ANTICANCER RESEARCH *43*: 4843-4853 (2023)
doi:10.21873/anticanres.16681

High Glucose-stimulated aPKC Activation Promotes Pancreatic Cancer Cell Progression Through YAP Signaling

TEPPEI SUNAGUCHI[1,2], YOSUKE HORIKOSHI[2], TAKEHIKO HANAKI[1], TERUHISA SAKAMOTO[1], KAZUHIRO NAKASO[2], CHIEKO SAKAI[3], KAZUNARI YAMASHITA[4], SHIGEO OHNO[5], YOSHIYUKI FUJIWARA[1] and TATSUYA MATSURA[2,6]

[1]*Division of Gastrointestinal and Pediatric Surgery, Department of Surgery, Faculty of Medicine, Tottori University, Yonago, Japan;*
[2]*Division of Biochemistry, Department of Pathophysiological and Therapeutic Sciences, Faculty of Medicine, Tottori University, Yonago, Japan;*
[3]*Department of Adult and Elderly Nursing, School of Health Sciences, Tottori University Faculty of Medicine, Yonago, Japan;*
[4]*Departments of Pathology and Oncology, Juntendo University School of Medicine, Tokyo, Japan;*
[5]*Laboratory of Cancer Biology, Institute for Diseases of Old Age, Juntendo University School of Medicine, Tokyo, Japan;*
[6]*Department of Nutritional Sciences, Faculty of Human Ecology, Yasuda Women's University, Hiroshima, Japan*

Abstract. *Background/Aim: Persistent hyperglycemia caused by diabetes mellitus is a risk factor for pancreatic cancer (PC). We have previously reported that aberrant activation of atypical protein kinase C (aPKC) enhances PC cell progression. However, no reports have elucidated whether hyperglycemia promotes PC cell progression and whether aPKC activation is related to PC cell progression mechanisms. Materials and Methods: We examined whether high-glucose stimulation accelerates PC cell proliferation, migration, and invasion. Furthermore, to determine whether PC cells activate aPKC upon high-glucose stimulation, we measured the phosphorylation of aPKC at T560 in PC cells. Results: High-glucose stimulation accelerated PC cell proliferation, migration, and invasion. High-glucose treatment increased aPKC's activated form, with T560 phosphorylation, in PC cells. However, aPKC knockdown attenuated these effects. aPKC reportedly induces cell transformation through Yes-associated protein (YAP) activation. YAP expression was increased in high glucose–treated PC cells but not in aPKC-knockdown cells. aPKC interacts with partitioning defective 3 (Par-3), which aids in establishing cell polarity and inhibits aPKC by binding as a substrate. In Par-3-knockdown PC cells, YAP expression increased independently of high-glucose treatment. Over-expression of Par-3 and aPKC-dominant negative mutants prevented the high glucose–stimulated nuclear localization of YAP. YAP forms a complex with the zinc finger E-box binding homeobox 1 protein (ZEB1), an activator of epithelial-mesenchymal transition. ZEB1 expression was increased by high glucose treatment or Par-3 knockdown, but aPKC knockdown suppressed this increase. Conclusion: High glucose–induced aPKC activation promotes PC progression by enhancing the YAP signaling pathway.*

Correspondence to: Yosuke Horikoshi, Division of Biochemistry, Department of Pathophysiological and Therapeutic Sciences, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago city, Tottori, Japan. Tel: +81 859386153, e-mail: horikoshiy@tottori-u.ac.jp; Takehiko Hanaki, Division of Gastrointestinal and Pediatric Surgery, Department of Surgery, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago city, Tottori, Japan. Tel: +81 859386567, e-mail: hanaki-ttr@umin.ac.jp

Key Words: High-glucose stimulation, atypical protein kinase C, Yes-associated protein, partitioning defective 3, pancreatic cancer, diabetes mellitus.

Early detection of pancreatic cancer (PC) is often difficult because of the lack of specific symptoms in the early stages, and 80%-85% of PC cases are already inoperable because of invasion or distant metastasis when detected (1). Thus, PC has the lowest 5-year survival rate of all cancers (9%) and an extremely high recurrence rate (2). Persistent hyperglycemia caused by diabetes mellitus is a risk factor for PC (3, 4). In 2021, approximately 537 million people worldwide had diabetes, and one in 10 adults would have diabetes mellitus, with the number increasing each year (4). Hence, the number of patients with both PC and diabetes

mellitus will probably continue to increase further in the future. Therefore, the novel regulators of PC progression must be urgently identified.

In high glucose–treated hepatocellular carcinoma, the oncogene Yes-associated protein (YAP), a member of the Hippo signaling, promotes cancer cell proliferation and tumorigenic potential (5, 6). The Hippo pathway involves upstream macrophage stimulating (Mst)1/Mst2; this gene phosphorylates and activates large tumor-suppressor kinase (Lats)1/Lats2, which can phosphorylate YAP to stimulate its cytoplasmic sequestration and exclusion from the nucleus (7). Unphosphorylated YAP, an active form of YAP, translocates to the nucleus where it primarily interacts with transcriptional enhanced associate domain (TEAD) to control gene transcription. The transcription factor zinc finger E-box binding homeobox 1 protein (ZEB1) activates the embryonic epithelial–mesenchymal transition (EMT) program, which is a major driver of tumor progression. ZEB1 and YAP/TEAD are key regulators of the expression of tumor-promoting target genes (8, 9).

The increased expression and activation of aPKC, a molecule of cell polarity regulatory signaling in patients with PC, are associated with a poor prognosis (10, 11). This molecule interacts with Mst1/2 and dissociates Mst1/2 from Lats1/2, leading to nuclear YAP accumulation and cell proliferation (12). However, no reports have clarified whether a similar regulation of YAP activity exists in PCs and whether this YAP activity regulation *via* aPKC activation is involved in PC cell progression.

We hypothesized that sustained hyperglycemia in patients with PC concurrent with diabetes mellitus induces PC progression by activating aPKC. Thus, this study aimed to investigate whether aPKC activation occurs under high-glucose stimulation in PC cells and promotes cell progression *via* YAP oncogene activation.

Materials and Methods

Cell lines and cell culture. The human PC cell lines MIA PaCa-2 and Panc-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) and supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine (FUJIFILM Wako Chemicals, Tokyo, Japan), and antibiotics [penicillin (100 U/ml) and streptomycin (100 μg/ml); Nacalai Tesque, Inc., Kyoto, Japan]. High-glucose treatment was performed 24 h after cell seeding. In addition, when cells were treated with 2-deoxy-D-glucose (2-DG) (Fujifilm Wako Chemicals, Tokyo, Japan), it was added together with high glucose.

Bromodeoxyuridine (BrdU) incorporation assay. Panc-1 or MIA PaCa-2 cells were seeded at 5×10^4 per well and cultured for 24 h. In addition, glucose at a specified concentration was added and cultured for 48 h. The 100 μM BrdU incorporated in the cells after a 2 h pulse was determined as described previously (13). BrdU-positive and -negative cells were visualized by immunofluorescence analysis (see below) using anti-BrdU antibody with 4',6-diamidino-2-phenylindole (DAPI) counterstains. More than 100 cells across several locations were counted.

Wound healing assay. Wound healing was assayed using culture inserts (ibidi Culture-Insert 2 well; ibidi GmbH, Martinsried, Germany). We seeded 4.2×10^4 cells into each culture insert. The cells were incubated for 24 h by adding the specified glucose concentration of 20 mM in DMEM containing 0.5% FBS. Then, the cells were imaged (3 fields per well) using a microscope (ECLIPSE Ts2-FL; Nikon Corp., Tokyo, Japan) and the NIS-Elements BR software (Nikon Corp.). The area of cell migration was measured and quantified using ImageJ software (version 1.53); it was calculated as follows: (mean area at 0 h – mean area at 24 h) or (mean area at 0 h – mean area at 48 h) (14).

