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Original Articles

p21-Activated kinase 3 promotes cancer stem cell phenotypes through activating the Akt-GSK3β-β-catenin signaling pathway in pancreatic cancer cells

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ABSTRACT

Relative to several other p21-activated kinase (PAK) family members, the role of PAK3 in regulating cancer cell functions remains unclear. Our study obtained evidence that PAK3 regulates the Akt-GSK3β-β-catenin signaling by acting as Ser^{473}-Akt kinase in several pancreatic cancer cell lines. Specifically, knockdown of PAK3 or overexpression of dominant-negative PAK3 inhibited the phosphorylation of Ser^{473}-Akt and GSK3β, resulting in the proteasomal degradation of β-catenin. Conversely, overexpression of PAK3 led to activation of Akt signaling and increased β-catenin expression. These changes, however, were not noted with the silencing and/or overexpression of PAK1, PAK2, or PAK4, which underlies the impetus of PAK3 as a key effector in governing malignant phenotypes in these pancreatic cancer cells, including cancer stem cell (CSC) expansion. Accordingly, PAK3 depletion effectively suppresses tumorsphere formation, ALDH activity, and the expression of CSC surface markers. Moreover, we used a stable knockdown clone of AsPC-1 cells to demonstrate the *in vivo* efficacy of PAK3 inhibition in suppressing tumorigenesis and xenograft tumor growth. Together, these findings suggest the potential role of PAK3 as a target for pancreatic cancer therapy, which warrants further investigations.

1. Introduction

Pancreatic cancer is the third leading cause of cancer-related deaths, with a dismal five-year survival rate, in the United States [1]. Approximately 75% of pancreatic cancer patients will die within the first year of diagnosis, and only 8% will survive more than five years [1]. While surgery might improve long term survival, over 80% of pancreatic cancer patients have unresectable tumors with metastasis at diagnosis [2]. Chemotherapy, alone or in combination with radiation, is the only option for these patients. However, despite decades of tremendous efforts in developing early diagnosis and novel therapies, progress has been limited in improving the overall survival rate. Therefore, development of more effective therapeutic strategies to improve the clinical response is urgently needed.

Accumulating evidence suggests the importance of cancer stem cells (CSCs) or cancer initiating cells in promoting tumorigenesis, cancer recurrence, metastasis, and resistance to chemo- and radiotherapy [3–7]. Although it accounts for less than 1% of all pancreatic cancer cells, pancreatic CSCs are considered responsible not only for tumor initiation, but also for metastasis and therapeutic resistance [8–11]. Aberrant activation of the Hedgehog, Notch, and/or Wnt signaling pathways has been implicated in the maintenance of pancreatic CSCs [10,12,13]. In principle, targeting these pathways to eliminate pancreatic CSCs might have therapeutic potential in pancreatic cancer treatment.

p21-Activated kinases (PAKs) are a family of six evolutionarily conserved serine/threonine protein kinases that act as downstream effectors of the small Rho GTPases Rac1 and Cdc42 in mammalian cells [14,15]. All PAKs are composed of an N-terminal regulatory domain and a highly conserved C-terminal kinase domain. Based on the

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structural and functional homology, these PAK proteins are classified into two groups, i.e., group I (PAK1-PAK3) and group II (PAK4-PAK6) [16]. The regulatory domain of the group I PAKs consists of p21 (Rac/Cdc42)-binding domain (PBD) and an overlapping auto-inhibitory domain (AID) [15,16]. In the inactive state, group I PAKs exist as a homodimer through trans-inhibition between the AID and the kinase domain [17,18]. Binding of activated GTPase (Rac or Cdc42) to the PBD leads to a series of conformational changes, which, in turn, activates group I PAKs by dissociating the AID from the kinase domain [18,19]. Numerous studies have shown that PAKs not only regulate cytoskeleton and cell motility, but also participate in cell proliferation, apoptosis, and mitosis [20–22]. Alterations in the expression of PAKs have been detected in many types of human tumors, including breast, colon, ovarian, bladder, pancreas, and brain, and the overexpression of PAKs is linked to tumor progression and drug resistance [23–26].

