Materials and method

Patients and samples collection

A total of 93 patients who had received radical surgery R0 resection with histological diagnosis of PDAC at the Tianjin Medical University Cancer Institute and Hospital, China from July 2011 to January 2015 were retrospectively collected in this study. Until the last follow-up date of October 23, 2019, the follow-up rate was 100%. Clinicopathological data of the 93 consecutive PDAC patients, including age, sex, tumor size, regional lymph node status, TNM stage, histological grade, differentiation and regional vessel invasion status were obtained. None of the patients had received neoadjuvant chemotherapy or radiotherapy before tissue samples were collected. Systemic gemcitabine-based chemotherapy was given to all the patients.

From January 2018 to November 2019, 39 consecutive cases of fresh PDAC tissues were prospectively collected during operation. The PDAC tissue mass was cut into two parts, one parts were grinded and digested into single cell suspension for flowcytometry, the paired parts were used for IHC detection of ESE3/EHF expression. The usage of these specimens and the patients' information were approved by the Ethics Committee of the Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). All patients provided written consent for the use of their specimens and disease information for future investigations according to the ethics committee and in accordance with recognized ethical guidelines of Helsinki.

Cell culture

Human PDAC cell lines PANC-1, BxPC-3 and SW1990 were obtained from the Type Culture Collection Committee of the Chinese Academy of Sciences (Shanghai, China) and the MiaPaca-2 cell line was obtained from ATCC in 2013. The murine PDAC cell line PANC02 was a gift from Prof. Yang SY (Moffitt Cancer Center, Tampa, FL, USA). The immortalized human PSC cell line ihPSC was established by retrovirus-mediated gene transfer for simian virus 40(SV40) T antigen and human telomerase reverse transcriptase (hTERT) into the human PSCs isolated from the resected pancreas tissue of a patient undergoing operation for pancreatic cancer. *Mycoplasma* contamination was excluded in these cell lines at the beginning of this study. These cells were cultured in DMEM and RPMI1640 basic medium supplemented with 10% Fetal Bovine Serum(FBS) at 37℃ in a humidified atmosphere of 95% air and 5% CO2.

Plasmid construction and lentiviral transduction

shCXCR4 sequences were designed by GeneCopeoia Company (China) and three recommended sequences for CXCR4 genes were synthesized and cloned into the psi-nU6.1 Vectors. Human CRISPR/dCas9-EHF knockout virus and murine CRISPR/dCas9-Ehf-knockout viruses were purchased from Genechemat company (China) and PANC-1-vector/EHF-KO, BxPC-3-vector/EHF-KO and PANC02-vector/Ehf-KO cell lines were constructed according to the manufacturer's instructions.

The establishment of primary cancer cell lines in pancreatic cancer

Fresh human PDAC tissues were obtained during surgery and immediately washed by PBS three times. Blood clots, dead tissues and other connective tissues were removed. PDAC tissues were cut into small pieces (1mm^3) and then PDAC pieces were transferred into 15ml Conical centrifugal tube(Corning) resuspended with a mix of 5ml enzymes buffer containing 1 mg/ml collagenase(Sigma-Aldrich,C2799), 2.5 U/ml hyaluronidase(Sigma-Aldrich, H3506) and 0.1 mg/ml DNase(Sigma-Aldrich DN25) in 37℃ water bath for 4~6 h. The mix were then filtrated in a 30um strainer (MACS Smart Strainer) to obtain single cell suspension. The primary cancer cells were centrifuged, cell pellet was re-suspended with fresh medium and seeded in 6-well plates. Genomic sequencing (including four highly mutated genes: TP53, KRAS, SMAD4 and p16) were performed to investigate the genomic background of primary cancer cell lines and detailed information of PDX1# and PDX2# were listed in supplementary table 4-5. Low-passage (<10 passages) primary cancer cells were used for later experiments.

Immunohistochemistry (IHC)

IHC analysis was performed in PDAC tissues using a DAB substrate kit (ORIGENE, ZLI-9019). All antibodies used in this study were list in supplementary table 9. The intensity of the staining was evaluated using the following criteria: 0, negative; 1, low; 2, medium; and 3, high. The extent of staining was scored as 0, 0% stained; 1, 1%–25% stained; 2, 26%–50% stained; and 3, 51%–100% stained. Five random fields (100×magnification) were evaluated under a light microscope. The final scores were calculated by multiplying the scores of the intensity with those of the extent and dividing the samples into four grades: 0, negative (-); 1–2, low staining (+); 3–5, medium staining (++); and 6–9, high staining (+++). IHC score was determined by two independent pathologists who were blinded to the patients' clinical features and outcomes.