Cell invasion assay. Cell invasion was analyzed using a transwell assay (8 μm pore size; Corning Inc., Corning, NY, USA). The upper side of the membranes was then coated with type I collagen (Koken, Tokyo, Japan) for 2 h at 37˚C to block the membrane pores. The human PC cells Panc-1 and MIA PaCa-2 (1×10^4/well) cultured in a serum-free medium for 24 h were seeded in the transwell inserts filled with DMEM containing 0.5% FBS and glucose. We incubated the cells for 48 h at 37˚C and then fixed the membranes in 2% paraformaldehyde for 20 min. After treatment with Triton X-100, the nuclei of cells that had traversed the cell-permeable membranes were stained with DAPI. Three visual fields were randomly selected from each membrane and photographed under a microscope (BZ53; Olympus, Tokyo, Japan) at 200× magnification. The experiments were conducted independently at least thrice.

Gene silencing. The genes aPKC and Par-3 were knocked down by RNA interference (RNAi), as previously described (13, 15). The target sequences of the human aPKC and Par-3 for siRNA were designed as follows: aPKCι #1 (GGAUCAAGUUGGUGAAGAA), aPKCι #2 (GGGAUAUCAUGAUAACACA), aPKCζ #1 (GAAG CAUGACAGCAUUAAA), aPKCζ #2 (GGACUU UGACCUAA UCAGA), Par-3 #1 (GGAGAAGGATAAAATGA AA), and Par-3 #2 (CGACAAATCTTATGATAAA). Custom siRNA (Sigma-Aldrich) was used to synthesize siRNA duplexes for aPKC and Par-3. We used Mission siRNA universal negative control (Sigma-Aldrich) as a negative control. Furthermore, cells were transfected twice using Lipofectamine RNAi MAX for efficient siRNA delivery (Thermo Fisher Scientific, Waltham, MA, USA).

Transient over-expression and immunofluorescence analysis. In a 24-well plate, 2.5×10^5 Madin-Darby canine kidney (MDCK) epithelial cells were seeded on coverslips. Hemagglutinin (HA)-tagged aPKCλ wild type (HA-aPKCλ WT), dominant-negative mutant (HA-aPKCλ KN), T7-tagged Par-3 wild type (T7-Par-3 WT), dominant-negative mutant of Par-3 S827A (T7-Par-3 SA) (16), and aPKC-binding deficient mutant of Par-3 S827/829A (T7-Par-3 2SA) (17) were transfected with Lipofectamine 3000 (Thermo Fisher Scientific). The control was a blank vector. For 24 h, these plasmid transfected cells were treated with 20 mM concentration of glucose. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 10% FBS in phosphate-buffered saline solution. Primary antibodies, anti-HA (Santa Cruz Biotechnology, Dallas, TX, USA), anti-T7 (MBL, Tokyo, Japan), and anti-YAP (Novus Biologicals,

Centennial, CO, USA and Santa Cruz Biotechnology, Dallas, TX, USA), and Alexa Fluor–conjugated secondary antibodies (Thermo Fisher Scientific) were used. Images were captured by laser scanning microscopy (LSM 780; Carl Zeiss, Jena, Germany).

Western blotting and nuclear fractionation. Western blotting was performed by 5-20% gradient or 12% sodium dodecyl–sulfate polyacrylamide gel electrophoresis (ATTO, Tokyo, Japan). Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) and blocked with 5% skimmed milk for 1 h at room temperature. Membranes were incubated with the primary antibody at 4˚C overnight, and the washed ones were incubated with the appropriate secondary antibody for 1 h at room temperature. Signals were detected using Immobilon Western chemiluminescent substrate (Millipore, Billerica, MA, USA). For visualization and quantification, we used Image Quant LAS 4000 mini system (GE Healthcare, Chicago, IL, USA) and ImageJ (version 1.53; National Institutes of Health, Bethesda, MD, USA), respectively. As an internal control for whole cell lysates, anti-GAPDH antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was used. For nuclear fractionation, samples were homogenized and nuclear proteins were concentrated using EzSubcell Extract (WSE-7421; ATTO, Tokyo, Japan). As an internal control for nuclear fraction, anti-Lamin-B1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was used.

Statistical analyses. All experiments were at least conducted in triplicate. Statistical analyses were performed with SPSS statistical software (version 25.0; IBM, Chicago, IL, USA). Data are presented as means±standard deviation. One-way analysis of variance or two-tailed Student's *t*-test was used to compare continuous variables between groups. The Tukey-Kramer test was used for *post hoc* analyses. Differences were considered significant at the $p<0.05$ level.

Results

High glucose-mediated activation of aPKC is involved in the regulation of PC cell progression. To confirm the progression of PC cells under hyperglycemic conditions, we examined cell proliferation, migration, and invasion induced by high-glucose stimulation following culture in a normal medium (containing 5 mM glucose). First, we assessed cell proliferation by measuring BrdU uptake into cells for 2 h; Compared with controls, MIA PaCa-2 stimulated with 10 and 20 mM concentrations of glucose for 48 h showed a marked increase in BrdU uptake in a glucose concentration-dependent manner (Figure 1A). Next, to clarify the effect of high-glucose stimulation on cell migration and invasion, we conducted wound healing and cell invasion assays. High-glucose treatment significantly enhanced MIA PaCa-2 migration dose-dependently at 48 h (Figure 1B). In the cell invasion assay, MIA PaCa-2 cell invasion was significantly enhanced in high glucose–treated cells compared with the control cell invasion (Figure 1C). Panc-1 cell, another PC cell, was phenocopied for cell proliferation, migration, and invasion following high-glucose treatment (data not shown). Various cancers, including PC, demonstrate aPKC over-expression or hyperactivation (10, 11). Through western blotting, we investigated whether high-glucose stimulation affects aPKC activation in PC cells. Treatment of MIA PaCa-2 cells with 10 and 20 mM concentrations of glucose for 24 h increased the activated form of aPKC, with T560 phosphorylation (Figure 1D and E). A previous study reported that aPKC is activated downstream of PI3K (18). To determine whether PC cells activate PI3K signaling upon high-glucose stimulation, we measured the phosphorylation of Ak strain transforming protein (Akt) at S473 in MIA PaCa-2 cells. High-glucose treatment increased Akt S437 phosphorylation, suggesting that the PI3K signal pathway was activated (Figure 1F). MIA PaCa-2 cells have a high expression of glucose transporter 1 (GLUT-1), which is involved in the intracellular uptake of glucose (19). The effect of glucose metabolism on aPKC activation was investigated by treating MIA PaCa-2 cells with 2-deoxy-D-glucose (2-DG), a glucose analog that inhibits glycolysis (19). The 2-DG treatment blocked the high glucose–induced T560 phosphorylation in aPKC (Figure 1G). Therefore, high-glucose stimulation may promote PC cell proliferation, migration, and invasion *via* aPKC activation.