Among various reported biological functions, the involvement of PAKs, especially PAK1 [27,28] and PAK4 [29,30] in regulating Akt phosphorylation in different cell systems, either by a kinase-dependent or kinase-independent scaffolding mechanism, is noteworthy. In this study, we report a novel function of PAK3 in regulating the Akt/GSK3β pathway by acting as Ser^{473}-Akt kinase in several pancreatic cancer cell lines examined, including AsPC-1, MiaPaCa-2, and SW1990. Specifically, shRNA-mediated knockdown of PAK3 or overexpression of dominant-negative PAK3 reduced the phosphorylation of Akt at Ser473 and GSK3β, resulting in downregulation of β-catenin. Conversely, overexpression of PAK3 led to activation of Akt, accompanied by increased β-catenin expression. These changes, however, were not noted with silencing of PAK1, PAK2, or PAK4, which underlies the impetus of PAK3 as a key effector in governing malignant phenotypes in these pancreatic cancer cells, among which CSC expansion is noteworthy. Moreover, we used a stable knockdown clone of AsPC-1 cells to demonstrate the *in vivo* efficacy of PAK3 inhibition in suppressing tumorigenesis and xenograft tumor growth. Together, these findings have translation potential to foster new strategies to eradicate pancreatic CSCs by targeting PAK3.

2. Materials and methods

2.1. Cell culture and reagents

Human pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-2, MiaPaCa-2, Panc-1, and SW1990 were obtained from the American Type Culture Collection (Manassas, VA, USA). With the exception of SW1990, these cell lines were maintained in recommended growth medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified incubator containing 5% CO_2. SW1990 cells were cultured in L15-based complete medium with no CO_2/air exchange. HPDE cells were obtained from Dr. Ming-Sound Tsao (University of Toronto) and maintained in growth factor-supplemented keratinocyte serum-free medium (Invitrogen). Antibodies used in this study and their sources were as follows: β-actin, PAK3, p-P70S6K, P70S6K (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Akt, c-Myc, GSK3β, GHA, PAK1, PAK2, p-^{473}S-Akt, p-^{9}S-GSK3β, Oct4, Nanog (Cell Signaling, Beverly, MA, USA); FLAG (Sigma-Aldrich, St. Louis, Mo, USA); PAK4 (Abcam, Cambridge, MA, USA); GAPDH (ProteinTech, Chicago, IL, USA); CD133, CD24, Cyclin D1 (GeneTex, Irvine, CA, USA), siRNAs used in this study and their sources were as follows: PAK4 (Santa Cruz Biotechnology).

2.2. shRNA lentivirus preparation and isolation of stable cell lines

shRNAs against PAK1, PAK2, and PAK3, and the pLAS.Void control shRNA were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The shRNA lentivirus particles were prepared by co-transfection with the packaging plasmid SPAX2 and enveloping plasmid MD2G into HEK293T cells, and the medium containing viral particles were collected after 72 h. After brief centrifugation, polybrene (8 μg/ml) was added to the supernatant to facilitate viral infection. Stable clones were selected by using puromycin for 7–10 days.

2.3. Co-immunoprecipitation (Co-IP)

Cells were harvested, incubated in IP lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100) for 30 min on ice, and then sonicated (3 times, 10 s each). After centrifugation, the supernatant was collected from each sample and then pre-cleared by incubation with 10 μl protein A/G agarose beads at 4 °C for 1 h with rocking. After removal of the protein A/G beads by centrifugation, protein contents in each sample were measured and aliquots containing 1500 μg of proteins were incubated with primary antibodies overnight at 4 °C with rocking, followed by 20 μl protein A/G agarose beads for 2 h at 4 °C. The immunoprecipitates were washed with IP lysis buffer, collected by centrifugation, and resuspended in 2X sample buffer for analysis by immunoblotting.

