

# Nigericin Suppresses the Wnt/ $\beta$ -catenin Signaling in Pancreatic Cancer through Targeting pre-miR-374b-PRKCA/HBP1 Axis

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## Research

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# Abstract

## Background

The polyether antibiotic nigericin has been demonstrated recently to have anti-tumor activity in multiple cancers. But the biochemical basis for its anti-cancer effects has not been fully elucidated. The objective of this study was to investigate the potential mechanisms of nigericin in pancreatic cancer (PC) cells.

## Methods

PC cells were exposed to increasing concentrations of nigericin at different time periods, and the corresponding IC<sub>50</sub> values were calculated. Then the effects on the biological functions of PC cells were evaluated. Subsequent experiments including the high-throughput RNA sequencing, qRT-PCR, western blot, TOP/FOP-Flash reporter, Co-Immunoprecipitation and luciferase report assays were employed to reveal the potential mechanisms of nigericin. In addition, the inhibitory effects of nigericin on PC cells were also accessed in the subcutaneous tumor model.

## Results

The data showed that nigericin was extremely sensitive to PC cells, and could influence the abilities of cell proliferation, colony formation, apoptosis, migration and invasion. The results *in vitro* implied that nigericin suppressed the Wnt/ $\beta$ -catenin signaling by up-regulating PRKCA and HBP1 mRNA expressions. Furthermore, the dual strands of pre-miR-374b were proved to down-regulate the PRKCA and HBP1 expressions coordinately, and over-expression of pre-miR-374b partly antagonized the suppressing effects of PC cells induced by nigericin. Meanwhile, the inhibitory effects of nigericin on PC cells were also confirmed in mice.

## Conclusion

These findings demonstrated that suppressing the Wnt/ $\beta$ -catenin signaling pathway by targeting pre-miR-374b-PRKCA/HBP1 axis might represent a novel molecular mechanism of nigericin in PC. Nigericin remained a candidate for a potential pre-clinical application for PC.

## Background

Nigericin, an antibiotic derived from *Streptomyces hygroscopicus*, is an ionophore and acts as an antiporter of  $K^+/H^+$  and raises the pH of acidic compartments. Early in 1987 and 1989, DNA synthesis in Ehrlich ascites tumor cells was found to be inhibited due to the decreased intracellular pH by the exogenous  $K^+/H^+$  antiporter nigericin [1–2]. Then a growing body of evidence supports that nigericin has

exhibited a promising anti-cancer activity in various types of cancers, including glioblastoma [3], lung cancer [4–5], breast cancer [6] and colorectal cancer [7]. Though the anti-cancer effects of this drug have been comprehensively discussed, the mechanisms are still largely a mystery. For example, nigericin could selectively target cancer stem cells (CSCs) in nasopharyngeal carcinoma, which could be considered as a candidate CSCs targeting drug for clinical evaluation [8]. In 2012, a study from Zhou et al. demonstrated that nigericin showed toxicity to colorectal cancer cells, and partly reversed the epithelial-mesenchymal transition (EMT) process during cell invasion and metastasis [7]. Mashima et al. revealed that the antibiotic nigericin inhibited AR-mediated activation of the PSA promoter and PSA production in prostate cancer cells [9]. Besides, nigericin might be exploited to enhance drug sensitivity when used in combination with other drugs, such as adriamycin [10], amiloride [11] and melphalan [12]. Our previous study demonstrated that nigericin could significantly suppress the tumor growth and metastasis of colorectal cancer cells by inhibiting Wnt/ $\beta$ -catenin signaling pathway and directly targeting the  $\beta$ -catenin destruction complex [13]. Similar reports were also found in ovarian cancer and chronic lymphocytic leukemia, which strongly indicated that the Wnt signaling pathway might be involved in the nigericin damage [14–15].

The Wnt signaling pathway is highly conserved throughout vertebrate species and has critical roles in embryonic development, tissue self-renewal and human diseases [16]. The role of Wnt signaling in carcinogenesis has most prominently been described for colorectal cancer [17], but aberrant Wnt signaling is also observed in many more cancer entities [18–19]. The Wnt signaling pathway is complex and includes canonical and noncanonical branches. The canonical pathway is activated by Wnt family ligands binding to Frizzled/LRP receptor complexes, which leads to the accumulation of non-phosphorylated  $\beta$ -catenin in the cytoplasm. After its translocation to the nucleus,  $\beta$ -catenin forms a complex with members of the TCF/LEF family of transcription factors, and activates target genes such as c-Myc, Cyclin D1 and Survivin [16, 20]. In 2018, in the United States, 55,440 new pancreatic cancer cases were diagnosed. By 2030, PC is projected to cause the second highest cancer-related mortality rate in the US. PC is a highly aggressive malignancy with few effective therapies and a very poor prognosis. The 5-year overall survival rate of PC is less than 5% [21–22]. The role of Wnt signaling in PC carcinogenesis, metastasis, and prognosis has been reported. Its aberrant activation resulting from activating or inactivating mutations in the CTNNB1 gene locus, or in the negative regulators AXIN and APC involving stabilization of  $\beta$ -catenin, and activation of target genes leads to a more aggressive phenotype of PC [23]. Meanwhile, the Wnt signaling drives drug resistance in PC in many ways [24]. Wnt signaling and the nuclear functions of  $\beta$ -catenin have been also reported to be critical for CSCs proliferation, differentiation, and maintenance [25–26].

The aim of this study was to test the anti-cancer activity of nigericin in human PC cells *in vitro* and *in vivo*. The drug was sensitive to PC cells, and the effects on the abilities of cell proliferation, colony formation, apoptosis, migration and invasion in PC cells were evaluated. Then the high-throughput RNA sequencing, qRT-PCR, western blot, TOP/FOP-Flash reporter and Co-Immunoprecipitation assays were performed, and the data revealed that nigericin could significantly suppress the Wnt/ $\beta$ -catenin signaling by up-regulating PRKCA and HBP1 mRNA expressions. Furthermore, the intramolecular stem-loop

structure of pre-miR-374b, which could be spliced into the mature miR-374b-5p and -3p, might simultaneously target the PRKCA and HBP1 3'-UTR sites. It was also shown that pre-miR-374b could reverse the inactivation of the Wnt/ $\beta$ -catenin pathway induced by nigericin *in vitro*. In addition, the inhibitory effects of nigericin on PC cells were also accessed in the subcutaneous tumor model.

## Methods

### Chemicals and cell culture

Human PC cell lines (PANC-1 and PL-45) were purchased from Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Cells were in the logarithmic stage of growth for experiments. Nigericin was purchased from Sigma Aldrich (#481990, USA) and stored in dimethyl sulfoxide (DMSO) at -20 °C.

### Cell viability and 50% inhibiting concentration (IC50) values

PC cells (PANC-1 and PL-45) were seeded in a 96-well plate at a density of  $3 \times 10^4$  cells and cultured at 37 °C for 24 h. After nigericin treatment with different concentrations (0, 0.125, 0.25, 0.5, 1, 2, 5, 10, 20 or 50  $\mu$ mol/L) at 0, 4, 8, 16, 32 or 48 h, cells were cultured with 20  $\mu$ l of 5 mg/ml MTT (Sigma, China) for 4 h at 37 °C, and then treated with 200  $\mu$ l of DMSO for 20 minutes. The absorbance of each well was measured by a Microplate Reader (Epoch, Winooski, USA) at 490 nm. IC50 values were measured at 4, 8, 16, 32 and 48 h respectively.

### Cell proliferation, colony formation and apoptosis assays

Treated cells were harvested and incubated with 100  $\mu$ l of culture medium in 96-multiwell plates ( $3 \times 10^3$  cells per well) for 24 h at 37 °C in 5% CO<sub>2</sub>. Then cell number was evaluated using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) for different time (0, 24, 48 and 72 h). CCK-8 (10  $\mu$ l) was added in each well. After 1 h incubation at 37 °C, the absorbance at 450 nm in each well was detected with plate reader. For colony formation assay,  $3 \times 10^3$  cells were plated in 6-well plates in complete culture medium containing 0.3% agar on the top of 0.6% agar in the same medium. After the plates were incubated at 37°C with 5% CO<sub>2</sub> for 14 days, the colonies were stained with 0.1% crystal violet. The colonies were counted in 5 random fields under the inverted microscope IX71 (Olympus, Japan). Colonies containing at least 50 cells were counted. For cell apoptosis, cells were double stained with Annexin-V-PE (BD Pharmingen, San Jose, CA, USA) and propidium iodide (PI) (Sigma, St. Louis, USA) following the manufacturer's instructions. Then cell apoptosis was determined by FACSscan flow cytometer.

### Migration and invasion assays

The migration and invasion assays were performed with transwell chambers (8  $\mu$ m pore size, 24-well insert, Corning Lowell, MA, USA). Cells were collected and suspended in the serum-free medium. Then 200  $\mu$ l serum-free medium containing  $1 \times 10^4$  cells was added to the upper chamber, while the 600  $\mu$ l

medium containing 10% FBS was added to the lower chamber as chemical attractant. Cells were incubated at 37°C for 48 h, and the non-invasive cells were removed with cotton swabs. Finally, the inserted membrane was fixed in methanol for 10 min and stained with 0.1% crystal violet for 20 min, and the infiltrated cells were counted and photographed under the inverted optical microscope IX71 (Olympus, Japan). For cell invasion evaluation, the insert of the chamber was prepared by Matrigel (100 µl, BD Biosciences, USA) covering the upper surface. Other methods were similar to the migration assay. The invaded cells were also counted and photographed under the microscope.