Multiplex fluorescent IHC and Multispectral imaging

Two sets of 93 PDAC tissues were used for immunological assessment of EHF, CD133 and ALDH1. All antibodies used in this study were list in supplementary table 9. CD133 or ALDH1 was labeled by Opal 520(494nm-525nm), EHF was labeled by Opal 650(627nm-650nm) (Perkin Elmer, 2395285). Isotype controls were used for all assays. Stained slides were scanned over the whole slide using the Vectra Polaris system (PerkinElmer). Phenochart slide reviewer (PerkinElmer) was used to systematically capture tissue heterogeneity in an unbiased manner. The selected images were then captured with a 20× lens using the Vectra Polaris system. Form cell Analysis software 2.4 (PerkinElmer) was used to evaluate the counts of EHF, ALDH1 and CD133 positive points per high power field (HPF; 200x). Tumor areas were manually outlined to exclude stromal nuclei. DAPI was used to identify nuclei. EHF was then measured on a cell-nucleus based mode. Five random fields (200×magnification) were evaluated. The count of EHF-positive points per high power field (HPF; 200X) ranged from 0 to 260, mean ± SD, 108.05 ± 65.13. EHF-positive counts/HPF > 108.05 was considered as high-EHF group; EHF-positive counts/HPF < 108.05 was considered as low-EHF group. The count of ALDH1-positive points per high power field (HPF; 200X) ranged from 0 to 30, mean ± SD, 17.61 ± 7.37. ALDH1-positive counts/HPF > 17.61 was considered as high-ALDH1 group; ALDH1-

positive counts/HPF < 17.61 was considered as low-ALDH1 group. The count of CD133-positive points per high power field (HPF; 200X) ranged from 0 to 52, mean ± SD, 27.99 ± 13.35. CD133-positive counts/HPF > 27.99 was considered as high-CD133 group; CD133-positive counts/HPF < 27.99 was considered as low-CD133 group.

Sphere formation assay

PDACs (5000 cells/ml) were cultured in ultra-low adhesion plates (Corning) in serum-free DMEM/F12 medium (GIBCO), which contains B27 (1:50, Invitrogen), 20ng/ml EGF (Proteintech), 10ng/ml FGF2 (Proteintech), 0.4% Bovine Serum Albumin (Sigma) and 5μg/ml insulin (Sigma). After 2 weeks, tumor spheres with diameter>75μm were counted.

CCK8 cell viability and cytotoxicity assay

PANC-1, BxPC-3, PDX1# and PDX2# cells were seeded in clear, flat-bottom 96-well plates (Corning) at a density of 1000 cells per well. The following day, cancer cells were treated with dilution range of ibuprofen(sigma,I4833) (0, 0.2mM, 0.4mM, 0.8mM, 1.6mM, 3.2mM and 6.4mM) or allopurinol(sigma, PHR1377) (0, 0.2mM, 0.5mM, 1mM, 2mM, 5mM, 10mM, 15mM and 20mM) (6 duplications for each concentration) for 72h. And then, culture media were replaced with fresh DMEM containing 10% CCK8 (Bimake, B34302) and plates were incubated for 3 h in an incubator. The absorbance was read at 595nm and the IC50 for ibuprofen or allopurinol were calculated.

Anchorage-independent growth assay

A total of 5000 cells were individualized through 40-μm porous strainers and seeded in a medium solution with 0.7% agar settled on a solidified 1.2% agar layer. Once solidified, fresh medium was added above the cell-containing layer and replaced 3 times a week. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. After 3 weeks in culture, medium was removed and replaced with 1 mg/mL 3-(4, 5-dimethylthiazolyl-2)-2 (MTT, M2128, Sigma-Aldrich) solution. Only the colonies formed by cells with metabolic activity are able to reduce MTT to formazan, forming dark blue crystals. Colonies with a diameter <100 μm were excluded from the analysis.