2.4. Immunocytochemical analysis

Cells were seeded onto round cover glasses in 6-well culture plates. After overnight attachment, cells were fixed with 4% paraformaldehyde (in PBS) for 20 min, and then permeabilized with 0.2% Triton X-100 (in PBS) for 10 min. These cells were blocked with 1% BSA in PBS for 1 h, and then incubated with primary antibody overnight at 4 °C. After washing with PBS, cells were incubated with secondary antibody for 1 h at room temperature. The cover glasses were mounted with VECTASHIELD® mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining.

2.5. Tumorsphere formation assays

A total of 10^4 AsPC-1, 10^3 MiaPaCa-2, and 10^4 SW1990 cells/well were seeded in ultra-low attachment 24-well plates (Corning Inc., Union City, CA, USA) and maintained in MammoCult™ Human Medium (STEMCELL Technologies; Vancouver, BC, Canada). After 8–10 days, the formation of tumorspheres containing > 30 cells was assessed at x40 magnification. All experiments were performed at least in triplicate.

2.6. Immunoblotting

Cell pellets were lysed in RIPA lysis buffer and sonicated, and the protein content of each sample was determined by using the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). An equal amount of proteins from each sample was loaded per lane, separated by SDS-PAGE, and then transferred onto nitrocellulose membranes. Transferred membranes were blocked with 5% non-fat milk for 1 h and then incubated with primary antibodies overnight at 4 °C. On the next day, membranes were washed with TBST and incubated with the corresponding secondary antibodies for 1 h at room temperature. Chemiluminescence Reagent Plus (Perkin- Elmer; Waltham, MA, USA) was used to detect signals.

2.7. RT -PCR analysis

Total RNA was isolated and reverse transcribed to cDNA using TRIzol reagent (Invitrogen) and the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), respectively, according to the manufacturer's instructions. The resulting PCR products were separated by electrophoresis in a 1% agarose gel. The following primers were used: β-catenin: 5′-ATGGCTACTCAAGCTGAT-3′ and 5′- ATTCTGCATGGTAC GTACAA -3'; β-Actin: 5'-GCTCGTCGTCGACAACGGCTC-3' and 5'-CAA