## Western blot analysis

Cells were lysed using the RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with proteinase inhibitor (Solarbio). Protein concentrations were quantified using the BCA protein assay (Pierce). The cell lysate containing 30 µg proteins was heat denatured and subjected to SDS-PAGE, followed by transfer to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) by semi-dry blotting. The membranes were blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h, and then incubated with the primary and secondary antibodies and visualized with the SuperSignal West Pico Chemiluminescent HRP substrate (Thermo). The primary antibodies used in this study were raised against  $\beta$ -catenin (#8480, CST), p- $\beta$ -catenin (#4176, CST), PRKCA (#2056, CST), GSK-3 $\beta$  (#9315, CST), HBP1 (sc-376831, Santa Cruz), TCF-4 (BS91324, Bioworld), Cyclin D1 (#2922, CST), AXIN2 (ab32197, Abcam), c-Myc (ab32072, Abcam),  $\beta$ -actin (ab82226, Abcam) and Histone (ab1791, Abcam). Protein levels were calculated relative to  $\beta$ -actin and Histone.

## TOP/FOP flash assay

Luciferase reporter assays of the Wnt/ $\beta$ -catenin signaling were performed with the TOP and FOP reporters that contained wild-type and mutated TCF/LEF DNA-binding sites, respectively. TOP/FOP-Flash was co-transfected into cells along with PRKCA (or HBP1) silence or pre-miR-374b overexpression vector. The TOP/FOP-Flash values were normalized to the *Renilla reniformis* (Promega) reading and the TOP/FOP ratio was measured, as previously described [13]. Experiments were performed in triplicate.

## High-throughput RNA sequencing and bioinformatics analysis

PANC-1 cells were exposed to nigericin (5 µmol/L) for different time periods (0, 8, 16 or 32 h), and the high-throughput RNA sequencing was performed as previously described [27]. These mRNAs and miRNAs with statistical significance were screened with p-value less than 0.05, false discovery rate (FDR) less than 0.05 and fold change (FC) more than 2.0 (or FC more than 1.5 for miRNA analysis). The common expressed elements among 3 compared groups (0 h vs 8 h, 0 h vs 16 h, 0 h vs 32 h) were analyzed using the venn analysis. The non-supervised hierarchical clustering was used in the form of heat map to present the expression patterns of the differentially expressed cancer-related mRNAs or miRNAs. Based on the results of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for the common differentially expressed mRNAs, the network between KEGG pathways and their corresponding genes was used to elucidate that these pathways including the Wnt signaling pathway. The common

differentially expressed miRNAs and mRNAs were subjected to the analysis and the putative targets of miRNAs were predicted by means of Targetscan (<http://www.targetscan.org/>). Then the miRNA-mRNA interaction network between up-regulated miRNAs and their corresponding down-regulated mRNAs (or down-regulated miRNAs and their corresponding up-regulated mRNAs) was conducted. High-throughput sequencing data reported herein had been deposited at the NCBI website (<https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA543685&go=go>) with the accession number PRJNA543685 (SRR9107550, SRR9107551, SRR9107552 and SRR9107553).

## **Small interfering RNA (siRNA), miRNA regents and plasmid vector construction**

The siRNAs targeting genes of PRKCA and HBP1 were designed using online siRNA programs (<http://sidirect2.rnai.jp/>). The Small interfering RNAs of PRKCA (Sense: AAGAAAAGUAACAAUUCAU; Anti-sense: GAAUUUGUUACUUUUUCUUGU) and HBP1 (Sense: UAUAUCCAGGUGAAGAUGGCA; Anti-sense: CCAUCUUCACCUGGAUAUAAC) were synthesized and obtained from Shanghai GenePharma Co., Ltd. MiR-374b-5p (or miR-374b-3p) mimic and inhibitor were also designed and synthesized from GenePharma along with the negative control (NC or anti-NC). Pre-miR-374b over-expression vector (pGCMV/EGFP/pre-miR-374b) was constructed and purchased from Genepharma (Shanghai, China), and a transfectant of the pGCMV/EGFP empty vector was used as a control. At 48 h after transfection, the transfection efficiency was estimated by observing the green fluorescent protein (GFP) signal under the inverted optical microscope IX71 (Olympus, Japan), and the PCR analysis was also used to detect the expression of pre-miR-374b in the collected cells. We also obtained stably transfected clones by blasticidin selection (Sigma, USA) for animal observation.

## **Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from PC cells using TRIzol (Invitrogen, USA) according to the manufacturer instructions, and then cDNA was generated from RNA using SuperScript III (Invitrogen). MRNAs and miRNAs expressions were evaluated using the SYBR green quantitative PCR kit (Life Technologies, USA) based on the manufacturer description. GAPDH and U6 were performed as a control internally for mRNAs and miRNAs respectively. QRT-PCR was performed on the ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems). Levels of relative expression were calculated and quantified with the  $2^{-\Delta\Delta Ct}$  method. Primers were presented in **Supplementary Table 1**.

## **Co-Immunoprecipitation (Co-IP)**

For immunoprecipitation, PANC-1 cells were treated by nigericin (1  $\mu\text{mol/L}$ ) or transfected with si-PRKCA or HBP1 for 48 h. Cells were washed twice with ice-cold PBS and lysed with lysis buffer containing phosphatase and protease inhibitor mixtures (Roche, Germany). Lysates were precleared for 2 h with Protein G-Dynabeads (Invitrogen) and then incubated overnight with anti-p- $\beta$ -catenin (#4176, CST), anti- $\beta$ -TrCP (#2056, CST), anti-HBP1 (sc-376831, Santa Cruz), anti-TCF-4 (BS91324, Bioworld ) or isotype

control prebound to Protein G-Dynabeads. The resin was washed three times with ice-cold lysis buffer, resuspended in 30  $\mu$ l of Laemmli sample buffer, boiled for 3 min and centrifuged at 14,000 g for 5 min. The immunoprecipitated samples were analyzed by immunoblotting under standard conditions.

## Luciferase reporter assay

The full-length of PRKCA (or HBP1) 3'UTR containing the predicted wild-type or mutated miR-374b-5p (or miR-374b-3p) binding sites were amplified by PCR and cloned into the downstream of the firefly luciferase reporter gene in pMIR (Ambion, USA) vectors to generate recombinant vectors PRKCA-wt and PRKCA-mut. The constructed luciferase reporter plasmids were co-transfected with miR-374b-5p (or miR-374b-3p) mimics or inhibitors or over-expressing pre-miR-374b vectors using Lipofectamine 2000 reagent (Invitrogen). After 48 h of incubation, the firefly and Renilla luciferase activities were quantified with a dual-luciferase reporter assay (Promega, USA).

## Tumor growth in nude mice

Four-week old male BALB/c-nude mice were purchased and maintained in a sterile environment (each group contained 5 mice). PANC-1 cells or that stably expressing pre-miR-374b were injected subcutaneously into the flanks of nude mice with a total volume of 200  $\mu$ l. Nigericin (4 mg/kg) was injected intraperitoneally every 2 days. Mice body weights and tumor volume (TV) were measured weekly, and TV was calculated as follows:  $TV (mm^3) = \text{length} \times \text{width}^2 \times 0.5$ . Bioluminescent imaging was performed on the 28th day after injection at which mice were anaesthetized and given D-luciferin potassium salt (150 mg/kg in phosphate-buffered saline, Sciencelight, Shanghai, China). After 20 min of injection, the bioluminescence was imaged with a charge-coupled device camera (IVIS; Lumina II, PerkinElmer). Signal was displayed as photons/s/cm<sup>2</sup>/sr. Mice were killed after the bioluminescent assay and the tumor mass were removed and stored at -80 °C for the subsequent PCR analysis. The animal experiment was approved by the Animal Care and Use Committee of the First Affiliated Hospital of Soochow University.

## Statistical analysis

All data analysis was performed using SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA). Measurement data were presented as mean  $\pm$  standard deviation (SD). Data differences between two groups were compared by the *t*-test, while differences among multiple groups were compared using one-way analysis of variance (ANOVA). *P* < 0.05 was considered statistically significant.

## Results

### PC cells were sensitive to nigericin by IC50 comparison

PANC-1 and PL-45 cells were exposed to increasing concentrations of nigericin for different time periods (0, 4, 8, 16, 32 or 48 h). As shown in Fig. 1A-B, nigericin treatment significantly suppressed the cell viabilities in a concentration dependent manner both in PANC-1 and PL-45 cells. Besides, the

corresponding IC50 values were also calculated, and our results showed that nigericin could also inhibit the cell viability in a time-dependent manner (Fig. 1C-D).

## **Nigericin inhibited the tumorigenic behaviors of PC cells *in vitro***

Since PC cells were sensitive to nigericin, we next determined whether nigericin exerted inhibitory effects on tumorigenic behaviors of PC cells *in vitro*. As calculated above, we chose 1  $\mu\text{mol/L}$  as the proper treated concentration of nigericin on PANC-1 and PL-45 cells (less than the IC50 values at 48 h for our subsequent experiments *in vitro*). We examined the cell proliferation ability using CCK-8 and colony formation assays. As demonstrated in Fig. 2A-D, nigericin significantly exerted the similar inhibitory effects on cell proliferation and colony forming abilities of PANC-1 and PL-45 cells. Conversely, nigericin treatment increased the apoptotic rates of PC cells (Fig. 2E-F). Based on the idea that nigericin served as a potential anti-cancer drug of PC, we further explored its effects on cell migration and invasion. We found that the migration and invasive abilities of PANC-1 cells were significantly suppressed, and similar results were also observed in PL-45 cells (Fig. 2G-J). These results indicated that nigericin exerted direct and anti-cancer effects on PC cells.

