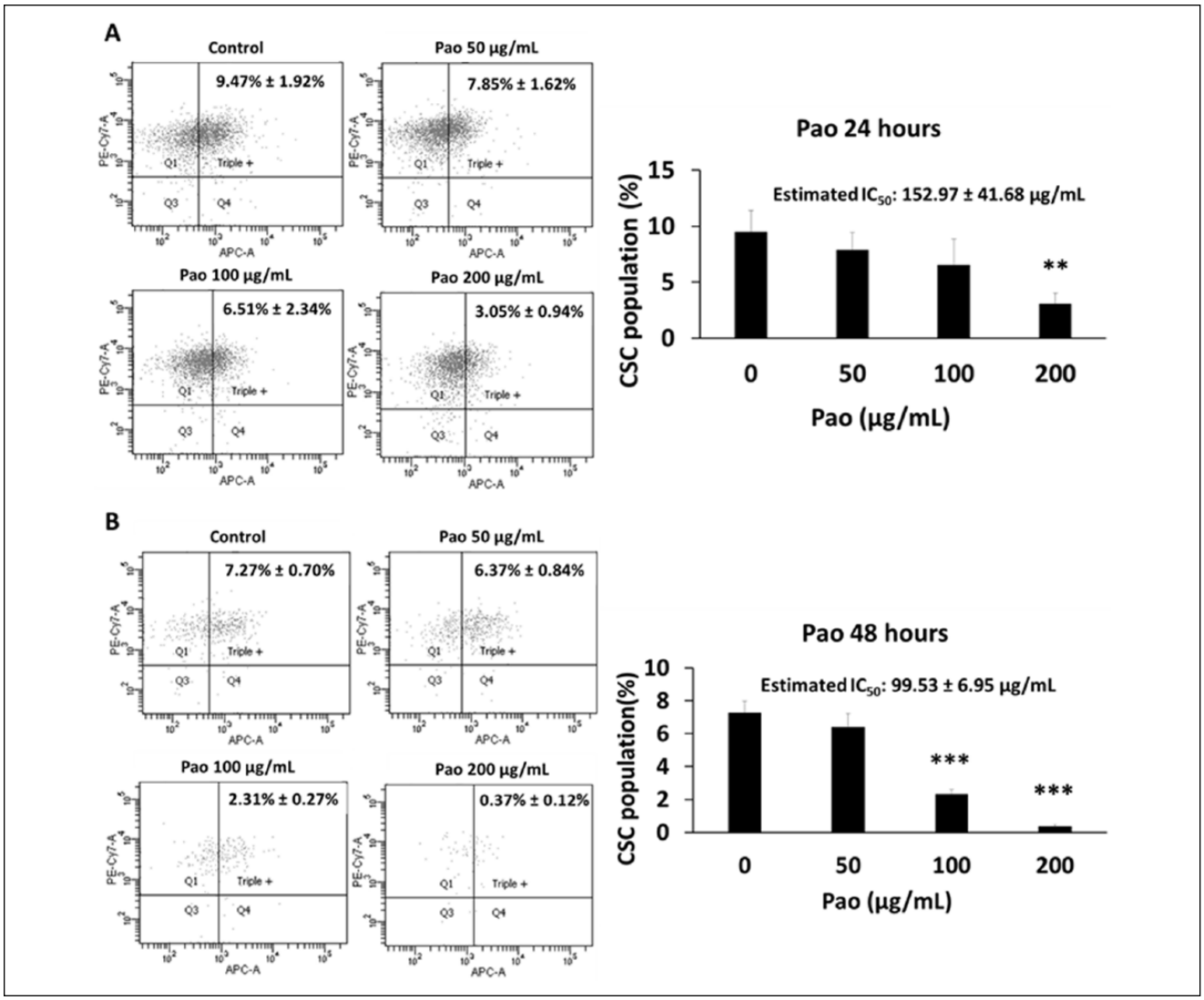

Figure 3. Inhibition of cancer stem-like cells (CSCs) populations by Pao. PANC-1 cells were treated with Pao for 24 hours (A) and 48 hours (B) at indicated concentrations. Cells were then stained with fluorescent conjugated antibodies for CD24, CD44, and EpCam, followed by flow cytometry analysis. Left panels show EpCam (APC) and CD44 (PE-Cy7) positive cells under CD24 (PE) positive gate. The percentages of CD24+ CD44+ EpCam+ cells were quantified and shown in the bar graph (mean ± SD of 3 experiments). The data were normalized to cell death. $^{**}P < .01$; $^{***}P < .001$ compared with untreated group.