ALDEFLUOR assay

The ALDEFLUOR™ kit (STEMCELL Technologies, 01700) was used to analyze the subpopulation with the high ALDH enzymatic activity which has been considered as the marker of pancreatic CSCs. The assay follows the protocols suggested by the manufacturer. 1×10^6 cells were suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA) and incubated in a cell incubator for 60 min at 37°C. For the negative control, each sample aliquot was treated with diethylamino benzaldehyde (DEAB), a specific ALDH inhibitor. The cell flow cytometric sorting gates were established using DEAB treated cells as negative controls.

Flowcytometry

To explore the relationship between the expression of EHF and percentages of pancreatic CSCs subsets in PDAC tissues, fresh PDAC specimens were collected and

divided into two parts, one part was immediately fixed in formalin buffer and embedded in paraffin for IHC of EHF; the other part were immediately prepared into the single cell suspension with 1 mg/ml collagenase (Sigma-Aldrich, C2799), 2.5 U/ml hyaluronidase (Sigma-Aldrich, H3506) and 0.1 mg/ml DNase (Sigma-Aldrich DN25). To determine the percentages of CSCs subsets of tumor tissues, single cell suspension was divided into three parts, three different combination of CSCs markers were used, including $ESA^+CD44^+CD24^+$ CSCs, $CD133^+$ CSCs and $ALDH^+$ CSCs. Related antibodies were used according the instructions.

The percentages of CSCs subsets in human pancreatic cancer cell lines were also detected by flowcytometry. Three different combination of CSCs markers was used, including $CD44^+CD24^+$ CSCs, $CD133^+$ CSCs and $ALDH^+$ CSCs.

Besides, the percentages of $CXCR4^+$ (biolegend, 306528) population in fresh PDAC specimens and pancreatic cancer cell lines were also determined by flowcytometry. Isotype controls were used as negative controls. The data were analyzed using soft Flow Jo 10.0.

In vivo limited dilution assay

Female 4–6-week-old NOD/SCID mice were purchased from SPF (Beijing) Biotechnology Company. All mice were maintained in specific pathogen–free conditions, and animal experiment procedures were approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital, in compliance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals.

To validate the effects of EHF on PDAC stemness, mice were randomized into four groups: (A) PANC-1-vector group, (B) PANC-1-EHF group, (C) PANC-1-scramble group, (D) PANC-1-shEHF group. In each group, cancer cells at a dilution range of 1×10^3, 1×10^5 and 1×10^6 were suspended in a 60μl mix of Matrigel plug (Corning,356234) and PBS at a 1:1 ratio and then subcutaneously injected into contralateral flanks of the mice. Primary PDX1#-vector, PDX1#-EHF, PDX1#-scramble and PDX1#-shEHF cell lines was also used to repeat the experiment. Xenograft tumors were observed and measured twice a week using a caliper. All the mice were euthanized at the end of two months. Subcutaneous tumors were harvested and tumor incidence were analyzed. Stem cell frequency were calculated on website http://bioinf.wehi.edu.au/software/elda/.

Reverse transcription PCR (RT-PCR)

The total RNA of the cells was extracted with TRizol (Invitrogen) according to the manufacturer's instructions. Then, the mRNA was reverse transcribed to single-stranded cDNAs using a Reverse Transcription PCR (RT-PCR) System (Takara Bio Inc.). Then, real-time fluorescent quantitative PCR was used to analyze the cDNA levels. Actin was used as a loading control. Related primers were listed in supplementary table10.

Western blotting

Whole-cell extracts were prepared by lysing the cells with SDS protein lysis buffer supplemented with proteinase inhibitor cocktail (bimake, B14001). Protein lysate was separated by SDS-PAGE, and then, the target proteins were detected by Western blotting with the following primary antibodies: anti-EHF (LSBio, LS-B11884,1:5000), anti-

Sox9(abcam,ab185230,1:1000), anti-Sox2(proteintech,66411-1-Ig,1:1000), anti-Nanog(abcam, ab109250,1:1000), anti-Oct4(abcam, ab18976,1:1000), anti-CXCR4(abcam, ab124824,1:1000), anti-E-cadherin(abcam,ab1416, 1:1000), anti-CK19(abcam,ab52625,1:1000) and anti-CA II (abcam,ab124687,1:1000). β-tubulin (Abmart,1:5000) was used as a loading control. Secondary antibodies: Goat anti-rabbit or mouse antibody at 1:5000(Abmart).