High glucose-mediated activation of aPKC enhanced YAP activation in PC cells. Next, we assessed the effect of high-glucose stimulation on the proliferation, migration, and invasion of aPKC-knockdown MIA PaCa-2 cells. The knockdown of aPKC in MIA PaCa-2 cells did not alter the high glucose-stimulated phosphorylation of Akt S473 (Figure 2A). To assess the effect of aPKC-knockdown on cell proliferation, we examined BrdU uptake in MIA PaCa-2 cells. Treatment with 20 mM concentration of glucose for 24 h significantly reduced BrdU uptake compared with the controls (Figure 2B). Furthermore, aPKC-knockdown inhibited the migration and invasion of MIA PaCa-2 cells treated with 20 mM glucose (Figure 2C and D). The activation of aPKC reportedly induces cell transformation *via* YAP activation (12). The phosphorylation of YAP at serine 127 by Lats1/2 prevents YAP from translocating into the nucleus, thereby inhibiting the transcriptional activity (7). Thus, by western blotting, we confirmed YAP phosphorylation or YAP translocation into the nucleus following the fractionation of MIA PaCa-2 cells treated with high glucose for 48 h. As a result, YAP phosphorylation decreased, but not YAP expression (Figure 2E). Moreover, the nuclear fraction of YAP increased in a glucose-dependent manner (Figure 2F). We performed aPKC-knockdown using specific siRNA to confirm whether aPKC is required for YAP expression and localization. Western blotting analysis showed that aPKC-knockdown reduced YAP expression compared with control knockdown (Figure 2G). Immunofluorescence analysis revealed that the translocation of YAP into the nucleus was increased by high-glucose treatment in control siRNA-transfected cells (Figure 2H). Conversely, aPKC-knockdown inhibited the translocation of YAP into the nucleus after the PC cells received high-glucose treatment (Figure 2H). Thus, YAP may be involved in the downstream pathway of aPKC.

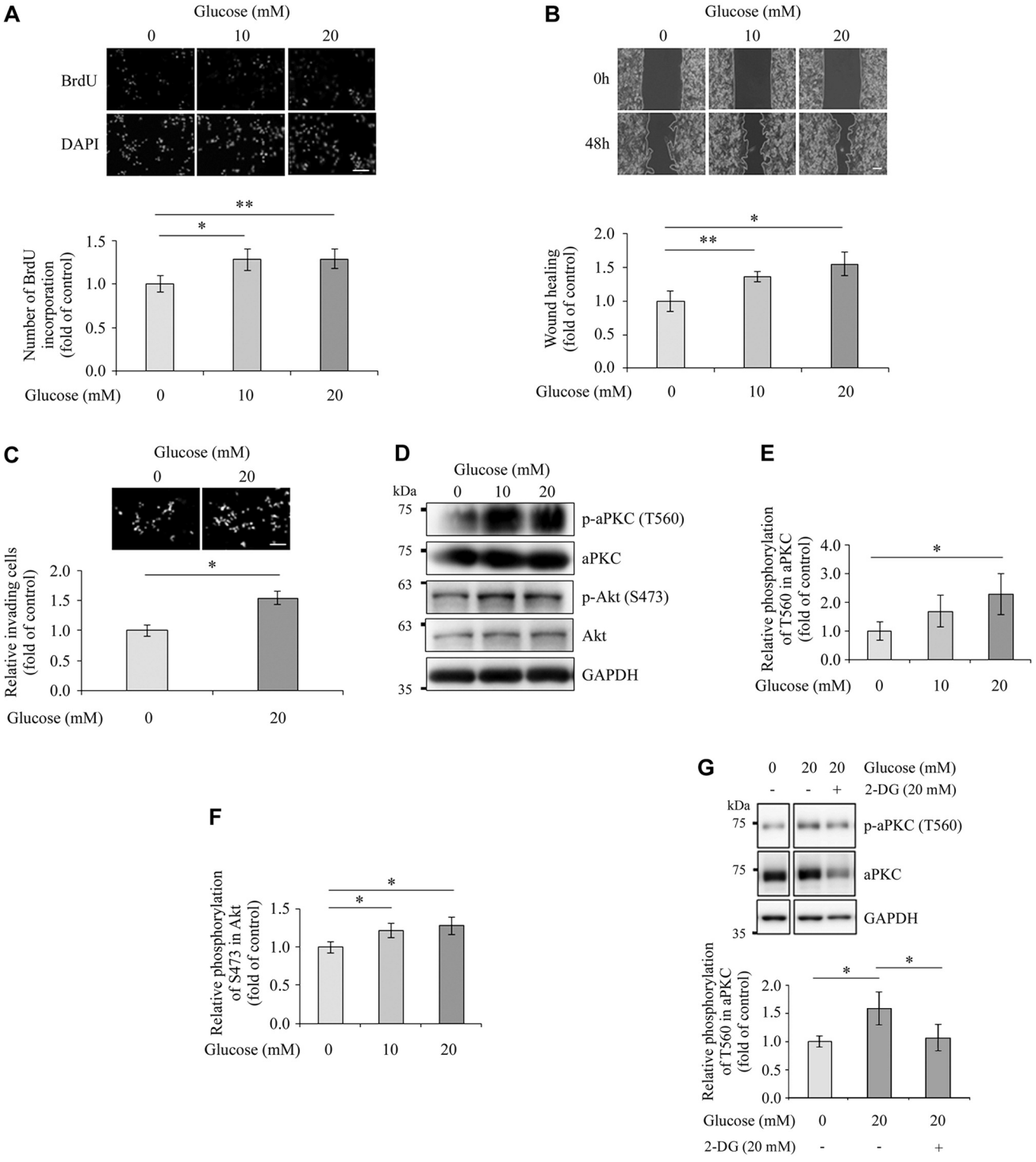

Figure 1. *High-glucose stimulation affects the proliferation, migration, and invasion of PC cells through aPKC activation. (A-C) Cells were incubated for 48 h with or without high-glucose treatment (10 or 20 mM). (A) The proliferation of MIA PaCa-2 cells was determined by BrdU assay. Scale bar=100 μm. (B) The migration rate of MIA PaCa-2 cells was analyzed by wound healing assay. Scale bar=200 μm. (C) The invasion of MIA PaCa-2 cells was analyzed by cell invasion assay. Scale bar=100 μm. (D-F) Cells were incubated for 24 h with or without high-glucose treatment (10 or 20 mM). (D) The expression levels of aPKC, aPKC T560p, Akt, and p-Akt were examined by western blotting. The results of densitometric analysis of phosphorylation for aPKC (E) and Akt (F) are shown as relative density. (G) To examine the effect of glucose metabolism on aPKC activation, we incubated 20 mM glucose–treated MIA PaCa-2 cells for 24 h with or without 2-DG treatment. The error bars indicated the standard deviation of 3 or 6 independent experiments. *p<0.05, **p<0.01.*