EpCAM were examined by immune staining and flow cytometry analysis. Pao reduced the CD44+ CD24+ EpCam+ population at both 24- and 48-hour treatment (Figure 3A and B). In the control group, CD44+ CD24+ EpCam+ cells constituted 7.5% to 9% of the whole population. At the concentration of 200 μg/mL, Pao significantly reduced CD44+ CD24+ EpCam+ cells to 3.05% at 24-hour treatment (Figure 3A), and to 0.37% at 48 hours (Figure 3B). At a lower concentration of 100 μg/mL, Pao reduced the triple positive cells to 2.31% at 48-hour treatment (Figure 3B), which was still a significant reduction compared with control (Figure 3B). We estimated that the IC_{50} value at 24-hour treatment was 152.97 ± 41.68 μg/mL, and at 48-hour treatment it was 99.53 ± 6.95 μg/mL (Figure 3A and B).

Canonical Wnt/β-catenin signaling pathway plays an important role in maintaining the self-renewal and spheroid formation capacities of CSCs.[17,33] Accumulation of β-catenin in the nucleus as a transcriptional factor is a hallmark of Wnt/β-catenin pathway activation.[34] Here, the cytoplasmic and nuclear fractions of the PANC-1 cells were each examined for β-catenin levels with or without Pao treatment. Pao 100 μg/mL at 24 and 48 hours reduced the level of β-catenin in both nucleus and cytoplasm, with more severe reduction in nucleus (Figure 4A). A panel of β-catenin downstream target genes, including BCL2L2,