Cell Sorting

To determine the effects of EHF on CSCs and non-CSCs, primary pancreatic cancer cell lines were used for cell sorting. PDX1# and PDX2# primary cell lines were stained with anti-human CD133 antibody (Miltenyi Biotec, 130-118-143) and ALDEFLUOR detection kit (STEMCELL Technologies, 01700), respectively; Then, CD133-, CD133+, ALDH- and ALDH+ cells were sorted by FACS. The purities of sorted $CD133^+$ cells and $ALDH^+$ cells were both higher than 95%. The purities of sorted $CD133^-$ cells and $ALDH^-$ cells were both nearly 100%. Cell viability was checked by Trypan blue dye exclusion. The sorted $CD133^+/CD133^-$ cells and $ALDH^+/CD133^-$ cells were transfected with EHF-overexpression plasmids or siEHF. 72 hours later, cells were collected for further experiments.

Preparation of PSC-conditioned medium

PSCs were grown to 70% to 80% confluence in 10cm dishes in complete culture media. Then the medium was replaced with FBS-free DMEM/F12 (1:1), and the cells were cultured for additional 48 h. The medium was collected, centrifuged at 1200 rpm for 5 min, and the supernatant was filtrated through a 0.22 μm filter (Millipore Corp., Billerica, MA, USA). PSC-CM was stored at −80 °C until further use.

To obtain the serum free single cytokine depletion conditional medium, PSCs were pre-treated with serum-free medium for 48h and then the PSCs-CM was collected. The serum free PSC-CM was incubated with the neutralizing antibody of IL6, IL8 and CXCL12, etc. for 48 h at 4 °C to obtain the serum free single cytokine-depleted PSC-CM. Isotype IgG was used as control. Related information of antibodies used was listed in supplementary table 9.

The evaluation of the increasement of PDAC stemness after treating by PSC-CM

For the medium in sphere formation assay, PSCs-CM was added into serum free medium (1:1), then the stem cells culture factors, including B27 (1:50), 20ng/ml EGF, 10ng/ml FGF2, 0.4% Bovine Serum Albumin and 5μg/ml insulin were added. For the control group, the medium was serum free medium with the stem cells culture factors of the same concentration. The increasement of the sphere between PSCs-CM group and control group was calculated in each cell lines. (PDAC-vector, PDAC-EHF, PDAC-scramble and PDAC-shEHF)

In order to investigate the increasement of the ability of PDACs to grow in suspension under the stimulation of PSCs-CM, their capacity to form colonies in solid agar was assessed. 500ul PSCs-CM was added to 500ul top agar, which was used to suspend PDAC-vector/ PDAC-EHF/ PDAC-scramble/ PDAC-shEHF. Control medium was used as control. The increasement of the number of clones between PSCs-CM group and control

group was calculated in each cell lines.

For the cell using in flowcytometry, PDAC-vector, PDAC-EHF, PDAC-scramble, PDAC-shEHF were seeded in 6-well plates (1×10^5 cells/well). The following day, the PDAC culture medium was replaced by a mix of PSC-CM and fresh 1640 medium (at a ratio of 1:1). Pure fresh 1640 medium was used as control. 48 hours later, cancer cells were collected for flowcytometry.

To validate the different effects of PSCs-CM on PDAC stemness in PDAC with different EHF expression, NOD/SCID mice were randomized into PANC-1-vector-control medium group, PANC-1-vector- PSCs-CM group, PANC-1-EHF-control medium group and PANC-1-EHF-PSCs-CM group. In each group, indicated PDAC cells at a dilution range of 1×10^3, 1×10^5 and 1×10^6 were suspended in a 60μl mix of Matrigel and PBS at a 1:1 and then subcutaneously injected into the contralateral flanks. Eight mice for each dilution. 200 ul PSCs-CM or culture medium were injected intratumorally three times a week. PDX1#-vector and PDX1#-EHF were also used to repeat the experiment. Seven mice for each dilution.

To determine EHF decreased the sensitivity of PDACs to PSCs derived CSC-supporting stimulus through CXCR4, cell lines of BxPC3-scramble-scramble, BxPC3-shEHF-scramble, BxPC3-scramble-shCXCR4, BxPC3-shEHF-shCXCR4 were set up. Mice were randomized into BxPC3-scramble-scramble-control medium, BxPC3-scramble-scramble-PSCs-CM, BxPC3-shEHF-scramble-control medium, BxPC3-shEHF-scramble-PSCs-CM, BxPC3-scramble-shCXCR4-control medium, BxPC3-scramble-shCXCR4-PSCs-CM, BxPC3-shEHF-shCXCR4-control medium, BxPC3-shEHF-shCXCR4-PSCs-CM. The increasements of the tumorigenicity between PDAC-PSCs-CM and PDAC-control medium were compared between cell lines.