## **Nigericin suppressed the Wnt/ $\beta$ -catenin signaling in PC cells**

To reveal the potential mechanisms of nigericin in PC, we used the high-throughput RNA sequencing analysis to screen the expression profile of mRNAs in PC cells after nigericin treatment at different time points (0, 8, 16 and 32 h). 172 mRNAs were commonly dys-regulated, and part of cancer-related mRNAs had been described in the hierarchical clustering analysis (**Supplementary Fig. 1A**). To ensure that our results were reliable, we assessed the expressions of 5 random mRNAs between the 0 h and 32 h group by qRT-PCR. Consistent with the data of RNA sequencing (**Supplementary Fig. 1B**), the results showed that the mRNA expressions of UGCG, HBP1 and MCL1 were significantly up-regulated at 32 h, compared to the 0 h group. Oppositely, other 2 mRNAs (CENPC and RPA2) were down-regulated (**Supplementary Fig. 1C**). These data were consistent well with the sequencing data, which demonstrated the high reliability and validity of the sequencing expression results. The network between KEGG pathways and their corresponding genes strongly implied that Wnt/ $\beta$ -catenin signaling was involved in the nigericin-mediated effects on PC cells (**Supplementary Fig. 1D**).

To identify the Wnt signaling that might play a role in nigericin damage, the expressions of downstream effectors of the pathway were determined. The western blot analysis demonstrated that the protein expression levels of total  $\beta$ -catenin, cytosolic  $\beta$ -catenin, nuclear  $\beta$ -catenin, Cyclin D1, AXIN2 and c-Myc were down-regulated after nigericin treatment at 48 h in a concentration-dependent manner. Whereas, the dose-dependent increased protein expressions of p- $\beta$ -catenin, PRKCA and HBP1 were also observed in PANC-1 and PL-45 cells (Fig. 3A-B). Besides, the transcriptional activities of  $\beta$ -catenin/TCF/LEF using the

TOP Flash cell reporter system were evaluated. The data showed that the relative TOP/FOP ratios were significantly down-regulated in a dose-dependent manner, compared to the non-nigericin treated PANC-1 and PL-45 cells (Fig. 3C-D). This was the preliminary evidence that nigericin could suppress the Wnt/ $\beta$ -catenin pathway in PC cells.

## **Nigericin suppressed the Wnt/ $\beta$ -catenin signaling through up-regulating PRKCA/HBP1 expressions**

PRKCA and HBP1 were two most-regulated and Wnt signaling-related molecules by the high-throughput RNA sequencing analysis. To further identify the potential roles of these two molecules in nigericin-mediated process, we firstly determined the mRNA or protein levels of PRKCA and HBP1 after small interfering RNAs respectively. The data showed that nigericin treatment significantly up-regulated the mRNA and protein levels of PRKCA or HBP1, and the small interfering RNAs which specifically targeted PRKCA or HBP1 were effective for the subsequent experiments (**Supplementary Fig. 2A-D**).

Regarding the inhibiting effects of Wnt/ $\beta$ -catenin pathway by nigericin, we also evaluated the roles of PRKCA or HBP1 in PC cells. The results of western blot analysis demonstrated that si-PRKCA could significantly up-regulated the protein expressions of PRKCA downstream effectors such as nuclear  $\beta$ -catenin, Cyclin D1, AXIN2 and c-Myc. Moreover, si-PRKCA treatment could also partly recover the expressions of these PRKCA downstream proteins induced by nigericin damage (Fig. 4A-B). Similar results were also found in HBP1 knocked down cells (Fig. 4C-D). Furthermore, the TOP Flash cell reporter system was also employed, and our data implied that the Wnt/ $\beta$ -catenin signaling was significantly inactivated by nigericin. In comparison with nigericin-treated cells, si-PRKCA, si-HBP1 or silencing these two molecules simultaneously could partly activate the pathway induced by nigericin in PANC-1 and PL-45 cells (Fig. 4E-F). These results indicated that PRKCA and HBP1 might be the key molecules in nigericin-mediated damage in PC cells. Besides, the co-IP assays also elucidated that PRKCA mediated  $\beta$ -catenin phosphorylation with  $\beta$ -TrCP was a novel mechanism regulating the Wnt/ $\beta$ -catenin pathway, and HBP1 physically interacted with TCF-4 and served as a negative regulation of Wnt/ $\beta$ -catenin pathway in PC cells. Interfering PRKCA or HBP1 expression presented a re-activated effects of the Wnt/ $\beta$ -catenin pathway induced by nigericin (**Supplementary Fig. 3A-D**).

## **Pre-miR-374b selectively targeted PRKCA and HBP1 in PC cells**

Since PRKCA and HBP1 played an essential role in nigericin-mediated damage in the perspective of mRNA levels, we hypothesized that these two molecules might be regulated singly or simultaneously by other potential molecules. Thus, we screened the expression profile of miRNAs from our high-throughput RNA sequencing at 4 different time points (0, 8, 16 and 32 h) (Fig. 5A). Three comparison groups (0 h vs 8 h, 0 h vs 16 h and 0 h vs 32 h) were set, and the venn analysis was performed to determine the common differentially expressed miRNAs among these 3 different comparison groups. A total of 74 common differentially expressed miRNAs (48 up- and 26 down-regulated) were found throughout the nigericin treatment (Fig. 5B-C). Then the changes of 5 random common differentially expressed miRNAs including

miR-2210, miR-449a, miR-374b-3p, miR-1268b and miR-29c-5p were presented from the high-throughput RNA sequencing in Fig. 5D, and the results of PCR analysis verified the reliability of the sequencing data (Fig. 5E). Besides, the interactions between miRNAs and their target genes from the sequencing data were theoretically predicted by conserved seed-matching sequence. The miRNA-mRNA interaction network between up-regulated miRNAs and their corresponding down-regulated mRNAs were presented (Fig. 5F). Similar network between down-regulated miRNAs and their corresponding up-regulated mRNAs was also constructed (Fig. 5G).

Subsequently, the venn analysis among PRKCA-predicted, HBP1-predicted and down-regulated miRNAs identified miR-374b as the potential upstream molecule of PRKCA and HBP1 (Fig. 6A). As described in Fig. 6B, has-pre-miR-374b had a stem-loop, which could be spiced into the mature miR-374b-5p and -3p by the Dicer protein. The results of PCR analysis demonstrated that nigericin could significantly down-regulate the pre-miR-374b expression in PC cells, and the expressions of mature miR-374b-5p and miR-374b-3p were also changed time-dependently by nigericin (Fig. 6C-E). To reveal the potential regulations between the mature miR-374b (miR-374b-5p and miR-374b-3p) and the Wnt-related genes (PRKCA and HBP1), the conserved seed-matching sequences were predicted (Fig. 6F). Then the results of luciferase report assays confirmed the potential relationships between miR-374b-5p (or miR-374b-3p) and PRKCA 3'-UTR sites (or HBP1 3'-UTR sites) (Fig. 6G-H). More importantly, when the pre-miR-374b over-expressing vector was co-transfected with a wild-type or mutant 3'-UTR fragment of PRKCA (or HBP1) pMIRREPORT luciferase vector into the HEK293T cells. The relative luciferase activity of pMIR/PRKCA (or HBP1) 3'-UTR WT, but not pMIR/PRKCA (or HBP1) 3'-UTR MUT, was significantly suppressed by pre-miR-374b (Fig. 6I). These data implied that pre-miR-374b might simultaneously regulate PRKCA and HBP1 mRNA expressions by the mature miR-374b-5p and miR-374b-3p respectively, which were also confirmed by the results of PRC and western blot analysis (Fig. 6J-O).

## **Nigericin suppressed the Wnt/ $\beta$ -catenin signaling via a pre-miR-374b-dependent mechanism *in vitro***

Based on the idea that PRKCA and HBP1 served as tumor suppressors in nigericin-mediated damage, and the data also strongly implied that pre-miR-374b could simultaneously regulate PRKCA and HBP1 mRNA expressions, we further performed a series of rescue assays to explore the key role of pre-miR-374b in PC cells. As shown in **Supplementary Fig. 4A-J**, nigericin treatment significantly suppressed the biological functions of PC cells, including the decreased abilities of cell proliferation, colony formation, migration and invasion, and the increased apoptotic rates. However, pre-miR-374b over-expression exerted a tumor promoting effects on PC cells. When compared to the nigericin-treated cells, pre-miR-374b could also effectively antagonize the suppressing effects of biological functions of PC cells induced by nigericin.

Since nigericin was proved to suppress PC cells through the Wnt/ $\beta$ -catenin signaling, we performed the western blot analysis to evaluate the expressions of Wnt/ $\beta$ -catenin downstream proteins in pre-miR-374b over-expression PC cells (**Supplementary Fig. 5A-B**). Simultaneously, the changes of TOP/FOP ratios were

also calculated and presented in **Supplementary Fig. 5C-D**. These data strongly demonstrated that pre-miR-374b over-expression effectively activated the Wnt/ $\beta$ -catenin pathway in PC cells, and could also counteract the inhibitory effects induced by nigericin. These data provided the strong evidence that nigericin suppressed the Wnt/ $\beta$ -catenin signaling via a pre-miR-374b-dependent mechanism *in vitro*.

## Nigericin inhibited the tumor growth of PC cells *in vivo*

Given the observed effects of nigericin on PC growth and metastasis *in vitro*, we subsequently determined whether nigericin suppressed tumorigenicity *in vivo*. Though the body weights of experimental mice were not influenced, the tumor mass observed by the bioluminescent assays on day 28 and tumor growth curves all indicated that nigericin could suppress the tumor growth of PC cells *in vivo*. On the contrary, subcutaneous injection of mice with pre-miR-374b over-expressing PANC-1 cells resulted in larger tumors, compared to the non-miR-374b over-expressing cells. Meanwhile, our results also confirmed that the inhibitory effects of nigericin could be significantly antagonized by pre-miR-374b over-expression (Fig. 7A-C). To further know the regulatory relationships among pre-miR-374b, miR-374b-5p, miR-374b-3p, PRKCA and HBP1, the PCR analysis was employed. The data indicated that nigericin treatment significantly reduced the expressions of pre-miR-374b, miR-374b-5p and miR-374b-3p, and up-regulated the PRKCA and HBP1 mRNA expressions in mice tumors. As expected, pre-miR-374b over-expression could antagonize the expressing levels of PRKCA and HBP1 in mice tumors induced by nigericin injection *in vivo*, compared to the nigericin treated cells (Fig. 7D-H). Thus, we concluded that nigericin might suppress the Wnt/ $\beta$ -catenin signaling in PC through targeting pre-miR-374b-PRKCA/HBP1 axis *in vitro* and *in vivo* (Fig. 7I).