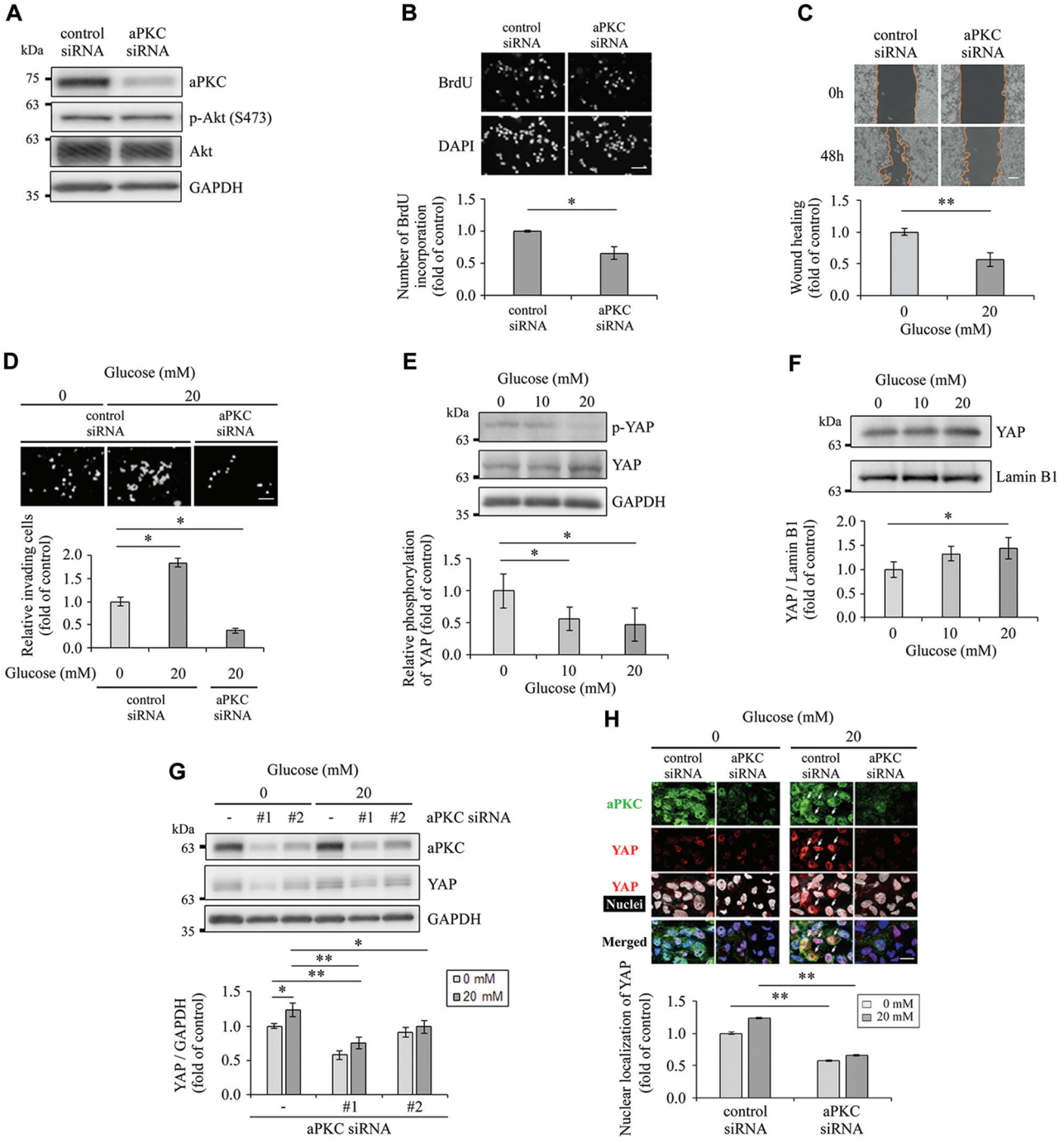

Figure 2. *aPKC promotes the proliferation, migration, and invasion of PC cells under a high-glucose environment via YAP activation. (A) MIA PaCa-2 cells were transfected with aPKC ι and ζ knockdown (aPKC siRNA) or control siRNA and incubated for 24 h in the presence of 20 mM glucose; the expression of aPKC, Akt, and p-Akt was analyzed by western blotting. After 48 h incubation with 20 mM glucose, the proliferation (B), migration (C), and invasion (D) of aPKC-knockdown MIA PaCa-2 cells were examined by BrdU assay, wound healing assay, and cell invasion assay, respectively, as described in Figure 1 legend. (E) MIA PaCa-2 cells were incubated for 24 h with or without high-glucose treatment (10 or 20 mM), and YAP phosphorylation was analyzed by western blotting and then quantified. (F) MIA PaCa-2 cells were incubated for 24 h with or without high-glucose treatment (10 or 20 mM), harvested, and then fractionated; the expression levels of YAP and LaminB1 in the nuclear fractions were determined by western blotting. (G) After 24 h incubation with 20 mM glucose, YAP expression in aPKC-knockdown MIA PaCa-2 cells were analyzed by western blotting and then quantified. (H) The nuclear localization of YAP in aPKC-knockdown MIA PaCa-2 cells was analyzed by immunofluorescence after 48 h incubation with 20 mM glucose. Arrows indicate cells with YAP-positive nuclei. Scale bar=20 μm. The error bars indicate the standard deviation of three or six independent experiments. *p<0.05, **p<0.01.*

These results suggest that aPKC activation followed by increased YAP expression contributed to tumor cell proliferation and invasion after high-glucose treatment. The nuclear localization of YAP through aPKC might be important in promoting high glucose–stimulated PC cell proliferation and progression.

Par-3 inhibits high glucose-induced YAP activation. We knocked down YAP and observed the proliferation, migration, and invasion of MIA PaCa-2 cells. BrdU uptake in YAP-knockdown MIA PaCa-2 cells was reduced 48 h after treatment with 20 mM glucose (Figure 3A). YAP knockdown inhibited the high glucose-mediated promotion of cell migration and invasion (Figure 3B and C).

The Par complex, a polarity regulatory protein complex, consists of Par-3, Par-6, and aPKC. As previously reported, Par-3 inhibits aPKC in the Par complex (20). Par-3 also regulates the access of aPKC to other substrates. The epithelial MDCK cell was transfected with HA-aPKCλ and T7-Par-3. Western blotting analysis showed that over-expressing wild-type Par-3 in PC cells did not lead to the evident suppression of YAP expression (data not shown). The cells were treated with high glucose for 24 h (Figure 3D and E). In immunohistochemical analysis, over-expressing wild-type aPKCλ promoted YAP translocation to the nucleus with high glucose for 24 h (control, 65.7%; HA-aPKCλ WT, 69.7%). However, the high glucose–induced nuclear localization of YAP was inhibited by HA-aPKCλ KN, a kinase-deficient mutant of aPKC (HA-aPKCλ KN, 37.5%). A similar phenotype to HA-aPKCλ KN was also observed when over-expressing wild-type Par-3 (T7-Par-3 WT, 46.9%). Moreover, over-expressing the dominant-negative mutant of Par-3 S827A, which is a non-phosphorylated mutant of aPKC, inhibited YAP activation (T7-Par-3 SA, 35.3%). On the other hand, YAP nuclear localization was not inhibited by over-expressing an aPKC-binding deficient point mutant of Par-3 S827/829A (T7-Par-3 2SA, 69.3%). Phosphorylation of Par-3 by aPKC may contribute to the release of inhibition by Par-3. Therefore, Par-3 may inhibit aPKC and YAP activation mediated by high glucose levels.

YAP signaling is regulated by the high glucose-mediated activation of aPKC in PC cells. The EMT transcription factor ZEB1 interacts with YAP to activate tumor-promoting gene expression (8, 9). YAP activation may positively affect PC progression through ZEB1 gene expression. As shown in Figure 4A, aPKC-knockdown cells decreased ZEB1 expression mediated by high-glucose treatment. Thus, aPKC-mediated YAP activation may play a critical role in cancer cell migration and invasion stimulated by high glucose.

We next examined Par-3 knockdown to determine whether Par-3 and aPKC also interact to inhibit YAP activation. Par-3 knockdown increased YAP expression in PC cells under normal culture without high-glucose treatment (Figure 4B and C). It also increased ZEB1 expression independent of high-glucose treatment (Figure 4B and D). These data demonstrated that aPKC-YAP signaling was involved in the high glucose-mediated up-regulation of PC cell proliferation, migration, and invasion.