The evaluation of the increasement of PDAC stemness after stimulated by CXCL12

To explore the difference of the effects of CXCL12 on sphere formation promoting in PDAC with different EHF expression, human recombinant CXCL12 were added into the serum-free medium for cancer cell sphere formation assay. The final concentration of CXCL12 was 100ng/ml.

To explore the difference of the effects of CXCL12 on promoting anchorage-independent growth in PDAC and PDAC-EHF, human recombinant CXCL12 (100ng/ml) was added into RPMI 1640 containing 20% FBS for further culture system. Cells were incubated for 3 weeks and the conditional medium was replaced every 3 times a week.

For the cell using in flowcytometry, PDAC-vector, PDAC-EHF, PDAC-scramble, PDAC-shEHF were seeded in 6-well plates (1×10^5 cells/well). The following day, the PDAC culture medium was replaced by a mix of 1640 contained with 100ng/ml CXCL12. 1640 medium was used as control. 48 hours later, cancer cells were collected for flowcytometry and western blot.

To validate the different effects of CXCL12 on PDAC stemness in PDAC with different EHF expression, NOD/SCID mice were randomized into PANC-1-vector-control medium group, PANC-1-vector-CXCL12 group, PANC-1-EHF- control medium group and PANC-1-EHF-CXCL12 group. In each group, indicated PDAC cells at a dilution range of 1×10^3, 1×10^5 and 1×10^6 were suspended in a 60μl mix of Matrigel and PBS at a 1:1 and then

subcutaneously injected into contralateral flanks. Eight mice for each dilution. Human recombinant CXCL12 or culture medium were injected intratumorally three times a week. PDX1#-vector and PDX1#-EHF were also used to repeat the experiment. Seven mice for each dilution. Tumor proliferation was monitored twice a week. Mice were sacrificed after 2 months. Subcutaneous tumors were harvested and tumor incidence were analyzed. Stem cell frequency was calculated on website. http://bioinf.wehi.edu.au/software/elda/.

Chromatin immunoprecipitation (Ch-IP) and luciferase analysis

Ch-IP assays were performed using Ch-IP kit (Millipore) according to the manufacturer's instructions. To detect if EHF directly bound to de promoter region of Sox2, Sox9, Nanog, Oct4, CXCR4, PANC-1 were immunoprecipitated with anti-EHF antibody (abcam, ab220113). Then the immunoprecipitated products were detected by PCR. Related primers were listed in supplementary table 10. To detect if EHF directly suppressed the transcriptional activity of CXCR4, luciferase analyses were conducted using dual-luciferase reporter assay kit (Promega, E1910). PANC-1 and 293T cells were transfected with pCDH-EHF plasmid or control vector (pCDH-vector), which were subsequently transfected with pGL3-CXCR4-EBS1-wt and pGL3-CXCR4-EBS1-mut, respectively. Forty-eight hours later, cells were subjected to dual luciferase analysis. Related sequences of the vectors for luciferase analysis were listed in supplementary table 11.

Similarly, to elucidate if PPARγ directly bound to the promoter region and suppressed the transcriptional activity of EHF, PANC-1 were immunoprecipitated with anti-PPAR γ (abcam, ab45036). The immunoprecipitated products were detected by PCR. PANC-1 was transfected with pGL3-EHF-PPRE(WT) plasmid or control vector(pGL3-vector).Then PANC-1 were treated with 10μM rosiglitazone for 24 hours. DMSO was used as negative control. Renilla reporter plasmid was used as internal control. Forty-eight hours later, cells were subjected to dual luciferase analysis.

The information of the related Ch-IP primers was listed in supplementary table 10. The results of luciferase analysis were expressed as a fold induction relative to the cells transfected with the control vector after normalization to Renilla activity.

In vitro drug-screening for EHF agonists among drugs of routine medication

For EHF agonists screening, $1x10^5$ PANC-1 were suspended with DMEM (10%FBS) and seeded in 6-well plates. 12 hours later, PANC-1 was adherent to the bottom of the plate, the medium was replaced with 1ml DMEM (10%FBS) with 10 μ M compounds added, the cells were cultured for additional 24 h. Then cells were collected for evaluating EHF expression by western blot. Detailed compounds information was listed in supplementary table 3.