## Discussion

Our recent study in 2018 proved that nigericin treatment significantly reduced tumor cell proliferation in dose- and time-dependent manners in colorectal cancer cells [13]. Then the role of nigericin was also investigated in PC cells after the high-throughput RNA sequencing. From the perspective of circRNAs through regulating parental gene transcriptions, we used the bioinformatics analyses to explore the potential mechanism of nigericin in PC cells [27]. In this study, we exposed PC cell lines (PANC-1 and PL-45) to increasing concentrations of nigericin for different time periods and the IC50 values were evaluated. Similar to the results in colorectal cancer cells, PC cells were also sensitive to nigericin in concentration- or time-dependent manners. Choosing a low and suitable concentration, the effects of nigericin on the tumorigenic behaviors of PC cells were evaluated. Our data demonstrated that nigericin exerted the anti-cancer effects on PC cells, and significantly influenced the abilities of cell proliferation, colony formation, apoptosis, migration and invasion.

To reveal the potential mechanisms of nigericin in PC, we used the high-throughput RNA sequencing analysis to screen the expression profile of mRNAs, and a total of 172 commonly dys-regulated mRNAs were identified throughout the nigericin-treated process. The reliability and validity of the sequencing

expression results were verified, and the network between KEGG pathways and their corresponding genes strongly implied that the Wnt/ $\beta$ -catenin signaling was involved in the nigericin-mediated effects in PC cells. Then the western blot and TOP/FOP-Flash reporter assays confirmed our hypothesis that nigericin could suppress the growth of PC cells by the Wnt/ $\beta$ -catenin pathway. Among these commonly dys-regulated mRNAs from the high-throughput RNA sequencing analysis, PRKCA and HBP1 were two key molecules, which might have a role in tumor suppression by inhibiting the Wnt signaling pathway. PKC $\alpha$ , which is encoded by PRKCA gene, is a member of the Protein Kinase C (PKC) family that includes ten serine/threonine kinases. Significant evidence indicates that PKC $\alpha$  acts as a tumor suppressor that PKC $\alpha$ -induced the phosphorylation of  $\beta$ -catenin, leading to its degradation by the proteasome [28–29]. In 2010, Lee et al. also reported that PKC $\alpha$ -induced phosphorylation of the orphan receptor ROR $\alpha$  results in  $\beta$ -catenin co-transcriptional activity inhibition and in the trans-repression of Wnt/ $\beta$ -catenin target genes [30]. High mobility group box transcription factor 1 (HBP1) belongs to the sequence-specific, HMG family of transcription factors. As a putative suppressor of the Wnt pathway, HBP1 may inhibit the transcription of TCF4 targets by directly blocking the binding of TCF4 with DNA [31–32]. As to the effects of PRKCA and HBP1 in the repression of the Wnt– $\beta$ -catenin pathway in cancers, we further investigated the potential role of these two molecules in the nigericin-mediated process. Our data demonstrated that silencing PRKCA or HBP1 by small interfering RNAs could inactivate the Wnt/ $\beta$ -catenin signaling in PC cells. More importantly, silencing PRKCA, HBP1 or these two molecules simultaneously could partly attenuate the inactivation of the Wnt signaling induced by nigericin in PANC-1 and PL-45 cells. Besides, the results of Co-IP assays further confirmed the significance of PRKCA and HBP1 in the nigericin damage.

MiRNAs are a class of endogenous noncoding RNAs, which are derived from longer transcripts termed pre-miRNAs, and recognize mainly the 3'-UTR region of messenger RNA (mRNA) and inhibit protein synthesis. Recent reports indicate that dys-regulation of the mature miRNAs and their target genes promote cancer initiation, progression and drug resistance, including PC [33–34]. More interestingly, the dual strands of certain pre-miRNAs, e.g., pre-miR-150 [35], pre-miR-139 [36] and pre-miR-145 [37], can act as anti-cancer miRNAs in several cancers, and each guide strand and passenger strand coordinately regulates oncogenic genes. The pre-miRNA signature led us to focus on the dual strands of pre-miR-374b, i.e., miR-374-5p (the guide strand) and miR-374-3p (the passenger strand). Our data showed that nigericin could coordinately down-regulate the expressions of pre-miR-374b, miR-374b-5p and miR-374b-3p in PC cells in a time-dependent manner. Analyzing the structure of pre-miR-374b and the predicted conserved seed-matching sequences between the miR-374b products and Wnt-related genes, we speculated that miR-374b-5p or -3p might selectively target the PRKCA and HBP1) 3'-UTR sites respectively. Fortunately, the subsequent experiments including the luciferase report assays, PRC and western blot analysis all confirmed our previous hypothesis, that pre-miR-374b might simultaneously regulate PRKCA and HBP1 mRNA expressions by the mature miR-374b-5p and miR-374b-3p respectively. Using a pre-miR-374b over-expressing vector, a series of rescue assays *in vitro* were also performed and the results demonstrated that pre-miR-374b could effectively antagonize the suppressing effects of biological functions of PC cells induced by nigericin. These findings provided strong evidence that nigericin might suppress the Wnt/ $\beta$ -

catenin signaling through a pre-miR-374b-dependent mechanism. In addition, the suppressing effects of nigericin on tumor growth, and the regulatory relationships between pre-miR-374b and target genes, were also discussed in a subcutaneous tumor model.

## Conclusions

In conclusion, the work presented here is the first to demonstrate the toxicology and anti-cancer effects of nigericin to PC cells. The high-throughput RNA sequencing was performed to screen the expression profile of mRNAs, and the results *in vitro* implied that nigericin could significantly suppress the Wnt/ $\beta$ -catenin signaling by up-regulating PRKCA and HBP1 mRNA expressions. Furthermore, the dual strands of certain pre-miR-374b were proved to down-regulate the expressions of PRKCA and HBP1 coordinately, and over-expression of pre-miR-374b might partly antagonize the suppressing effects of PC cells induced by nigericin. These findings demonstrated that suppressing the Wnt/ $\beta$ -catenin signaling pathway by targeting pre-miR-374b-PRKCA/HBP1 axis might represent a novel molecular mechanism of nigericin in PC. Nigericin remained a candidate for a potential pre-clinical application for PC.

## Abbreviations

PC: Pancreatic cancer; CSCs: Cancer stem cells; EMT: Epithelial-mesenchymal transition; DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; IC50: 50% inhibiting concentration; PI: Propidium iodide; BSA: Bovine serum albumin; FDR: False discovery rate; FC: Fold change; KEGG: Kyoto Encyclopedia of Genes and Genomes; siRNA: Small interfering RNA; QRT-PCR: Quantitative real-time polymerase chain reaction; Co-IP: Co-Immunoprecipitation; TV: Tumor volume; PKC: Protein Kinase C; HBP1: High mobility group box transcription factor 1.

## Declarations

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We are grateful to the patients for their contributions to this study.

### Authors' contributions

QZ, DZ and YX designed the study. QZ, DZ, DW, YH, FL and ZX conducted the experiments and data analysis. DW and MJ provided technical support. QZ and DZ wrote the manuscript. All authors read and approve the final manuscript.

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### Availability of data and materials

Not applicable.

### **Ethics approval and consent to participate**

The human materials were obtained with the consent of patients and approved by the ethics committee of the First Affiliated Hospital of Soochow University.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no conflict of interest.