Discussion

Diabetes mellitus is a lifestyle-related disease and a risk factor for cancer progression (3, 4). The number of patients with diabetes has been increasing in recent years (4). However, no studies have examined the association of PC progression with diabetes mellitus, which is a persistent hyperglycemic condition. Therefore, we determined whether a persistent high glucose level affects the progression of PC. First, we found that high-glucose treatment of PC cells enhanced cell proliferation, migration, and invasive potential (Figure 1). This result supports our hypothesis that a sustained state of high-glucose stimulation is involved in PC cell progression.

PC exhibits mutational activation of the Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) oncogene and inactivation of tumor-suppressor genes, including cyclin-dependent kinase inhibitor 2A, tumor protein 53, SMAD family member 4, and breast cancer gene 2 (21). KRAS mutation increases the expression of GLUT-1 and rate-limiting glycolytic enzymes, including hexokinase, phosphofructokinase 1, and lactate dehydrogenase A, which promote glycolytic activity and increase lactate production (22). In cancer cells, glycolysis is an important process that provides energy and biomass for cell growth (23). The 2-DG is a glucose analog that can inhibit glycolysis by competitively inhibiting hexokinase 2, which suppresses the growth and metastasis of cancer cells (24, 25). The glycolytic intermediate 3-phosphoglycerate is the major pathway for serine synthesis in vivo and is required for cancer cell growth and proliferation (26). Serine is also important for the synthesis of phosphatidylserine (PS), a lipid messenger in cell signaling pathways. The PS is an important activator of the PKC family of enzymes, including the aPKC family (27, 28). Inhibition of the glycolytic system by 2-DG treatment suppressed the activation of both aPKC and YAP (Figure 1). The high glucose–mediated activation of aPKC may be caused by PS generation from glucose uptake by PC cells. Therefore, increased PS production from glucose metabolism in PC cells may contribute to aPKC activation, but the details require further investigation.

Glucose uptake into cancer cells by GLUT-1 has been reported. GLUT-1 is highly expressed in the cell lines of pancreatic ductal adenocarcinoma (BxPC-3 and Panc-1) (19). Inhibiting the aPKC activity by the myristoylated PKC peptide reduced the amount of GLUT-1 at the plasma membrane (29). Thus, aPKC activation may promote the cellular uptake of glucose by facilitating GLUT-1 localization to the plasma membrane, thereby further accelerating the proliferation and progression of PC cells.

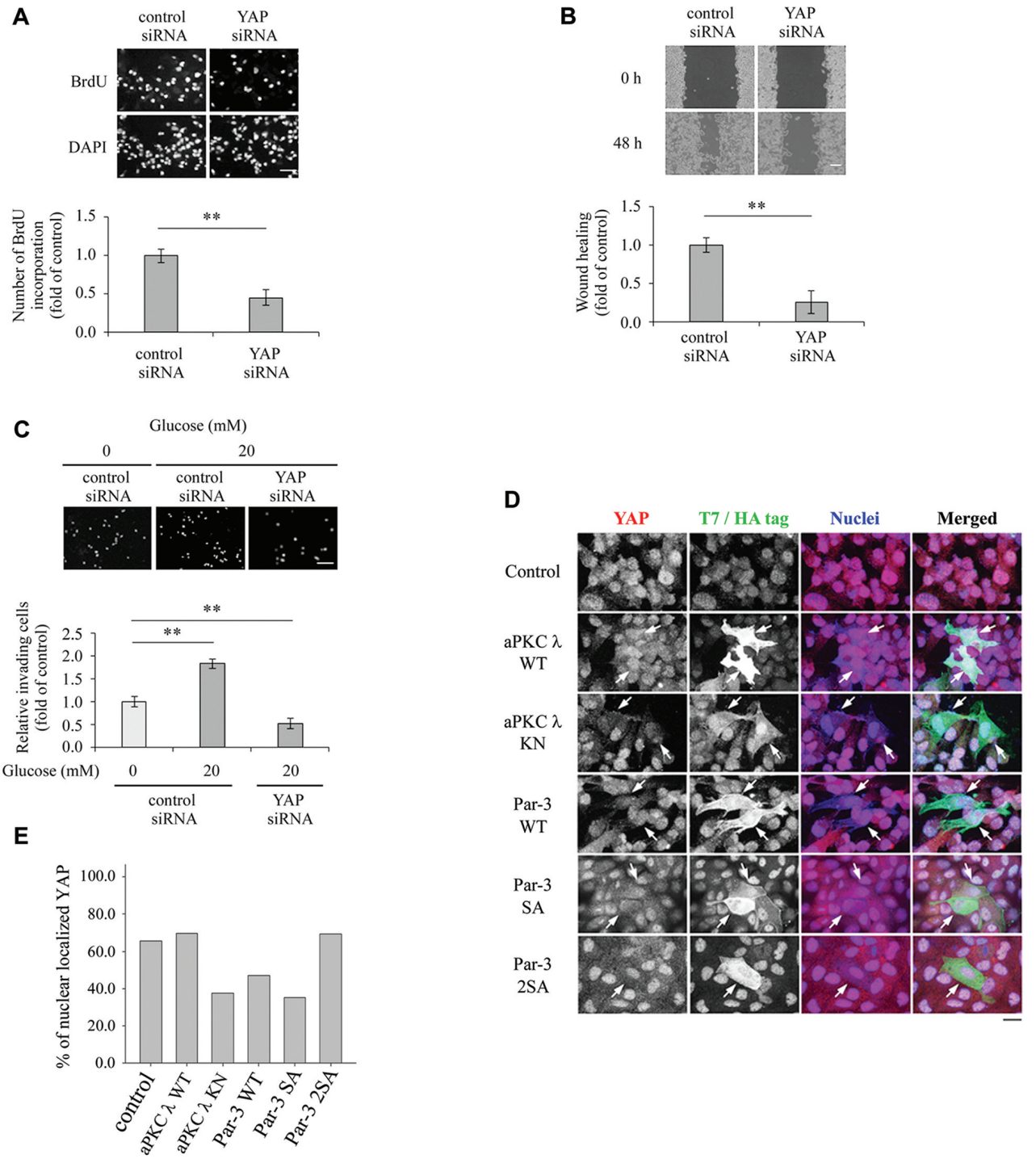

Figure 3. *Par-3 inhibits high glucose–induced YAP activation. (A) YAP siRNA- or control siRNA-transfected MIA PaCa-2 cells were incubated for 48 h in the presence of 20 mM glucose. Cell proliferation was measured by BrdU assay. Scale bar=100 μm. (B) The migration rate of PC cells was analyzed by wound healing assay. Scale bar=200 μm. (C) Cell invasion was analyzed by cell invasion assay. Scale bar=100 μm. (D) MDCK epithelial cells were transfected with the control, HA-aPKC λ WT, HA-aPKC λ-dominant-negative mutant (KN), T7-Par-3 WT, dominant-negative mutant of T7-Par-3 S827A (SA), aPKC-binding deficient mutant of T7-Par-3 S827/829A (2SA) and immunostained for over-expressed cells (green, arrows) and YAP (red). HA-aPKC λ WT over-expressed cells, but not HA-aPKC λ KN cells, promoted the high glucose–stimulated translocation of YAP to the nucleus. In contrast, high glucose–stimulated YAP translocation to the nucleus was inhibited in cells with T7-Par-3 WT and T7-Par-3 SA expression, but not in aPKC-binding deficient mutant of T7-Par-3 2SA. (E) Quantification of the results shown in (D). Percentage of the nuclear location of YAP in cells expressing HA-aPKC λ or T7-Par-3. Scale bar=20 μm. The error bars indicate the standard deviation of 3 or 6 independent experiments. *p<0.05, **p<0.01.*