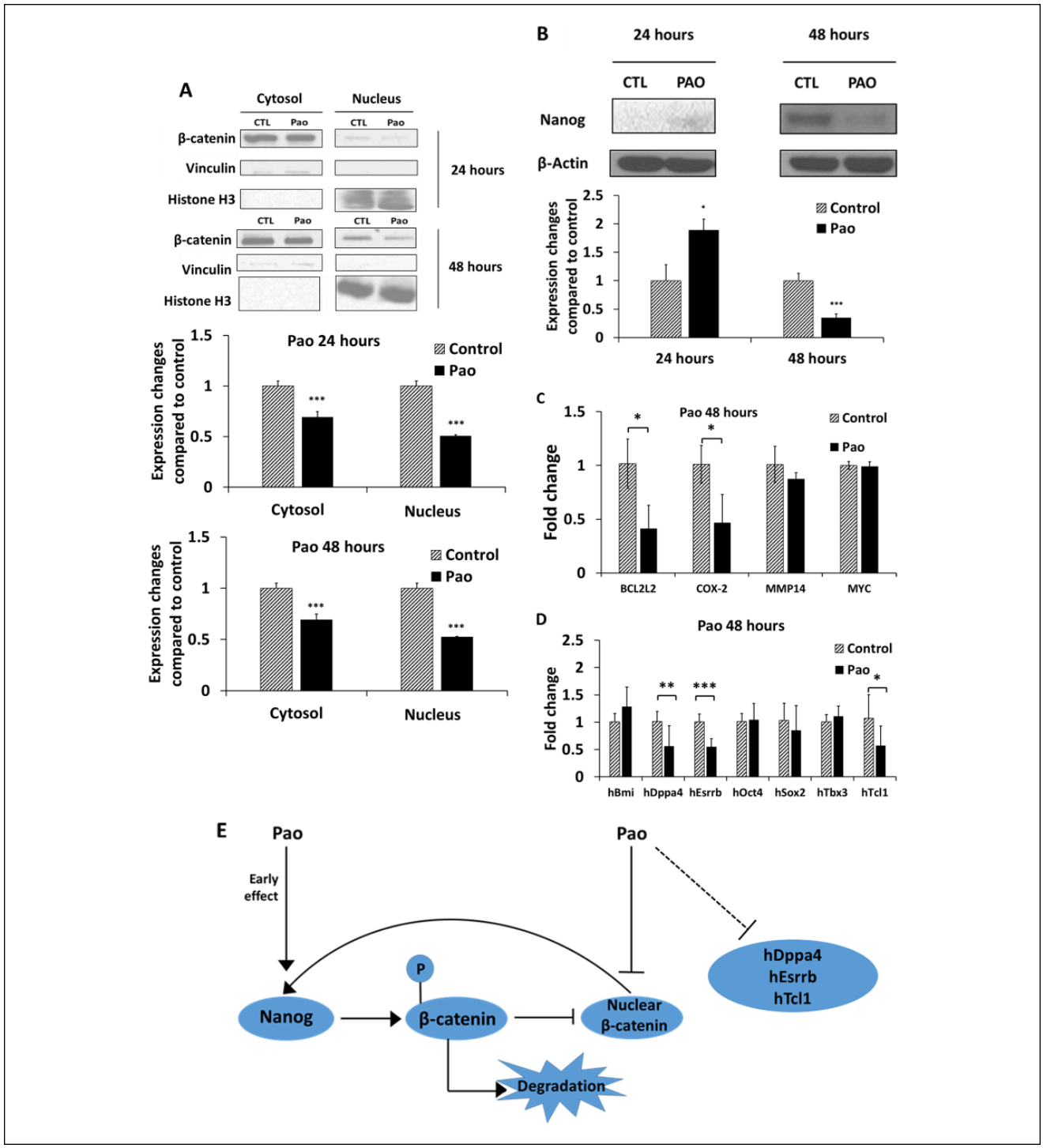

Figure 4. Decrease of gene expression related to cancer stem-like cells (CSCs) by Pao. PANC-1 cells were treated with Pao at 100 μg/mL for 24 and 48 hours. (A) β-Catenin levels in cytoplasmic and nuclear fractions, detected by Western blot. Vinculin was a loading control for cytoplasmic proteins, and histone H3 was a loading control for nuclear fraction. Bar graph shows band density normalized to loading control and compared to untreated cells. (B) The expression of Nanog, detected by Western blots. Bar graph shows band density normalized to loading control and compared to untreated cells. (C) The expression of β-catenin downstream target genes at 48 hours of Pao treatment, detected by RT-qPCR. (D) The expression of CSC-related genes after 48 hours Pao treatment, detected by RT-qPCR. (E) Postulated mechanism of Pao inhibiting Nanog and nuclear β-catenin. Pao treatment has an early effect in increasing Nanog expression, which leads to β-catenin phosphorylation and degradation, therefore represses nuclear β-catenin level. The decreasing nuclear β-catenin level negatively influences Nanog expression. Pao treatment may also directly inhibit β-catenin nuclear accumulation. Both can result in an overall suppression of both Nanog and nuclear β-catenin levels. Pao also inhibited the RNA level of CSC-related genes, such as Dppa4, Esrrb and Tcl1. **P* < .05; ***P* < .01; ****P* < .001 compared with the untreated control group.

COX-2, MMP14, and MYC, were examined by RT-qPCR (Figure 4C). None of these genes were changed at 24-hour treatment. However, at 48-hour treatment, the expression of BCL2L2 and COX-2 was significantly decreased, consistent with Wnt/β-catenin signaling pathway inhibition.

Studies have shown that a stem cell related gene Nanog can induce β-catenin phosphorylation and therefore enhance its degradation, and consequently inhibit Wnt signaling pathway.[35] We therefore examined the expression of Nanog by Western blot. Nanog was increased at 24 hours of Pao treatment but was decreased at 48 hours of Pao treatment (Figure 4B). We postulate that increase in Nanog at the earlier time point suppressed nuclear β-catenin levels, and then the feedback from decreasing β-catenin levels caused inhibition in Nanog expression at a later time point.[36,37] As a result, both Nanog and the Wnt signaling pathway were inhibited by Pao. A panel of other CSC-related genes were also examined by RT-qPCR, which are reported to be important for CSC initiation and maintenance.[27] Data showed that the expressions of Dppa4, Esrrb, and Tcl1 were inhibited with 48-hour Pao treatment (Figure 4D).