## **References**

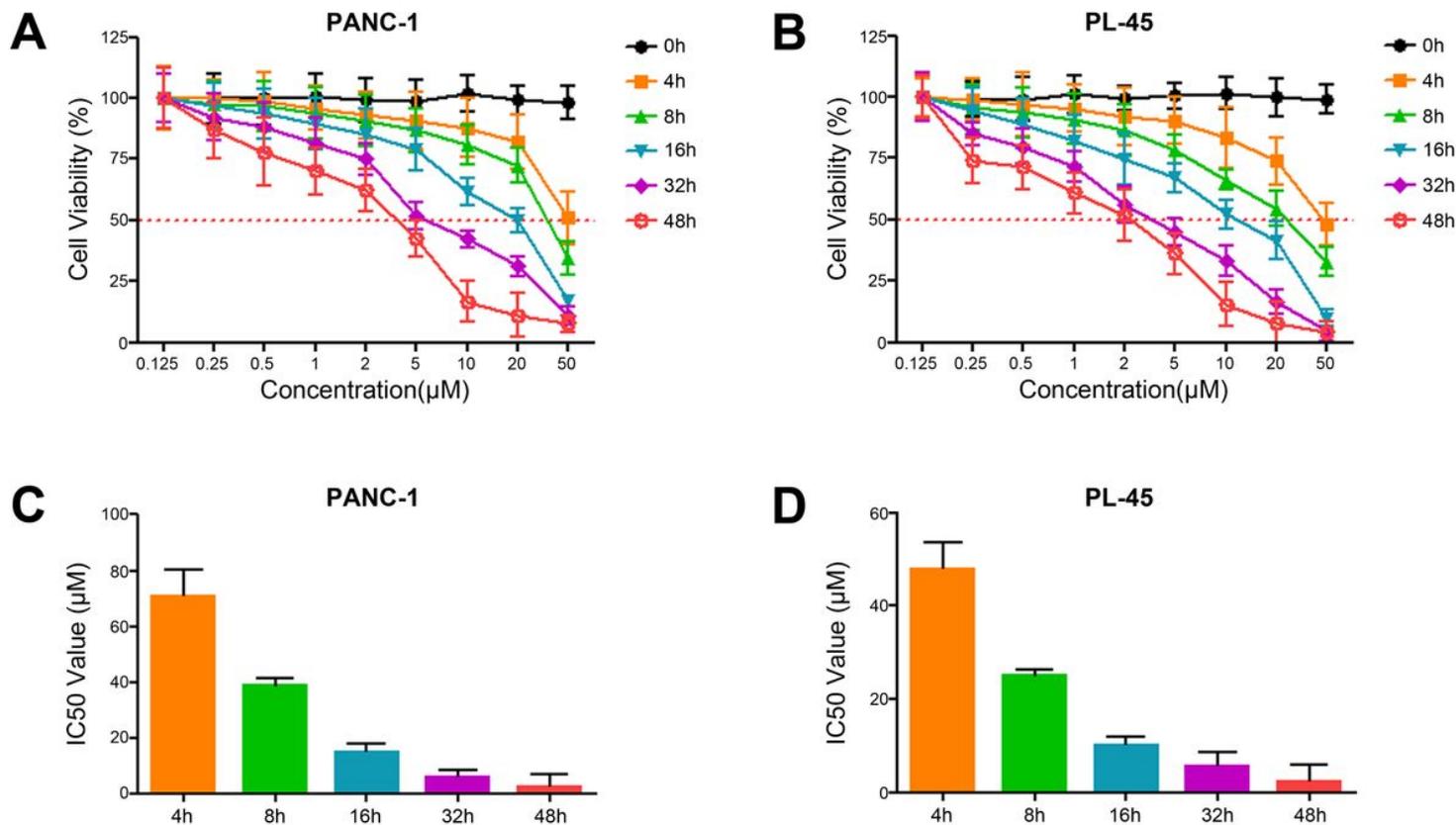
1. Margolis LB, Rozovskaja IA, Skulachev VP. Acidification of the interior of Ehrlich ascites tumor cells by nigericin inhibits DNA synthesis. *FEBS Lett.* 1987, 220(2): 288-90.
2. Margolis LB, Novikova I Yu, Rozovskaya IA, Skulachev VP. K<sup>+</sup>/H<sup>+</sup>-antiporter nigericin arrests DNA synthesis in Ehrlich ascites carcinoma cells. *Proc Natl Acad Sci U S A.* 1989, 86(17): 6626-9.
3. Leulmi N, Sighel D, Defant A, Khenaka K, Boulahrouf A, Mancini I. Nigericin and grisorixin methyl ester from the Algerian soil-living *Streptomyces youssoufiensis* SF10 strain: a computational study on their epimeric structures and evaluation of glioblastoma stem cells. *Nat Prod Res.* 2019, 33(2): 266-73.
4. Varnes ME, Bayne MT, Menegay HJ, Tuttle SW. Effect of the K<sup>+</sup>/H<sup>+</sup> ionophore nigericin on response of A549 cells to photodynamic therapy and tert-butylhydroperoxide. *Free Radic Biol Med.* 1993, 15(4): 395-405.
5. Yakisich JS, Azad N, Kaushik V, O'Doherty GA, Iyer AK. Nigericin decreases the viability of multidrug-resistant cancer cells and lung tumor spheres and potentiates the effects of cardiac glycosides. *Tumour Biol.* 2017, 39(3): 1010428317694310.
6. Schindler M, Grabski S, Hoff E, Simon SM. Defective pH regulation of acidic compartments in human breast cancer cells (MCF-7) is normalized in adriamycin-resistant cells (MCF-7adr). *Biochemistry.* 1996, 35(9): 2811-7.
7. Zhou HM, Dong TT, Wang LL, Feng B, Zhao HC, Fan XK, Zheng MH. Suppression of colorectal cancer metastasis by nigericin through inhibition of epithelial-mesenchymal transition. *World J Gastroenterol.* 2012, 18(21): 2640-8.
8. Deng CC, Liang Y, Wu MS, Feng FT, Hu WR, Chen LZ, Feng QS, Bei JX, Zeng YX. Nigericin selectively targets cancer stem cells in nasopharyngeal carcinoma. *Int J Biochem Cell Biol.* 2013, 45(9): 1997-2006.

9. Mashima T, Okabe S, Seimiya H.  
Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth. *Mol Pharmacol.* 2010, 78(5): 846-54.
10. Marquardt D, Center MS. Drug transport mechanisms in HL60 cells isolated for resistance to adriamycin: evidence for nuclear drug accumulation and redistribution in resistant cells. *Cancer Res.* 1992, 52(11): 3157-63.
11. Hasuda K, Lee C, Tannock IF. Antitumor activity of nigericin and 5-(N-ethyl-N-isopropyl)amiloride: an approach to therapy based on cellular acidification and the inhibition of regulation of intracellular pH. *Oncol Res.* 1994, 6(6): 259-68.
12. Kuin A, Aalders M, Lamfers M, van Zuidam DJ, Essers M, Beijnen JH, Smets LA. Potentiation of anti-cancer drug activity at low intratumoral pH induced by the mitochondrial inhibitor m-iodobenzylguanidine (MIBG) and its analogue benzylguanidine (BG). *Br J Cancer.* 1999, 79(5-6): 793-801.
13. Liu F, Li W, Hua S, Han Y, Xu Z, Wan D, Wang Y, Chen W, Kuang Y, Shi J, Zhi Q.  
Nigericin Exerts Anticancer Effects on Human Colorectal Cancer Cells by Inhibiting Wnt/ $\beta$ -catenin Signaling Pathway. *Mol Cancer Ther.* 2018, 17(5): 952-65.
14. Wang W, Zhao Y, Yao S, Cui X, Pan W, Huang W, Gao J, Dong T, Zhang S. Nigericin Inhibits Epithelial Ovarian Cancer Metastasis by Suppressing the Cell Cycle and Epithelial-Mesenchymal Transition. *Biochemistry (Mosc).* 2017, 82(8): 933-41.
15. Lu D, Choi MY, Yu J, Castro JE, Kipps TJ, Carson DA. Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. *Proc Natl Acad Sci U S A.* 2011, 108(32): 13253-7.
16. Clevers H, Nusse R. Wnt/ $\beta$ -catenin signaling and disease. *Cell.* 2012, 149(6): 1192-205.
17. Schatoff EM, Leach BI, Dow LE. Wnt Signaling and Colorectal Cancer. *Curr Colorectal Cancer Rep.* 2017, 13(2): 101-10.
18. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. *Oncogene.* 2017, 36(11): 1461-73.
19. Murillo-Garzón V, Kypta R. WNT signalling in prostate cancer. *Nat Rev Urol.* 2017, 14(11): 683-96.
20. Lecarpentier Y, Schussler O, Hébert JL, Vallée A. Multiple Targets of the Canonical WNT/ $\beta$ -Catenin Signaling in Cancers. *Front Oncol.* 2019, 9: 1248.
21. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin.* 2018, 68(1): 7-30.
22. Saad AM, Turk T, Al-Husseini MJ, Abdel-Rahman O.  
Trends in pancreatic adenocarcinoma incidence and mortality in the United States in the last four decades; a SEER-based study. *BMC Cancer.* 2018, 18(1): 688.
23. Javadinia SA, Shahidsales S, Fanipakdel A, Joudi-Mashhad M, Mehramiz M, Talebian S, Maftouh M, Mardani R, Hassanian SM, Khazaei M, Ferns GA, Avan A. Therapeutic potential of targeting the Wnt/ $\beta$ -catenin pathway in the treatment of pancreatic cancer. *J Cell Biochem.* 2018, Oct 28.

24. Cui J, Jiang W, Wang S, Wang L, Xie K. Role of Wnt/ $\beta$ -catenin signaling in drug resistance of pancreatic cancer. *Curr Pharm Des.* 2012, 18(17): 2464-71.
25. Chen S, Huang J, Liu Z, Liang Q, Zhang N, Jin Y. FAM83A is amplified and promotes cancer stem cell-like traits and chemoresistance in pancreatic cancer. *Oncogenesis.* 2017, 6(3): e300.
26. Mohammed MK, Shao C, Wang J, Wei Q, Wang X, Collier Z, Tang S, Liu H, Zhang F, Huang J, Guo D, Lu M, Liu F, Liu J, Ma C, Shi LL, Athiviraham A, He TC, Lee MJ. Wnt/ $\beta$ -catenin signaling plays an ever-expanding role in stem cell self-renewal, tumorigenesis and cancer chemoresistance. *Genes Dis.* 2016, 3(1): 11-40.
27. Xu Z, Shen J, Hua S, Wan D, Chen Q, Han Y, Ren R, Liu F, Du Z, Guo X, Shi J, Zhi Q. High-throughput sequencing of circRNAs reveals novel insights into mechanisms of nigericin in pancreatic cancer. *BMC Genomics.* 2019, 20(1): 716.
28. Dupasquier S, Blache P, Picque Lasorsa L, Zhao H, Abraham JD, Haigh JJ, Ychou M, Prévostel C. Modulating PKC $\alpha$  Activity to Target Wnt/ $\beta$ -Catenin Signaling in Colon Cancer. *Cancers (Basel).* 2019, 11(5): 693.
29. Kim S, Chun SY, Kwon YS, Nam C. Crosstalk between Wnt signaling and Phorbol ester-mediated PKC signaling in MCF-7 human breast cancer cells. *Biomed Pharmacother.* 2016, 77: 114-9.
30. Lee JM, Kim IS, Kim H, Lee JS, Kim K, Yim HY, Jeong J, Kim JH, Kim JY, Lee H, Seo SB, Kim H, Rosenfeld MG, Kim KI, Baek SH. ROR $\alpha$  attenuates Wnt/ $\beta$ -catenin signaling by PKC $\alpha$ -dependent phosphorylation in colon cancer. *Mol Cell.* 2010, 37(2): 183-95.
31. Sampson EM, Haque ZK, Ku MC, Tevosian SG, Albanese C, Pestell RG, Paulson KE, Yee AS. Negative regulation of the Wnt- $\beta$ -catenin pathway by the transcriptional repressor HBP1. *EMBO J.* 2001, 20(16): 4500-11.
32. Watanabe N, Kageyama R, Ohtsuka T. Hbp1 regulates the timing of neuronal differentiation during cortical development by controlling cell cycle progression. *Development.* 2015, 142(13): 2278-90.
33. Madurantakam Royam M, Ramesh R, Shanker R, Sabarimurugan S, Kumarasamy C, Ramesh N, Gothandam KM, Baxi S, Gupta A, Krishnan S, Jayaraj R. miRNA Predictors of Pancreatic Cancer Chemotherapeutic Response: A Systematic Review and Meta-Analysis. *Cancers (Basel).* 2019, 11(7): 900.
34. Qadir MI, Faheem A. miRNA: A Diagnostic and Therapeutic Tool for Pancreatic Cancer. *Crit Rev Eukaryot Gene Expr.* 2017, 27(3): 197-204.
35. Okato A, Arai T, Kojima S, Koshizuka K, Osako Y, Idichi T, Kurozumi A, Goto Y, Kato M, Naya Y, Ichikawa T, Seki N. Dual strands of pre-miR-150 (miR-150-5p and miR-150-3p) act as antitumor miRNAs targeting SPOCK1 in naïve and castration-resistant prostate cancer. *Int J Oncol.* 2017, 51(1): 245-56.
36. Yonemori M, Seki N, Yoshino H, Matsushita R, Miyamoto K, Nakagawa M, Enokida H. Dual tumor-suppressors miR-139-5p and miR-139-3p targeting matrix metalloproteinase 11 in bladder cancer. *Cancer Sci.* 2016, 107(9): 1233-42.