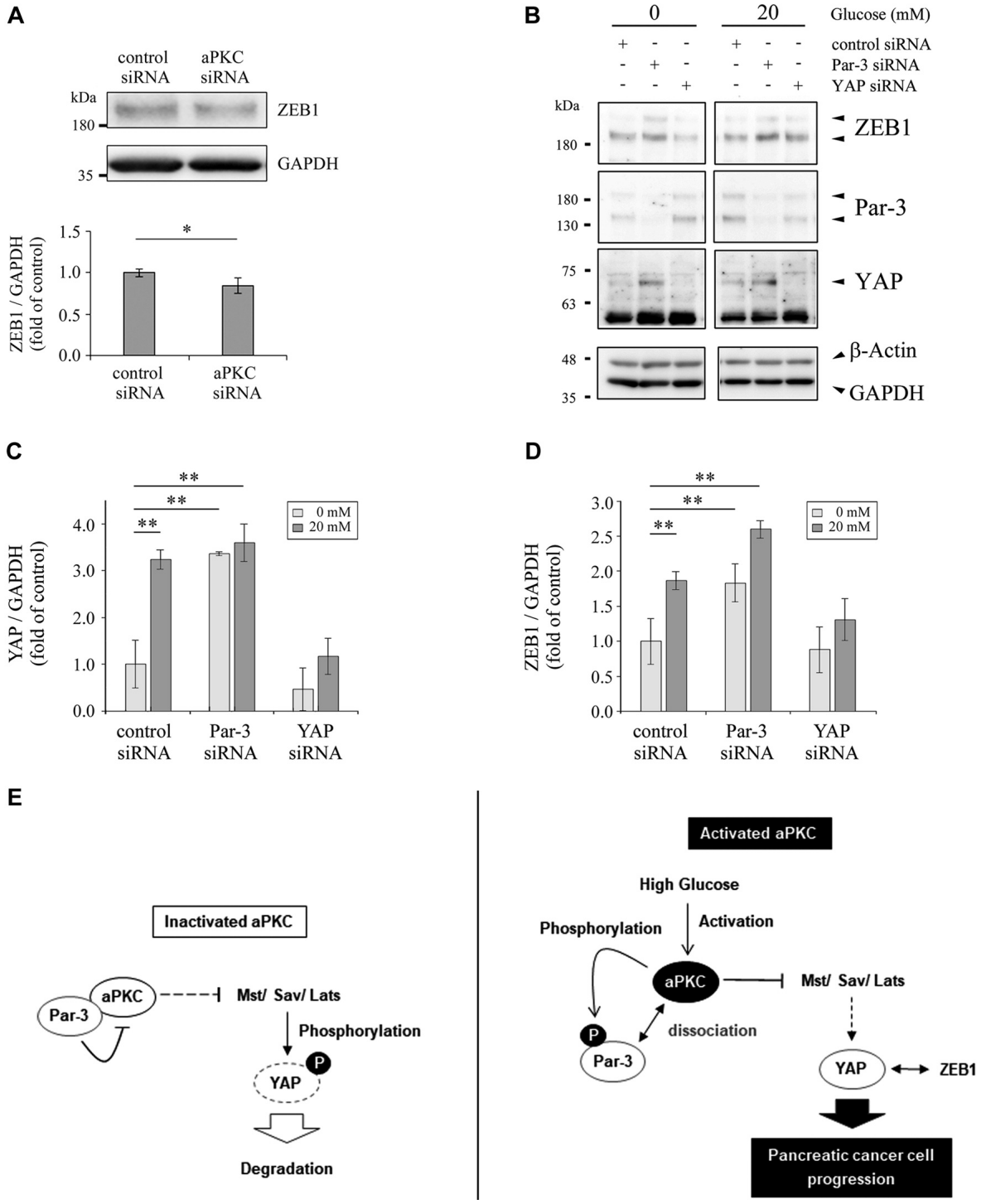

Figure 4. *aPKC/YAP/ZEB1 signaling is involved in the high glucose–mediated progression of PC cells. (A) To examine the effect of aPKC silencing on ZEB1 expression, we transfected MIA PaCa-2 cells with aPKC siRNA or control siRNA and incubated them for 24 h in the presence of 20 mM glucose; ZEB1 expression was analyzed by western blotting and then quantified. (B) YAP- or Par-3-knockdown MIA PaCa-2 cells were incubated for 24 h with or without 20 mM glucose, and the expression of ZEB1, Par-3, and YAP was examined using western blotting. The results of densitometric analysis for YAP (C) and ZEB1 (D) are shown as relative density. (E) Model for the mechanism of high-glucose–induced PC cell progression. A potential mechanism underlying the regulation of PC cell progression by aPKC, YAP, and Par-3. Par-3 is involved in PC progression inhibition by regulating the aPKC activity (left panel). High glucose–induced aPKC activation leads to PC cell progression via YAP signal (right panel). The error bars indicate the standard deviation of three or six independent experiments. *p<0.05, **p<0.01.*

Glucose metabolites are involved in the activation of the YAP signaling pathway through the O-GlcNAcylation of YAP (5, 6). Glucose starvation or 2-DG treatment dramatically reduces YAP O-GlcNAcylation and dephosphorylation/activation (6). Thus, a crosstalk between glucose metabolism and YAP signaling is important in cancer progression. Our analysis confirms that high-glucose treatment enhances PC cell proliferation, migration, and invasive potential in a YAP activation–dependent manner. We hypothesize that aPKC enhances the uptake of glucose into the cells, thereby accelerating and then activating YAP glycosylation (5, 6).

Most of the metabolic effects of insulin are mediated by aPKC and protein kinase B (or Akt), which are both downstream effectors of PI3K (30). In many cancers, including PC, aPKC is over-expressed (10, 11). The aPKC is an important downstream regulator of KRAS, and aPKC activation is associated with promoting cancer progression (31, 32). In fact, aPKC regulates EMT and cell migration and may contribute to a poor prognosis in patients with cancer (10, 32, 33). We found that aPKC is activated by stimulating PC cells with high glucose (Figure 1D and 1E). In addition, aPKC-knockdown inhibited the proliferation, migration, and invasion of MIA PaCa-2 cells (Figure 2). YAP complexes with other transcription factors to regulate the expression of target genes are essential for tumorigenesis. ZEB1 interacts directly with YAP to form an activator complex and to induce the expression of target genes as key signaling hubs in the EMT process (8). We found that ZEB1 involved in cancer progression is up-regulated downstream of YAP activation by aPKC (Figure 4A). In addition, aPKC or YAP knockdown in PC cells prevented the high glucose–induced expression of ZEB1 (Figure 4). Thus, high-glucose stimulation promotes PC cell progression upon YAP activation *via* the aberrant activation of aPKC. The activation of aPKC reportedly induces cell transformation *via* YAP activation (12). The aberrant activation of aPKC causes the disruption of epithelial cell adhesion structures (32-34). YAP nuclear localization is suppressed by cell–cell junctional structures (7). The disruption of intercellular adhesion structures by the aberrant activation of aPKC may be involved in regulating YAP activation and nuclear localization in PC progression. To regulate cell polarity, aPKC forms a complex with Par-3 and Par-6. Soriano et al. has shown that Par-3 within the Par complex inhibits aPKC kinase activity or substrate interaction (20). Par-3 may be involved in regulating aPKC activity or accessing other aPKC substrates. Importantly, in some human cancers, Par-3 loss is associated with accelerated tumor progression, suggesting that Par-3 functions as a tumor-suppressor gene (35). Par-3 knockdown experiments have shown that Par-3 knockdown leads to a glucose-independent activation of YAP (Figure 4A and C). In contrast, immunofluorescent staining analysis revealed that wild-type Par-3 over-expression inhibited the high glucose–induced translocation of YAP to the nucleus (Figure 3D and E). Furthermore, over-expression of the dominant-negative mutant of Par-3 S827A, a non-phosphorylated mutant by aPKC, inhibited YAP activation. Conversely, the aPKC-binding deficient mutant of Par-3 (S837/829A) did not inhibit the nuclear localization of YAP. Par-3 phosphorylation by aPKC may be required to suppress Par-3 inhibition. These findings suggest that Par-3 is involved in regulating PC progression by modulating the aPKC activity (Figure 4E). Supporting these findings, Par-3 reportedly plays a role in regulating the YAP activity (36).