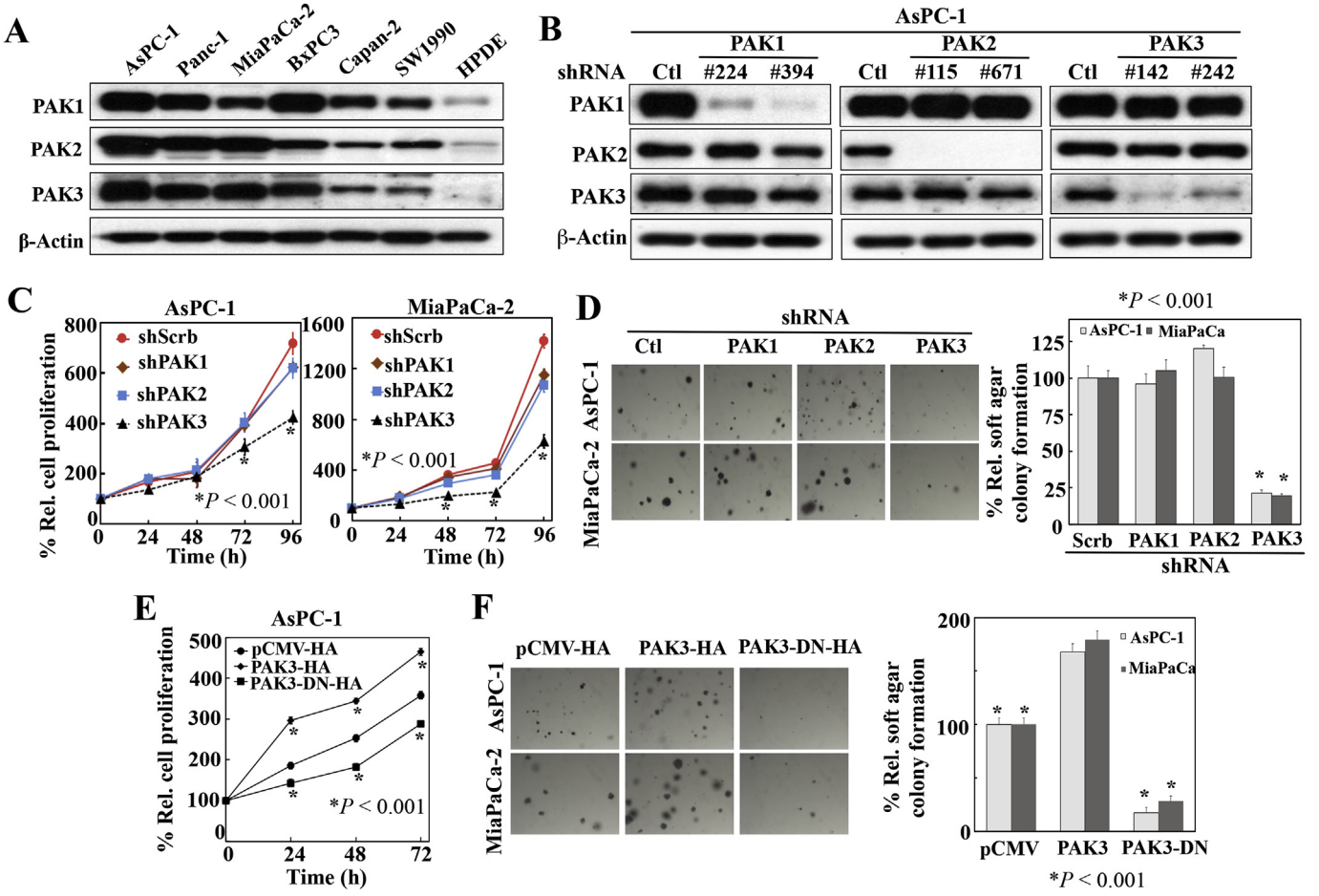

Fig. 1. PAK3 is involved in the regulation of pancreatic cancer cell growth. (A) Western blot analysis of the basal expression levels of PAK1, PAK2, versus PAK3 in six human pancreatic cancer cell lines vis-à-vis immortalized HPDE cell. **(B)** Western blot analysis of the expression levels of PAK1, PAK2, versus PAK3 in the stable knockdown clones of individual isoforms, each with two different shRNAs, in AsPC-1 cells. **(C & D)** Differential effects of the stable knockdown of PAK1, PAK2, versus PAK3 on cell proliferation **(C)** and soft agar colony formation **(D)** in AsPC-1 and MiaPaCa-2 cells. Cell proliferation was determined by MTT assays. Data, means ± S.D.; $n = 3$, $^*p < 0.001$. **(E & F)** Effects of the ectopic expression of empty plasmid (pCMV-HA), wild type PAK3 (PAK3-HA) and dominant negative PAK3 (PAK3-DN-HA) on cell proliferation **(E)** and soft agar colony formation **(F)** in AsPC-1 and/or MiaPaCa-2 cells. Data, means ± S.D.; $n = 3$, $^*p < 0.001$.

ACATGATCTGGGTCATCTTCTC-3'.

2.8. Soft agar colony formation assays

5 ml of medium containing 0.75% agarose was placed in 6-cm dishes as bottom layer. 3 ml of cell suspension with 3×10^4 cells in medium containing 0.36% agarose was placed on top of solidified bottom layer. After three weeks, colonies were stained with crystal violet solution (0.04% crystal violet and 2% ethanol in PBS) and counted. Cell survival is expressed as a percentage and was determined from the numbers of colonies.