Taken together, Pao treatment has an early effect in increasing Nanog expression, which leads to β-catenin phosphorylation and degradation, thereby repressing nuclear β-catenin level. The decreasing nuclear β-catenin level negatively influences Nanog expression. Pao treatment may also directly inhibit β-catenin nuclear accumulation. Both can result in an overall suppression of both Nanog and nuclear β-catenin levels (Figure 4E). The full mechanism of Pao-induced CSC inhibition is worth further investigation.

Pao Inhibits Pancreatic Cancer Stem-Like Cells In Vivo

Tumorigenicity was examined in immunocompromised mice to evaluate the inhibitory activity of Pao against pancreatic CSCs in vivo. A one-time treatment was performed first using inoculation of different numbers of PANC-1 cells at limited dilutions. Respectively, 2×10^4 cells, 2×10^5 cells, and 1×10^6 cells were mixed with 200 μg/mL Pao and injected subcutaneously into the left flanks of nude mice (N = 10). As control, the same number of cells were mixed with PBS and inoculated into the right flanks of the same mouse. At all 3 numbers of cell injections, neither a delay nor a reduction of rate in tumor formation was found (Figure 5A, C, and E). The one-time Pao treatment tended to reduce the size of tumors at the 2×10^4 and 2×10^5 cells groups, but there was no significant difference compared with control groups (Figure 5B, D, and F).

As the one-time Pao treatment failed to reduce the rate of tumor formation, we conducted repeated treatment with oral administration of Pao. The cell number was selected to be 2×10^5 per injection. Mice (N = 10) were injected subcutaneously at both left and right flanks with PANC-1 cells mixed with 200 μg/mL of Pao. Treatment started the next day and lasted for 3 weeks with oral gavage of 20 mg/kg Pao, 5 times per week. Control mice (N = 10) were inoculated with the same number of cells mixed with PBS and were gavaged with equivalent volumes of saline.

Both the rate and time of tumor formation were significantly reduced by Pao treatment (Figure 6A). At day 6, tumor formation rate in control group reached 80%, while in Pao-treated group it was only 10%. At day 20, when the treatment stopped, all mice in control group were bearing tumors on both flanks (100% tumor formation), while the Pao-treated group only had 30% tumor formation. All mice were kept for 2 more months after treatment had stopped. At the end of the experiment, the Pao treatment group had a maximum of 65% tumor formation, compared with the 100% tumor formation in the control group. These data indicate that Pao administration at 20 mg/kg orally eliminated CSCs in 35% of the injection sites.

Growth of the formed tumors was also inhibited by Pao treatment compared with the control group (Figure 6B). A long-term inhibitory effect in tumor growth was observed after treatment had stopped (Figure 6B). Adverse effects were monitored during the treatment and no adverse effects were observed. Body weight showed no difference between the treated and the control group (Figure 6C).

Discussion

CSCs are a small population in the bulk of cancer cells that are responsible for generation of new tumors. They possess self-renewal ability and are able to give rise to all cell types found in a particular bulk of tumor.[16] Traditional antitumor chemotherapy drugs lack the ability to eliminate CSCs, which survive and later raise recurrent tumors, often at metastatic sites.[6,7] CSCs are also responsible for drug resistance.[18,38] The mechanism by which CSCs become drug resistant is not very clear, but it is probably due to the upregulated expression of ABCG2 transporters, which facilitate efflux of chemotherapeutic drugs from the cytosol.[38] Other properties may also contribute to CSCs' drug resistance, such as overexpression of detoxifying enzymes and antiapoptotic proteins, and enhanced DNA repair ability.[38] Therefore, drugs inhibiting CSCs hold the promise to comprehensively inhibit tumor growth, metastasis, recurrence, and conquer drug resistance. In this study, we demonstrated both in vitro and in vivo that the extract of the plant Pao Pereira (Pao) inhibited pancreatic CSCs. Previously, we have reported that Pao induces apoptosis in pancreatic cancer cells and sensitizes pancreatic cancer cells to gemcitabine treatment.[25] Independent of its apoptosis-inducing activity, inhibition of CSCs could be another reason contributing to Pao-induced gemcitabine sensitivity. Taken together, the benefits of Pao in pancreatic cancer