Conclusion

In conclusion, high glucose-stimulated aPKC activation leads to PC cell progression, which may be mediated by aPKC-induced YAP activation. These data strongly suggest that aPKC and YAP inhibition is an effective therapeutic target for PC. Further studies are required to determine whether an inhibitor of aPKC and/or YAP can suppress the growth, migration, and invasion of PC cells.

Conflicts of Interest

There are no conflicts of interest to declare in relation to this study. The authors declare that they have not used any type of generative artificial intelligence for the writing of this manuscript nor for the creation of images, graphics, tables, or their corresponding captions.

Authors' Contributions

Teppei Sunaguchi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-review & editing. Yosuke Horikoshi: Conceptualization, Formal analysis, Funding acquisition, Resources, Methodology, Project administration, Supervision, Validation, Visualization, Writing–original draft, Writing-review & editing. Takehiko Hanaki: Conceptualization, Supervision, Funding acquisition, Resources, Writing-review & editing. Teruhisa Sakamoto: Resources, Validation. Kazuhiro Nakaso: Resources, Validation. Chieko Sakai: Resources, Validation. Kazunari Yamashita: Methodology, Resources, Validation. Shigeo Ohno: Methodology, Resources. Yoshiyuki Fujiwara: Supervision, Writing-review & editing. Tatsuya Matsura: Supervision, Writing-review & editing.

Acknowledgements

The Authors would like to thank Wataru Miyauchi for insightful discussions. This study was partly performed using the facilities of the Tottori BioFrontier managed by the Tottori prefecture (https://www.bio-frontier.jp/english).

Funding

This work was partly supported by Tottori University's President's Discretionary Fund and Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers 19K07505 (Y.H.), and 21K16421 (T.H.).

References

1 Qin C, Yang G, Yang J, Ren B, Wang H, Chen G, Zhao F, You L, Wang W, Zhao Y: Metabolism of pancreatic cancer: paving the way to better anticancer strategies. Mol Cancer 19(1): 50, 2020. DOI: 10.1186/s12943-020-01169-7

2 Zhang L, Sanagapalli S, Stoita A: Challenges in diagnosis of pancreatic cancer. World J Gastroenterol 24(19): 2047-2060, 2018. DOI: 10.3748/wjg.v24.i19.2047

3 Zhang YB, Pan XF, Lu Q, Wang YX, Geng TT, Zhou YF, Liao LM, Tu ZZ, Chen JX, Xia PF, Wang Y, Wan ZZ, Guo KQ, Yang K, Yang HD, Chen SH, Wang GD, Han X, Wang YX, Yu D, He MA, Zhang XM, Liu LG, Wu T, Wu SL, Liu G, Pan A: Associations of combined healthy lifestyles with cancer morbidity and mortality among individuals with diabetes: results from five cohort studies in the USA, the UK and China. Diabetologia 65(12): 2044-2055, 2022. DOI: 10.1007/s00125-022-05754-x

4 Hu Y, Zeng N, Ge Y, Wang D, Qin X, Zhang W, Jiang F, Liu Y: Identification of the shared gene signatures and biological mechanism in type 2 diabetes and pancreatic cancer. Front Endocrinol (Lausanne) 13: 847760, 2022. DOI: 10.3389/fendo.2022.847760

5 Zhang X, Qiao Y, Wu Q, Chen Y, Zou S, Liu X, Zhu G, Zhao Y, Chen Y, Yu Y, Pan Q, Wang J, Sun F: The essential role of YAP O-GlcNAcylation in high-glucose-stimulated liver tumorigenesis. Nat Commun 8: 15280, 2017. DOI: 10.1038/ncomms15280

6 Peng C, Zhu Y, Zhang W, Liao Q, Chen Y, Zhao X, Guo Q, Shen P, Zhen B, Qian X, Yang D, Zhang JS, Xiao D, Qin W, Pei H: Regulation of the Hippo-YAP pathway by glucose sensor O-GlcNAcylation. Mol Cell 68(3): 591-604.e5, 2017. DOI: 10.1016/j.molcel.2017.10.010

7 Yu FX, Zhao B, Guan KL: Hippo pathway in organ size control, tissue homeostasis, and cancer. Cell 163(4): 811-828, 2015. DOI: 10.1016/j.cell.2015.10.044

8 Lehmann W, Mossmann D, Kleemann J, Mock K, Meisinger C, Brummer T, Herr R, Brabletz S, Stemmler MP, Brabletz T: ZEB1 turns into a transcriptional activator by interacting with YAP1 in aggressive cancer types. Nat Commun 7: 10498, 2016. DOI: 10.1038/ncomms10498

9 Krebs AM, Mitschke J, Lasierra Losada M, Schmalhofer O, Boerries M, Busch H, Boettcher M, Mougiakakos D, Reichardt W, Bronsert P, Brunton V, Pilarsky C, Winkler TH, Brabletz S, Stemmler MP, Brabletz T: The EMT-activator Zeb1 is a key factor for cell plasticity and promotes metastasis in pancreatic cancer. Nat Cell Biol 19(5): 518-529, 2017. DOI: 10.1038/ncb3513

10 Scotti ML, Bamlet WR, Smyrk TC, Fields AP, Murray NR: Protein kinase Ciota is required for pancreatic cancer cell transformed growth and tumorigenesis. Cancer Res 70(5): 2064-2074, 2010. DOI: 10.1158/0008-5472.CAN-09-2684

11 Kasai T, Tamori S, Takasaki Y, Matsuoka I, Ozaki A, Matsuda C, Harada Y, Sasaki K, Ohno S, Akimoto K: High expression of PKCλ and ALDH1A3 indicates a poor prognosis, and PKCλ is required for the asymmetric cell division of ALDH1A3-positive cancer stem cells in PDAC. Biochem Biophys Res Commun 669: 85-94, 2023. DOI: 10.1016/j.bbrc.2023.05.086

12 Archibald A, Al-Masri M, Liew-Spilger A, McCaffrey L: Atypical protein kinase C induces cell transformation by disrupting Hippo/Yap signaling. Mol Biol Cell 26(20): 3578-3595, 2015. DOI: 10.1091/mbc.E15-05-0265

13 Hanaki T, Horikoshi Y, Nakaso K, Nakasone M, Kitagawa Y, Amisaki M, Arai Y, Tokuyasu N, Sakamoto T, Honjo S, Saito H, Ikeguchi M, Yamashita K, Ohno S, Matsura T: Nicotine enhances the malignant potential of human pancreatic cancer cells *via* activation of atypical protein kinase C. Biochim Biophys Acta 1860(11 Pt A): 2404-2415, 2016. DOI: 10.1016/j.bbagen.2016.07.008

14 Kurashiki T, Horikoshi Y, Kamizaki K, Sunaguchi T, Hara K, Morimoto M, Kitagawa Y, Nakaso K, Otsuki A, Matsura T: Molecular mechanisms underlying the promotion of wound repair by coenzyme Q10: PI3K/Akt signal activation *via* alterations to cell membrane domains. J Clin Biochem Nutr 70(3): 222-230, 2022. DOI: 10.3164/jcbn.21-141