2.9. Cell proliferation assays

Cells were seeded onto 96-well plates at a density of 5×10^3 cells per well in the presence of 10% FBS, and cells were incubated with MTT (Biomatik, Wilmington, DE, USA) for a 1 h for the indicated time intervals. The medium was then removed from each well and replaced with DMSO to dissolve the reduced MTT dye for subsequent colorimetric measurement of absorbance at 560 nm. Cell proliferation is expressed as percentages of viable cells relative to the corresponding control cells.

2.10. Plasmid construction and transient transfection

The PAK3-KD-HA (PAK3-K297L-HA) plasmid was generated by site-directed mutagenesis of the HA-tagged wild-type PAK3 plasmids by using the QuickChange II XL site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). The Flag-tagged wild-type PAK1 plasmid was prepared by subcloning PAK1 cDNA into p3XFLAG-CMV36 (Sigma-Aldrich) by NotI/KpnI sites. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.11. ALDH staining

ALDH activity was performed using ALDEFLUOR™ reagent kit (STEMCELL Technologies) according to the manufacturer's protocol. Briefly, 1×10^6 cells were incubated in Aldefluor buffer containing the substrate of ALDH for 30 min at 37 °C. Cells that could catalyze the substrate to its fluorescent product were considered $ALDH^+$. Sorting gates for FACS were drawn relative to cell baseline fluorescence, which was determined by the addition of the ALDH-specific inhibitor diethylaminobenzaldehyde (DEAB) during the incubation. The data were analyzed by Cell Quest Pro (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (FlowJo LLC, Ashland, OR, USA).