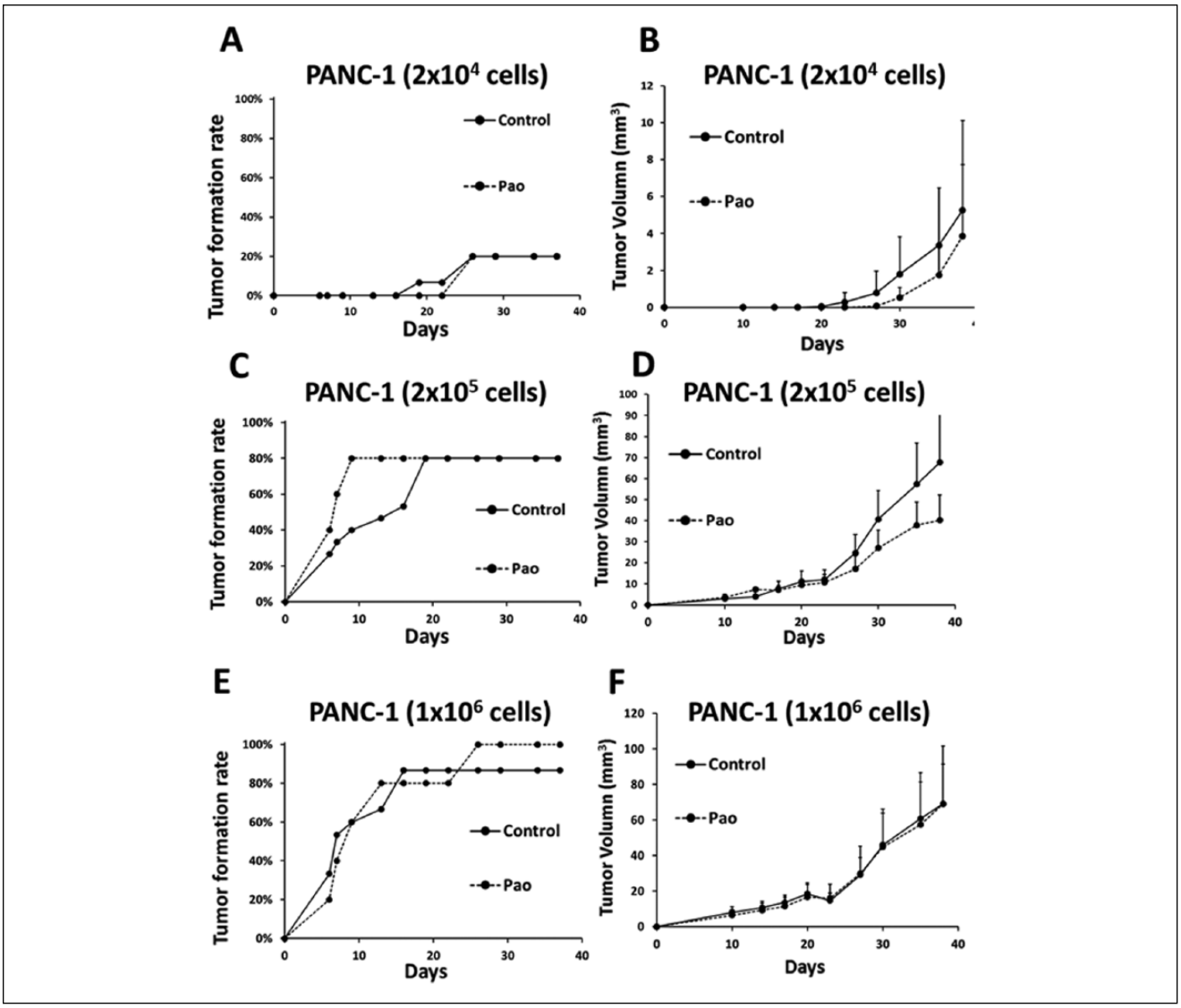

Figure 5. Effects of one-time Pao treatment on PANC-1 tumor formation in nude mice. (A, B) 2 × 10^4 PANC-1 cells, (C, D) 2 × 10^5 PANC-1 cells, and (E, F) 1 × 106 PANC-1 cells were mixed with 200 μg/mL Pao, and then inoculated into the left flank of each mouse. The same number of PANC-1 cells were mixed with phosphate buffered saline (PBS) and inoculated into the right flank of each mouse. A total of 10 mice were used for each cell number. The tumor formation rate (A, C, E) was defined as the number of tumors observed at a specific day / 10 × 100%. Tumor size (B, D, F) was measured by caliper, and the tumor volume was calculated using the formulation: tumor volume = width × width × length/2.

treatment are worth investigation clinically, especially in combination with current chemotherapies.

To date, there has been no efficient method to pinpoint a pancreatic CSC and maintain and amplify it for drug development purposes. Functional assays such as tumor spheroid assay and tumorigenicity in mice are commonly used.[39] The use of chromosomal dye[30,31] or several cell surface markers are powerful to identify and isolate a subpopulation enriched with stem-like features.[32] In our studies here, we did not only rely on CSCs isolated and separately treated. First, it is difficult to obtain and maintain a pure CSC population.[40] Second, isolated CSCs might lose their natural environment in the bulk population.[16] Instead, we treated the bulk of pancreatic cancer cells, and a side population, and examined the CSC specific outcomes. The inhibitory results from our studies are not likely due to the general cytotoxicity of Pao to the bulk of cancer cells, because Pao has an IC_{50} value of 300 μg/mL in 48 hours of treatment toward the bulk of PANC-1 cells and has a much lower IC_{50} value of 153 μg/mL for the reduction of CD44+ CD24+ EpCam+ cells at a shorter treatment time of 24 hours, and 99.53 μg/mL at 48 hours. Furthermore, in the tumor spheroid formation assay,