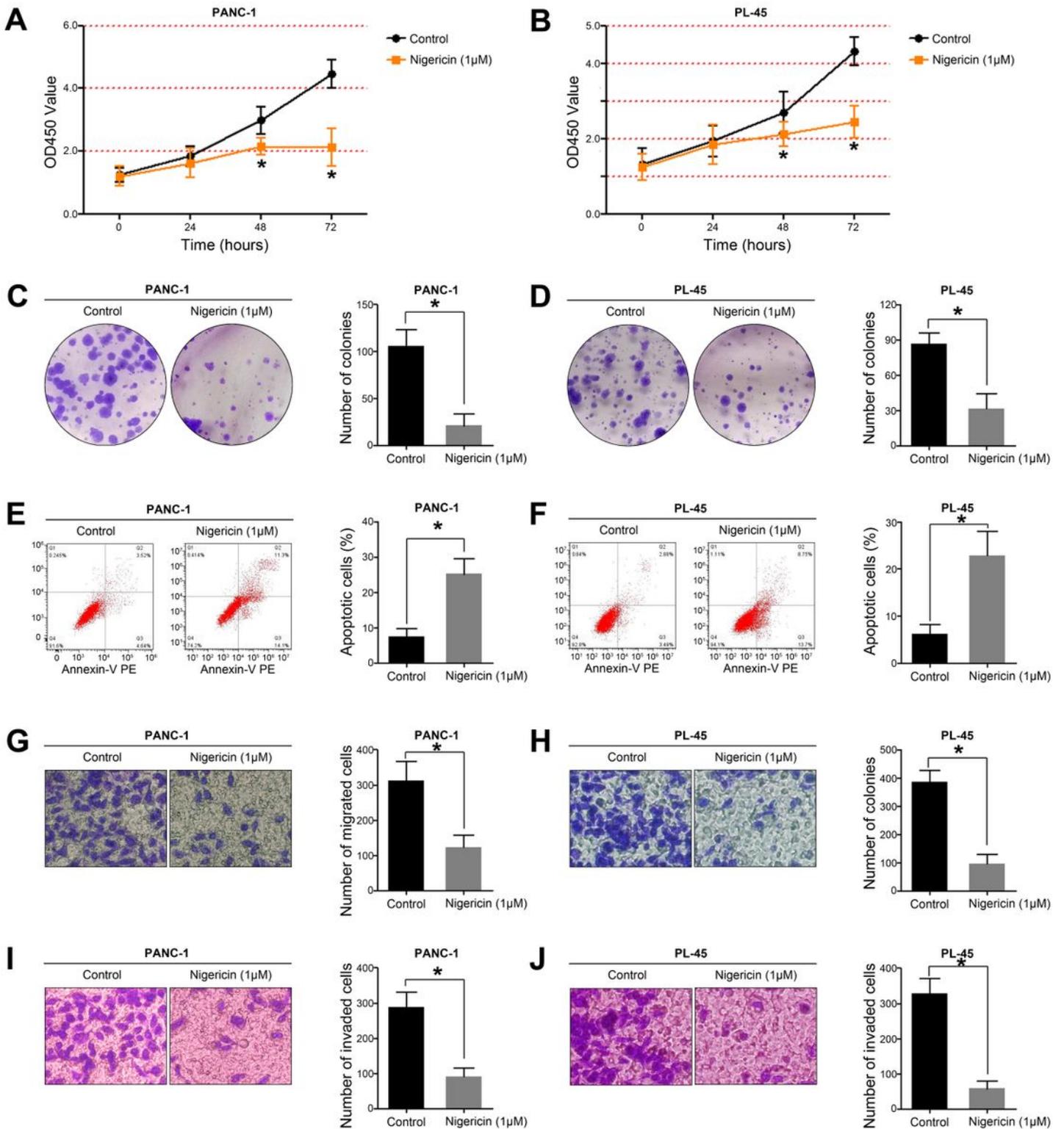
37. Matsushita R, Yoshino H, Enokida H, Goto Y, Miyamoto K, Yonemori M, Inoguchi S, Nakagawa M, Seki N. Regulation of UHRF1 by dual-strand tumor-suppressor microRNA-145 (miR-145-5p and miR-145-3p): Inhibition of bladder cancer cell aggressiveness. *Oncotarget*. 2016, 7(19): 28460-87.

## Figures



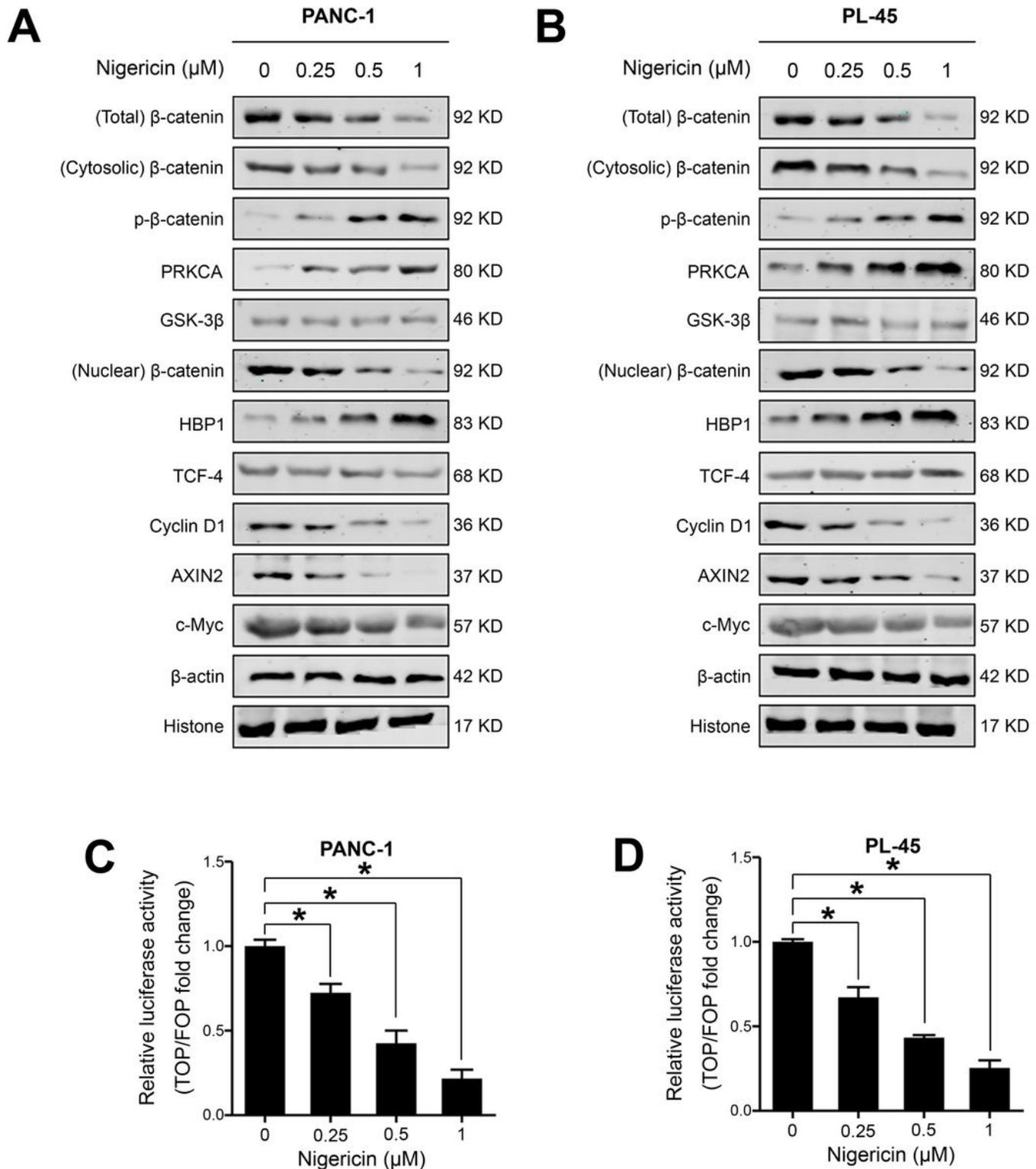
**Figure 1**

PC cells were sensitive to nigericin by IC50 comparison. (A-B) PANC-1 and PL-45 cells were exposed to increasing concentrations of nigericin (0, 0.25, 0.5, 1, 2, 5, 10, 20 and 50 µmol/L) for different time periods (0, 4, 8, 16, 32 or 48h). (C-D) The corresponding IC50 values were calculated.