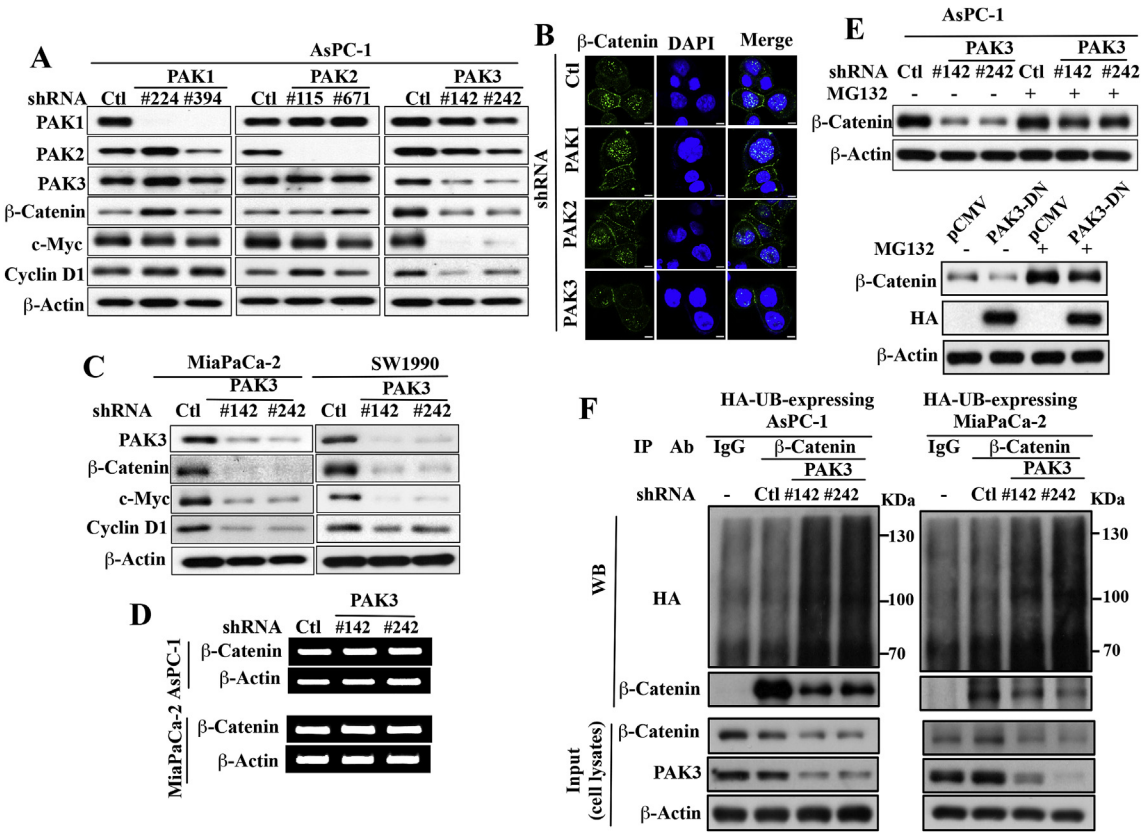

Fig. 2. Evidence that PAK3 regulates β-catenin protein stability in pancreatic cancer cells. (A) Western blot analyses of the differential effects of the stable knockdown of PAK1, PAK2, and PAK3, each with two different shRNAs, on the expression levels of β-catenin, and its target gene products c-Myc, and cyclin D1 in AsPC-1 cells. **(B)** Immunocytochemical analysis of the effects of the stable knockdown of PAK1, PAK2, versus PAK3 on the expression and cellular distribution of β-catenin in AsPC-1 cells. Scale bar = 5 μm. **(C)** Western blot analysis of the effects of stable knockdown of PAK3 on the expression of β-catenin, c-Myc, and cyclin D1 in MiaPaCa-2 and SW1990 cells. **(D)** RT-PCR analysis of the effect of PAK3 knockdown on mRNA expression of β-catenin in AsPC-1 and MiaPaCa-2 cells. **(E)** Western blot analyses of the protective effect of 10 μM MG-132 on PAK3 knockdown- (upper) and PAK3-DN-HA-mediated (lower) downregulation of β-catenin in AsPC-1 cell. **(F)** Co-immunoprecipitation analyses of the effect of stable PAK3 knockdown on β-catenin ubiquitination in AsPC-1 and MiaPaCa-2 cells ectopically expressing HA-ubiquitin (HA-Ub). Equal amounts of cell protein lysates were immunoprecipitated with β-catenin antibody and protein A/G agarose beads followed by immunoblotting with anti-HA antibodies.

2.12. Animal models

All experimental procedures using mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Academia Sinica (AS IACUC). For the xenograft tumor growth model, stable clone of control (Ctl) or PAK3 knockdown (shPAK3 #242; AsPC-1-PAK3^{KD}) AsPC-1 cells (1×10^6 cells in 0.1 ml; 50% Matrigel in PBS) was subcutaneously injected in 8-week-old female athymic nude mice obtained from National Laboratory Animal Center (NLAC, Taipei, Taiwan) ($n = 5$). Tumor volumes were calculated weekly from caliper measurements (with^2 x length x 0.52). For the tumorigenicity model, different amounts of control or PAK3^{KD} AsPC-1 cells (1×10^2, 1×10^3, and 1×10^4 cells in 0.1 ml; 50% Matrigel in PBS) were subcutaneously injected into both flanks of the mice ($n = 8$). After 2–9 weeks post-injection, the tumor incidence in mice receiving PAK3^{KD} xenografts is compared to the respective control groups.

2.13. Statistics analysis

In vitro experiments were performed at least three times and quantitative data are presented as means ± S.D. Data from *in vivo* tumor volume experiments are expressed as means ± S.E.M. Group means were compared using Student's *t*-test under assumptions of normality and equal variance. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). *P*-values less than 0.05 were considered significant.