In vitro evaluation of the therapeutic effect of rosiglitazone

To evaluate the effects of rosiglitazone on the maintenance of the stemness of PDACs, adherent cancer cells were pre-treated with 5μM rosiglitazone(Sigma,R2408) for 48 hours (DMSO was used as control) , which were collected for sphere formation assays. To avoid cellular toxicity, no continuous infusion of rosiglitazone was added into sphere formation system. After 2 weeks, tumor spheres with diameter>75μm were counted.

In vivo evaluation of the therapeutic effect of rosiglitazone

To evaluate the effects of rosiglitazone on PDAC stemness under the stimulus of CXCL12, NOD/SCID mice were randomized into four groups (DMSO was used as the control for rosiglitazone, culture medium was used as the control for CXCL12): (A) control medium +DMSO, (B) control medium +rosiglitazone, (C) CXCL12+DMSO, (D) CXCL12+rosiglitazone. In each group, PANC-1 cells at a dilution range of 1×10^3, 1×10^5 and 1×10^6 were suspended in a 60μl mix of Matrigel and PBS at a 1:1 ratio and then subcutaneously injected into mice. One week later, rosiglitazone (100mg/kg/day; DMSO was used as control) and human recombinant CXCL12 protein (20μg/mouse/day; control medium was used as control) were intratumorally injected three times a week. Tumor proliferation was monitored twice a week. Mice were sacrificed after 2 months; subcutaneous tumors were harvested and tumor incidence were analyzed. Stem cell frequency were also calculated on website http://bioinf.wehi.edu.au/software/elda/.

To better determine the effects of rosiglitazone on PDAC, 5×10^5 PANC02-luc cell was resuspended using a 40ul mix of matrigel plug and PBS(1:1) per mouse for the establishment of orthotopic tumor models. BABL/C nude were randomized into two groups :(A) DMSO control group(n=8), (B) rosiglitazone treatment group(n=8). One week after tumor inoculation, rosiglitazone (100mg/kg/day) or DMSO were injected intraperitoneally three times a week and continued to one month. Tumour growth was analysed by bioluminescent imaging (BLI) twice a week. Pancreatic tumours were harvested for further experiments at the end of one month. To evaluate the effects of rosiglitazone on $ALDH^+$ cells, fresh tumour tissues were prepared into the single cell suspension with 1 mg/ml collagenase, 2.5 U/ml hyaluronidase and 0.1 mg/ml DNase and then cells were stained with ALDEFLUOR kit for flowcytometry analysis (PI was used to exclude dead cells and CD45 was used to exclude leukocytes). IHC and western blot on tumor tissues were carried out to detect the expression of EHF and stemness markers (Sox9, Sox2, Nanog and Oct4). Another cohort of orthotopic tumor models were also established which received the same treatment in each groups Motilities of the mice were recorded and survival curves were plotted.

To determine if rosiglitazone suppressed the stemness of PDAC mostly via EHF, PANC02-ctrl-luc and PANC02-EHF-KO-luc cell lines were established. BABL/C nude mice were randomized into two groups: (A) DMSO control group, (B) rosiglitazone treatment group. In each group, pancreatic orthotopic tumor models was established using a 40ul mix of matrigel plug and PBS(1:1) per mouse containing 5×10^5 PANC02- ctrl-luc(n=8) or PANC-EHF-KO(n=8) cell lines. One week later, rosiglitazone (100mg/kg/day) or DMSO were injected intraperitoneally three times a week and drug treatment was continued to one month. Tumour growth was analysed by bioluminescent imaging (BLI) twice a week. Motilities of mice was recorded and survival curves were plotted.

Breeding and genotyping analysis for KPC mice model

LSL-KrasG12D/+ mice, LSL-Trp53R172H/+ and Pdx1-Cre mouse model was a gift from the Stephen J. Simpson Lab (Broadway Research Building at Johns Hopkins University, USA) and mice were maintained in specific pathogen-free conditions. The breeding

strategy to get KPC mice often started with crossing LSL-Kras$^{G12D/+}$ mice with LSL-Trp53$^{R172H/+}$ mice to obtain LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$ (KP) mice. LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$ mice (KP) male mice were then crossed with Pdx-1-Cre female mice to generate LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$; Pdx-1-Cre (KPC) mice. Genomic DNA of mice tails were purified by Mouse direct PCR kit (Bimake, B40013) and then polymerase chain reaction (PCR) was performed for the genotyping of KPC mice. The primer sequences and PCR conditions for the genotyping of KPC mice were listed in supplementary table 7-8. All animal experiment procedures were approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital, in compliance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals.