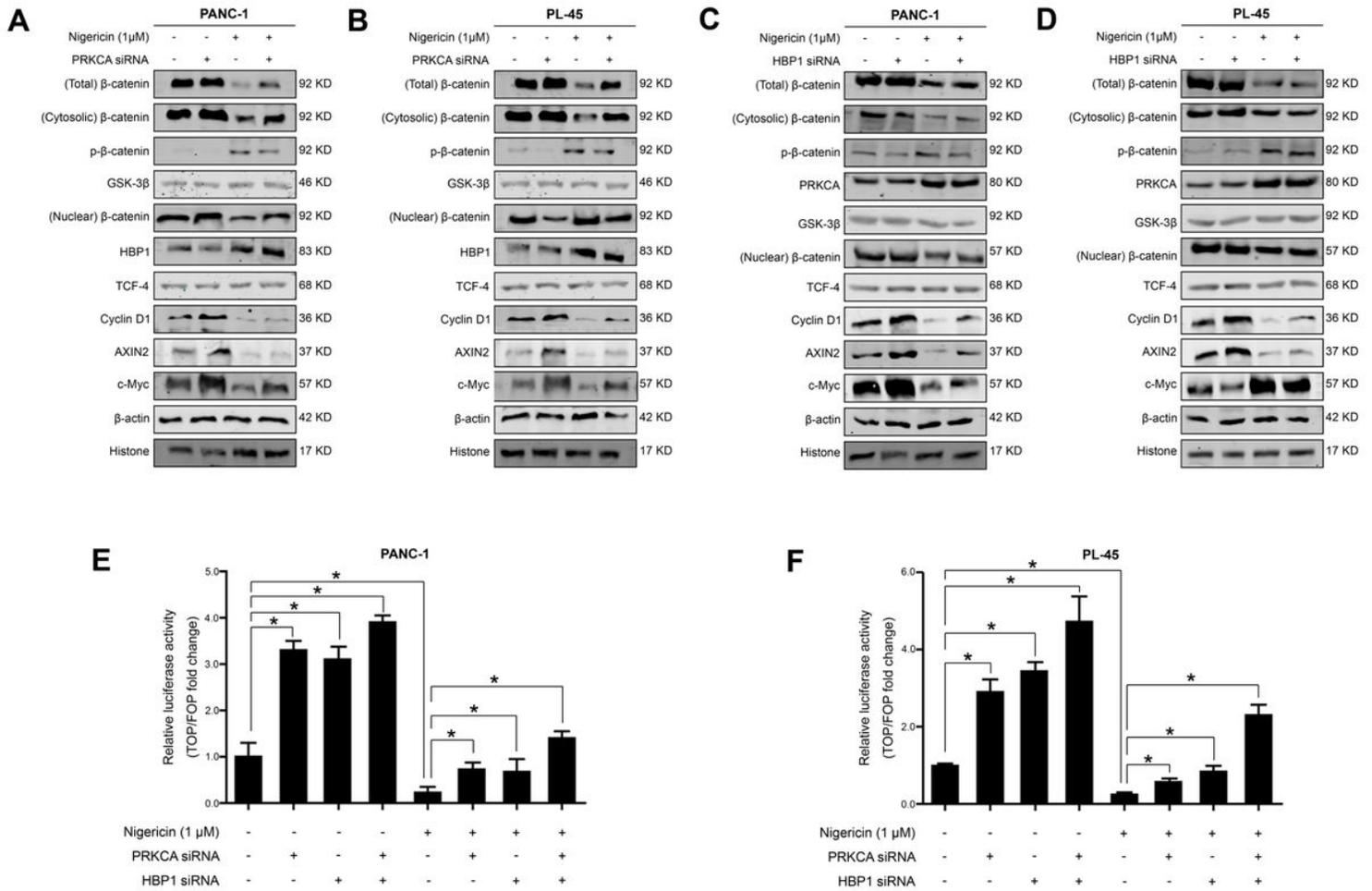
**Figure 2**

Nigericin inhibited the tumorigenic behaviors of PC cells in vitro. (A-B) Cell proliferation using the CCK-8 assays. (C-D) Colony formation assays. (E-F) Cell apoptosis by the FACS. (G-J) The migration and invasion abilities. (\* $P < 0.05$ )



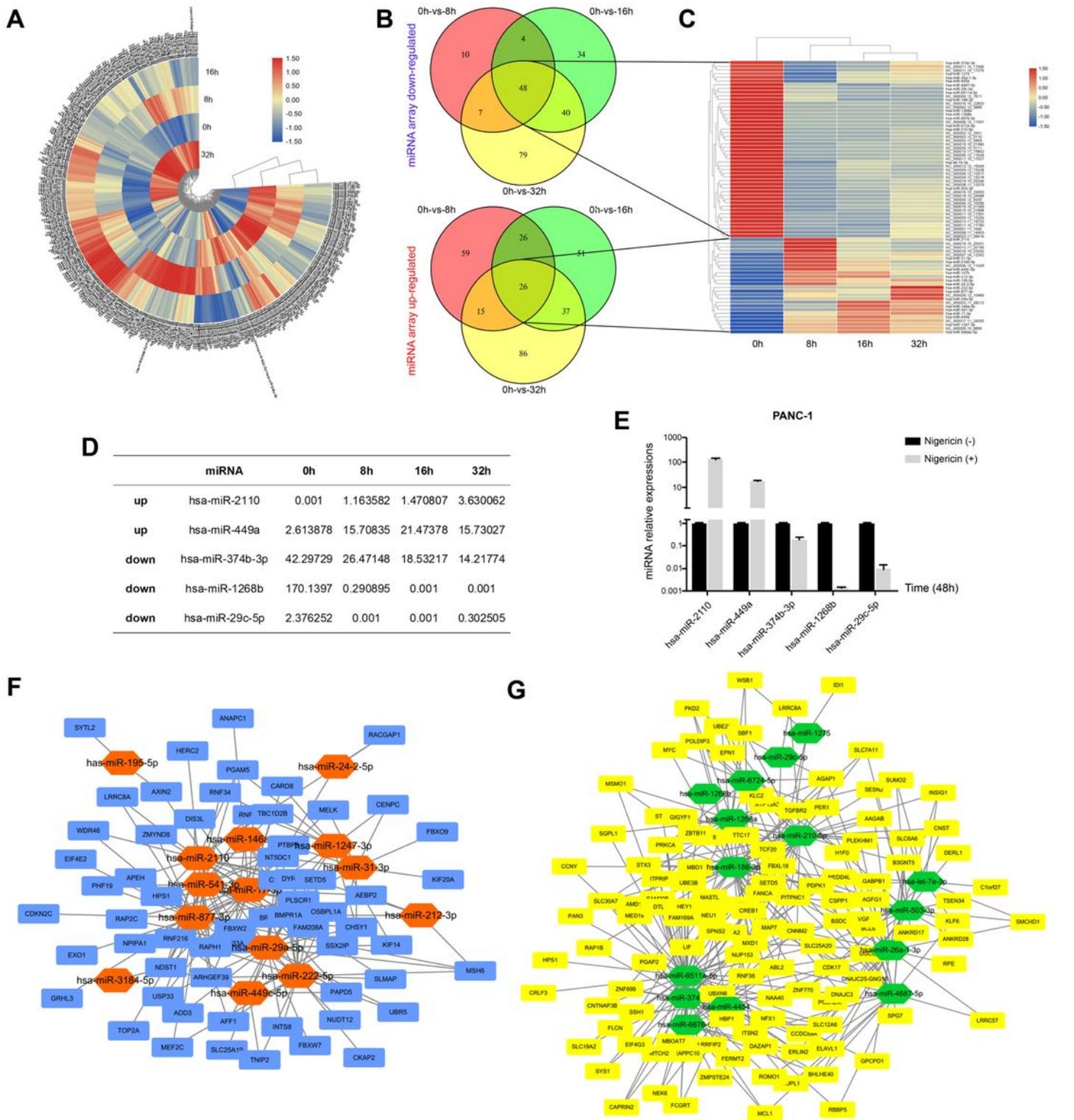
**Figure 3**

Nigericin suppressed the Wnt/ $\beta$ -catenin signaling of PC cells in a dose-dependent manner. (A-B) The expressions of downstream effectors of the Wnt/ $\beta$ -catenin pathway were determined by western blot analysis. (C-D) The relative TOP/FOP ratios using the TOP Flash cell reporter system evaluated the activation of Wnt/ $\beta$ -catenin signaling. (\* $P < 0.05$ )