3. Results

3.1. Evidence that PAK3 is involved in the regulation of pancreatic cancer cell proliferation

To shed light onto the biological function of PAK3 in pancreatic cancer cells, we first examined the relative expression levels of group I PAKs (PAK1-3) in six different pancreatic cancer cell lines versus normal human pancreatic ductal epithelium (HPDE) cells by Western blotting. While all of these pancreatic cancer cell lines exhibited high abundance of PAK1, PAK2 and PAK3 were also upregulated in AsPC-1, Panc-1, MiaPaCa-2, and BxPC-3 cells, and to a lesser extent, in Capan-2 and SW1990 cells relative to that of HPDE cells (Fig. 1A). To discern the functional role of group I PAKs, we conducted shRNA-mediated knockdown of individual isoforms, each with two different shRNAs, to examine the consequent effects on cell proliferation and soft agar colony formation in AsPC-1 and MiaPaCa-2. This genetic silencing was isoform-specific as no cross-inhibition was noted with shRNAs for individual isoforms in three cell lines examined, including AsPC-1 (Fig. 1B), MiaPaCa-2 (Supplementary Fig. S1), and SW1190 cells (Supplementary Fig. S1). It is noteworthy that among these three isoforms, only knockdown of PAK3 could significantly suppress the cell proliferation and colony-forming ability of AsPC-1 and MiaPaCa-2 cells ($P < 0.05$), while depletion of PAK1 or PAK2 displayed no significant inhibition in either functional assay (Fig. 1C and D). Consistent with the effect of PAK3 knockdown, enforced expression of HA-tagged dominant