Ultrasound imaging for pancreatic tumor in KPC mice

Pancreatic tumor of KPC mice were monitored by the VEVO2100 ultrasound imaging system (Visual Sonics). KPC mice were anesthetized and taped to the imaging stage. KPC mice were imaged in supine position; then, images of pancreas were captured using the abdominal package in B-Mode and long diameter/short diameter of pancreatic tumor were measured. Tumor volume(V) was calculated by the following formula: V= (long diameter) × (short diameter)^2/2.

Preclinical animal cohorts

Pancreatic tumor volume of KPC mice were monitored twice a week by the VEVO2100 ultrasound imaging system (Visual Sonics).When pancreatic tumor of KPC mice initiated and reached 20~60mm^3 , mice were then randomized into four groups: (A) vehicle (Corn oil purchased from Sigma, C8267; 1.0ml/kg once a day by oral gavage), (B) gemcitabine (purchased from MCE, HY-17026; 25mg/kg intraperitoneally once a week), (C) rosiglitazone (purchased from Sigma, C2408; rosiglitazone were pre-dissolved in olive oil; 100mg/kg once a day by oral gavage), (D) gemcitabine +rosiglitazone. Mice were separated in 2 sets. For set 1(8 mice per group), drug was administrated when the tumor reached 20~60mm^3 until death. In set 2 (6 mice per group), mice were treated like in set 1 but killed after 8 weeks of treatment to compare tissues. The volume of pancreatic tumor was monitored twice a week by the VEVO2100 ultrasound imaging system. For mice in set 2, pancreatic tumor tissues were harvested and weighed. Tumor tissues were divided into three parts: first part was immediately made into single cell suspensions and prepared for detection of ALDH activity by flow cytometry (PI was used to exclude dead cells and CD45 was used to exclude leukocytes); second part of the tumor tissues were immediately fixed in buffered formalin and embedded in paraffin and another part were kept at -80℃ for protein extraction and western blot analysis. Tissue slides(5μm) were prepared and Hematoxylin & Eosin (H&E) staining were performed for histopathological analysis according to instructions. IHC of Ki67 (abcam, ab16667) staining were performed to evaluate proliferation status of tumor tissues. Tissues protein from vehicle group and rosiglitazone group were prepared for western blot to analyze the expression of EHF and stemness markers (Sox9, Sox2, Nanog and Oct4).

In vitro evaluation of the therapeutic effect of ibuprofen and allopurinol

To evaluate the effects of ibuprofen and allopurinol on the maintenance of the stemness of PDACs, adherent cancer cells were pre-treated with 1mM ibuprofen (sigma, I4833) or 5 mM allopurinol (sigma, PHR1377) for 48 hours (DMSO was used as control), which were collected for flowcytometry analysis of $CD44^+CD24^+$ cells, western blot of stemness genes (Sox9, Sox2, Nanog and Oct4) and sphere formation assays. To avoid cellular toxicity, no continuous infusion of ibuprofen or allopurinol was added into sphere formation system. After 2 weeks, tumor spheres with diameter>75μm were counted.

In vivo evaluation of the therapeutic effect of ibuprofen and allopurinol

To evaluate the effects of ibuprofen or allopurinol on PDAC stemness, NOD/SCID mice were randomized into two groups (DMSO was used as the control): (A) DMSO group (B) ibuprofen or allopurinol group, PANC-1 cells at a dilution range of 1×10^3, 1×10^5 and 1×10^6 were suspended in a 60μl mix of Matrigel and PBS at a 1:1 ratio and then subcutaneously injected into 4-6-week-old NOD/SCID mice. One week later, ibuprofen (45mg/kg/day; DMSO was used as control) or allopurinol (30mg/kg/day; DMSO was used as control) were peritumorally injected three times a week. Tumor proliferation was monitored twice a week. Mice were sacrificed after 2 months; subcutaneous tumors were harvested and tumor incidence were analyzed. Stem cell frequencies were also calculated on website http://bioinf.wehi.edu.au/software/elda/. Proportion of $ALDH^+$ cells in subcutaneous tumors were analyzed by flowcytometry.