15 Horikoshi Y, Kamizaki K, Hanaki T, Morimoto M, Kitagawa Y, Nakaso K, Kusumoto C, Matsura T: α-Tocopherol promotes HaCaT keratinocyte wound repair through the regulation of polarity proteins leading to the polarized cell migration. BioFactors 44(2): 180-191, 2018. DOI: 10.1002/biof.1414

16 Nagai-Tamai Y, Mizuno K, Hirose T, Suzuki A, Ohno S: Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells. Genes Cells 7(11): 1161-1171, 2002. DOI: 10.1046/j.1365-2443.2002.00590.x

17 Horikoshi Y, Suzuki A, Yamanaka T, Sasaki K, Mizuno K, Sawada H, Yonemura S, Ohno S: Interaction between PAR-3 and the aPKC-PAR-6 complex is indispensable for apical domain development of epithelial cells. J Cell Sci 122(Pt 10): 1595-1606, 2009. DOI: 10.1242/jcs.043174

18 Akimoto K, Takahashi R, Moriya S, Nishioka N, Takayanagi J, Kimura K, Fukui Y, Osada S, Mizuno K, Hirai S, Kazlauskas A, Ohno S: EGF or PDGF receptors activate atypical PKClambda through phosphatidylinositol 3-kinase. EMBO J 15(4): 788-798, 1996. DOI: 10.1002/j.1460-2075.1996.tb00414.x

19 Maher JC, Savaraj N, Priebe W, Liu H, Lampidis TJ: Differential sensitivity to 2-Deoxy-D-glucose between two pancreatic cell lines correlates with GLUT-1 expression. Pancreas 30(2): e34-e39, 2005. DOI: 10.1097/01.mpa.0000153327.46945.26

20 Soriano EV, Ivanova ME, Fletcher G, Riou P, Knowles PP, Barnouin K, Purkiss A, Kostelecky B, Saiu P, Linch M, Elbediwy A, Kjær S, O'Reilly N, Snijders AP, Parker PJ, Thompson BJ, McDonald NQ: aPKC inhibition by Par3 CR3 flanking regions controls substrate access and underpins apical-junctional polarization. Dev Cell 38(4): 384-398, 2016. DOI: 10.1016/j.devcel.2016.07.018

21 Rozenblum E, Schutte M, Goggins M, Hahn SA, Panzer S, Zahurak M, Goodman SN, Sohn TA, Hruban RH, Yeo CJ, Kern SE: Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res 57(9): 1731-1734, 1997.

22 Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, Locasale JW, Son J, Zhang H, Coloff JL, Yan H, Wang W, Chen S, Viale A, Zheng H, Paik JH, Lim C, Guimaraes AR, Martin ES, Chang J, Hezel AF, Perry SR, Hu J, Gan B, Xiao Y, Asara JM, Weissleder R, Wang YA, Chin L, Cantley LC, DePinho RA: Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. Cell 149(3): 656-670, 2012. DOI: 10.1016/j.cell.2012.01.058

23 McDonald OG, Li X, Saunders T, Tryggvadottir R, Mentch SJ, Warmoes MO, Word AE, Carrer A, Salz TH, Natsume S, Stauffer KM, Makohon-Moore A, Zhong Y, Wu H, Wellen KE, Locasale JW, Iacobuzio-Donahue CA, Feinberg AP: Epigenomic reprogramming during pancreatic cancer progression links

anabolic glucose metabolism to distant metastasis. Nat Genet 49(3): 367-376, 2017. DOI: 10.1038/ng.3753
24 Singh S, Pandey S, Chawla AS, Bhatt AN, Roy BG, Saluja D, Dwarakanath BS: Dietary 2-deoxy-D-glucose impairs tumour growth and metastasis by inhibiting angiogenesis. Eur J Cancer 123: 11-24, 2019. DOI: 10.1016/j.ejca.2019.09.005
25 Cay O, Radnell M, Jeppsson B, Ahrén B, Bengmark S: Inhibitory effect of 2-deoxy-d-glucose on liver tumor growth in rats. Cancer Res 52(20): 5794-5796, 1992.
26 Chaneton B, Hillmann P, Zheng L, Martin ACL, Maddocks ODK, Chokkathukalam A, Coyle JE, Jankevics A, Holding FP, Vousden KH, Frezza C, O'Reilly M, Gottlieb E: Serine is a natural ligand and allosteric activator of pyruvate kinase M2. Nature 491(7424): 458-462, 2012. DOI: 10.1038/nature11540
27 Nakanishi H, Exton JH: Purification and characterization of the zeta isoform of protein kinase C from bovine kidney. J Biol Chem 267(23): 16347-16354, 1992. DOI: 10.1016/S0021-9258(18)42008-X
28 Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258(5082): 607-614, 1992. DOI: 10.1126/science.1411571
29 Bosch RR, Bazuine M, Span PN, Willems PH, Olthaar AJ, van Rennes H, Maassen JA, Tack CJ, Hermus AR, Sweep CG: Regulation of GLUT1-mediated glucose uptake by PKClambda-PKCbeta(II) interactions in 3T3-L1 adipocytes. Biochem J 384(Pt 2): 349-355, 2004. DOI: 10.1042/BJ20040797
30 Farese RV, Sajan MP, Standaert ML: Atypical protein kinase c in insulin action and insulin resistance. Biochem Soc Trans 33(Pt 2): 350-353, 2005. DOI: 10.1042/BST0330350
31 Murray NR, Jamieson L, Yu W, Zhang J, Gökmen-Polar Y, Sier D, Anastasiadis P, Gatalica Z, Thompson EA, Fields AP: Protein kinase Ciota is required for Ras transformation and colon carcinogenesis in vivo. J Cell Biol 164(6): 797-802, 2004. DOI: 10.1083/jcb.200311011
32 Imamura N, Horikoshi Y, Matsuzaki T, Toriumi K, Kitatani K, Ogura G, Masuda R, Nakamura N, Takekoshi S, Iwazaki M: Localization of apkc lambda/iota and its interacting protein, lgl2, is significantly associated with lung adenocarcinoma progression. Tokai J Exp Clin Med 38(4): 146-158, 2013.
33 Du GS, Wang JM, Lu JX, Li Q, Ma CQ, Du JT, Zou SQ: Expression of P-aPKC-ι, E-Cadherin, and β-Catenin related to invasion and metastasis in hepatocellular carcinoma. Ann Surg Oncol 16(6): 1578-1586, 2009. DOI: 10.1245/s10434-009-0423-7
34 Horikoshi Y, Kitatani K, Toriumi K, Fukunishi N, Itoh Y, Nakamura N, Ohno S, Matsura T, Takekoshi S: Aberrant activation of atypical protein kinase C in carbon tetrachloride–induced oxidative stress provokes a disturbance of cell polarity and sealing of bile canalicular lumen. Am J Pathol 185(4): 958-968, 2015. DOI: 10.1016/j.ajpath.2014.12.015
35 Atashrazm F, Ellis S: The polarity protein PARD3 and cancer. Oncogene 40(25): 4245-4262, 2021. DOI: 10.1038/s41388-021-01813-6
36 Zhou PJ, Wang X, An N, Wei L, Zhang L, Huang X, Zhu HH, Fang YX, Gao WQ: Loss of Par3 promotes prostatic tumorigenesis by enhancing cell growth and changing cell division modes. Oncogene 38(12): 2192-2205, 2019. DOI: 10.1038/s41388-018-0580-x

Received September 13, 2023
Revised October 4, 2023
Accepted October 5, 2023