**Figure 4**

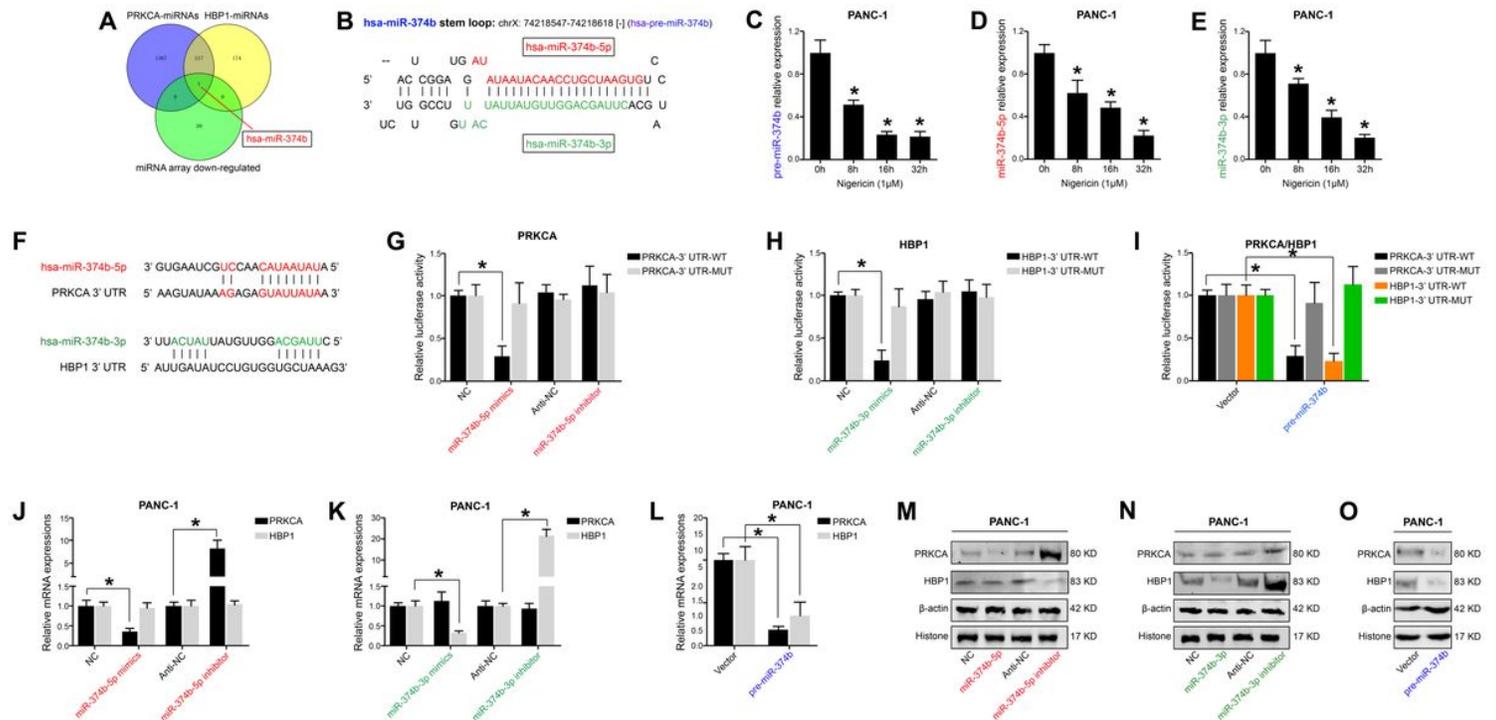
Nigericin suppressed the Wnt/ $\beta$ -catenin signaling through up-regulating PRKCA/HBP1 expressions. (A-D) The changes of protein expressions of PRKCA or HBP1 downstream effectors were determined by the western blot analysis. (E-F) The relative TOP/FOP ratios using the TOP Flash cell reporter system evaluated the activation of Wnt/ $\beta$ -catenin signaling when si-PRKCA or si-HBP1 was transfected into the nigericin-treated PC cells. (\* $P < 0.05$ )



**Figure 5**

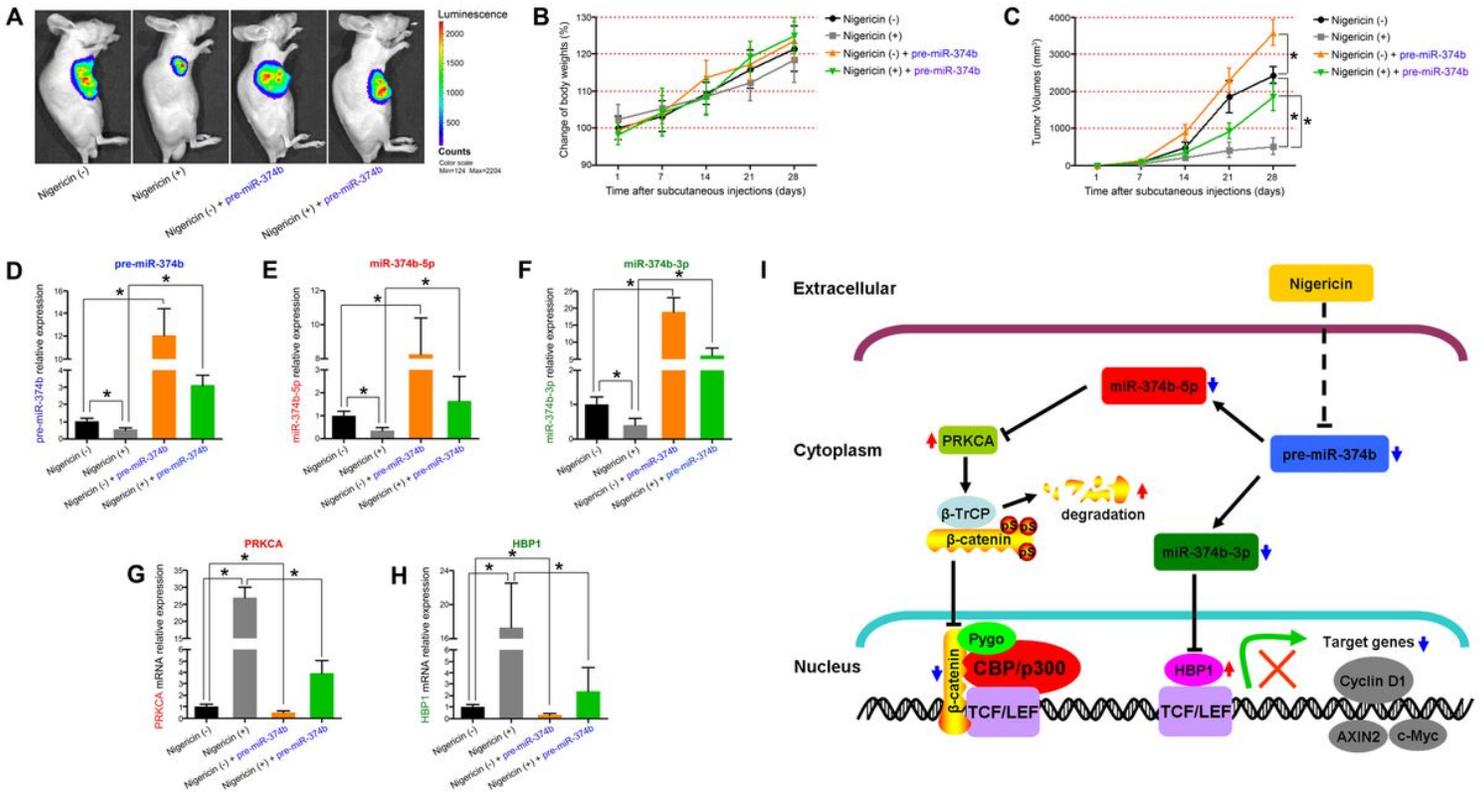
The high-throughput RNA sequencing analysis presented the expression profile of miRNAs in the nigericin-mediated damage. (A) The expression profile of miRNAs at 4 different time points (0, 8, 16 and 32h) was described in the hierarchical clustering analysis. (B-C) The venn analysis was performed and a total of 74 common differentially expressed miRNAs were identified throughout the nigericin treatment. (D-E) The expressions of 5 random miRNAs from the RNA sequencing were presented and verified by the

PCR analysis. (F-G) Getting rid of the undefined miRNAs, the miRNA-mRNA interaction network between up-regulated miRNAs and their corresponding down-regulated mRNAs were presented. Similar network between down-regulated miRNAs and their corresponding up-regulated mRNAs was also constructed. (\*P<0.05)



**Figure 6**

The dual strands of pre-miR-374b were proved to down-regulate the expressions of PRKCA and HBP1 coordinately. (A) The venn analysis among PRKCA-predicted, HBP1-predicted and down-regulated miRNAs identified miR-374b as the potential upstream molecule of PRKCA and HBP1. (B) The stem-loop structure of pre-miR-374b. (C-E) The results of PCR analysis demonstrated that nigericin could significantly down-regulate the pre-miR-374b, miR-374b-5p and miR-374b-3p expressions time-dependently. (F) The conserved seed-matching sequences between miR-374b-5p (or miR-374b-3p) and PRKCA (or HBP1) were predicted. (G-I) The results of luciferase report assays demonstrated that miR-374b-5p and miR-374b-3p could selectively target the 3'-UTR sites of PRKCA and HBP1 respectively, and pre-miR-374b coordinately target the 3'-UTR sites of PRKCA and HBP1. (J-L) The results of PCR analysis implied that pre-miR-374b might simultaneously regulate PRKCA and HBP1 mRNA expressions by the mature miR-374b-5p and miR-374b-3p. (M-O) The PRKCA and HBP1 protein expressions were also determined by western blot. (\*P<0.05)



**Figure 7**

Nigericin inhibited the tumor growth of PC cells in vivo. (A) Cells were injected subcutaneously into the flanks of nude mice, and the tumor mass on day 28 was observed by the bioluminescent assays. (B-C) The weights of experimental mice and tumor growth curves were calculated per week. (D-H) The relative expressions of pre-miR-374b, miR-374b-5p, miR-374b-3p, PRKCA and HBP1 in tumor masses were determined by the PCR analysis. (I) We concluded that nigericin suppressed the Wnt/ $\beta$ -catenin signaling in PC through targeting pre-miR-374b-PRKCA/HBP1 axis. (\* $P < 0.05$ )

## Supplementary Files

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