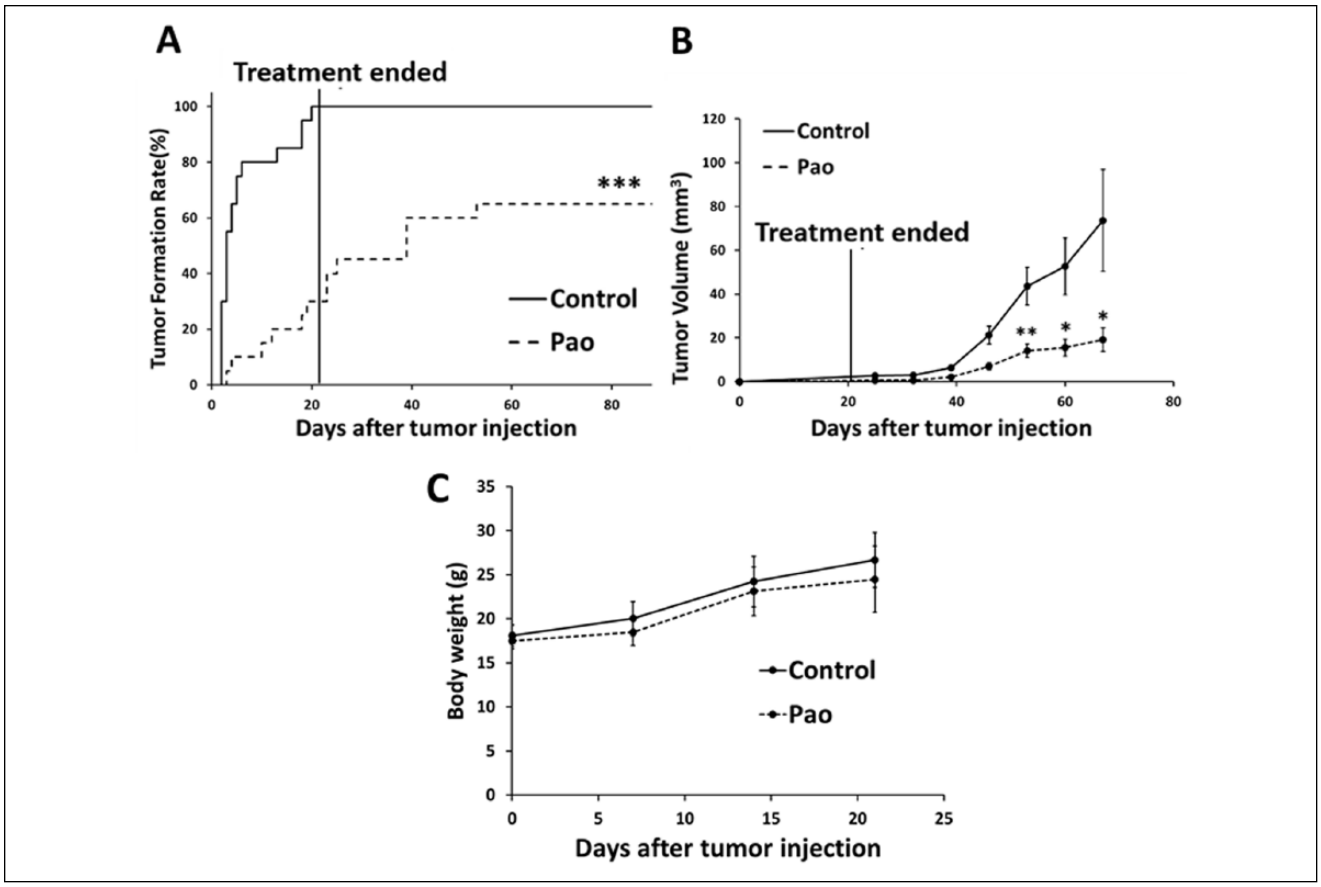

Figure 6. Effects of repeated Pao treatment on PANC-1 tumor formation and tumor growth in nude mice. (A) Tumor formation rate. A total of 2×10^5 PANC-1 cells were mixed with 200 μg/mL Pao (Pao) or phosphate buffered saline (PBS; control), and inoculated at both flanks of nude mice (N = 10 for each group). Treatment started the next day and lasted for 3 weeks with oral gavage of 20 mg/kg Pao (Pao) or saline (control) 5 times per week. ***$P < .001$ by log-rank test. (B) Longitudinal tumor growth. Tumor size was measured every 3 days by a caliper. Tumor volume = width × width × length/2. (C) Body weight of mice during treatment of 3 weeks. *$P < .05$; **$P < .01$; ***$P < .001$ compared with untreated control group.

Pao has an IC_{50} of 27 μg/mL in inhibiting the number of spheroids. These data suggest that Pao has a preferential inhibitory activity toward pancreatic CSCs.

The mechanism(s) by which Pao induces CSC inhibition needs to be further investigated. Our study showed that Pao reduced both Nanog and nuclear β-catenin level of PANC-1 cells, which are important in stem cell initiation and maintenance. Pao also reduced mRNA levels of several CSC-related genes, namely Dppa4, Esrrb, and Tcl1. The mechanism through which Pao interacts with Nanog, β-catenin singling pathway and/or the other CSC genes needs to be further investigated in depth. Moreover, as this plant preparation contains a complex mixture of natural compounds, it is possible that Pao affects other molecular targets and pathways that lead to CSC inhibition.

Previous studies on the extract of Pao showed the inhibitory effect on proliferation on pancreatic, ovarian and prostate cancers.[20,24-26] Our animal data here showed promising effects of Pao in inhibiting tumorigenicity and tumor growth, at a dose and administration route that can be easily translated into clinical use. No toxic side effects were observed in mice at this dosage. The inhibition in tumorigenicity implies a possible role of Pao in the prevention of cancer, in addition to data indicating a treatment role. Given that the extracts of Pao Pereira are consumed by the American public as a health supplement, the safety, toxicity, and effects of Pao as an anticancer agent should be further investigated clinically.

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Authors' Note

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Declaration of Conflicting Interests

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