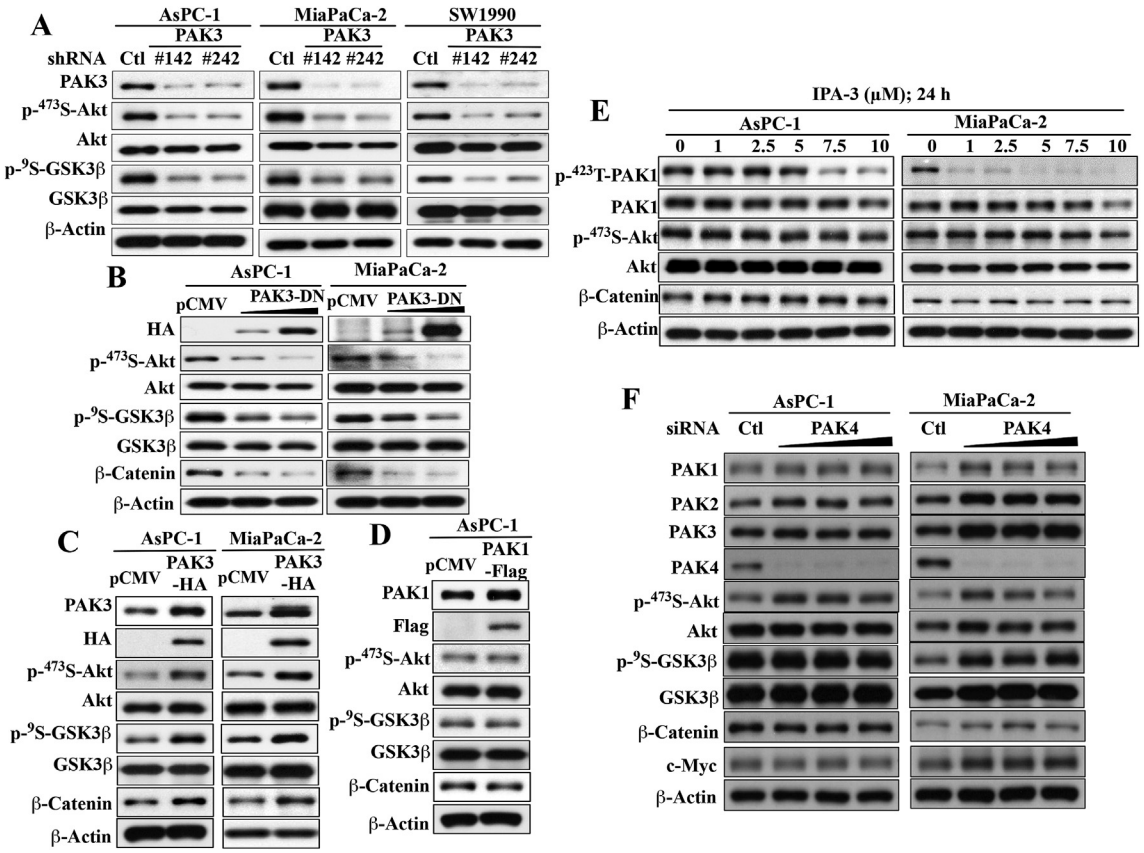

Fig. 3. Evidence that PAK3 regulates β-catenin stability via the Akt/GSK3β signaling pathway. (A) Suppressive effect of stable PAK3 knockdown on the protein expressions/phosphorylation of Akt, Ser^{473}-Akt, GSK3β, and Ser^{9}-GSK3β in AsPC-1, MiaPaCa-2, and SW1990 cells. **(B & C)** Effects of the ectopic expression of PAK3-DN-HA **(B)** and PAK3-HA **(C)** versus the pCMV vector control on the phosphorylation/expression of Ser^{473}-Akt, Akt, Ser^{9}-GSK3β, GSK3β, and β-catenin in AsPC-1 and MiaPaCa-2 cells. **(D & E)** Effect of the ectopic expression of PAK1-Flag versus the pCMV vector control **(D)** and the PAK-1 inhibitor IPA-3 **(E)** on the phosphorylation/expression of Ser^{473}-Akt, Akt, Ser^{9}-GSK3β, GSK3β, and β-catenin in AsPC-1 and/or MiaPaCa-2 cells. **(F)** Effect of siRNA-mediated knockdown of PAK4 on the expression/phosphorylation of PAK1, PAK2, PAK3, PAK4, Ser^{473}-Akt, Akt, Ser^{9}-GSK3β, GSK3β, β-catenin, and c-Myc in AsPC-1 and MiaPaCa-2 cells.

negative kinase dead PAK3 (PAK3-K297L-HA or PAK3-DN-HA) [17] significantly suppressed cell proliferation and soft agar colony formation in AsPC-1 and/or MiaPaCa-2 cells, suggesting the dependence of PAK3 kinase activity. Moreover, overexpression of wild type PAK3 (PAK3-HA) displayed a stimulating effect in both assays (Fig. 1E and F; Western blotting of PAK3-DN or PAK3 overexpression, please see Fig. 3B and C).

3.2. PAK3 regulates β-catenin stability via Akt/GSK3β signaling

Evidence suggests a mechanistic link between PAK1 and Wnt signaling via β-catenin stabilization in different cell systems [23], including gastric mucosa cells [28], colorectal cancer cells [31], and breast epithelial cells [32]. Pursuant to these reports, we examined the effect of the knockdown of PAK3 vis-à-vis PAK1 and PAK2, each with two different shRNA, on the protein expression of β-catenin and its downstream targets c-Myc and cyclin D1 in AsPC-1 cells by Western blotting. Among these three isoforms, depletion of PAK3, but not that of PAK1 or PAK2, caused parallel decreases in these biomarkers (Fig. 2A). This discriminative knockdown effect on β-catenin expression was confirmed by immunocytochemical analysis (Fig. 2B) as well as Western blotting of fractionated cytoplasmic versus nuclear fractions (Supplementary Fig. S2), which showed that silencing of PAK3 not only suppressed the expression of β-catenin, but also facilitated its nuclear exclusion. In contrast, knockdown of PAK1 or PAK2 had no appreciable effect on the expression or cellular distribution of β-catenin (Fig. 2B). This PAK3 knockdown-induced downregulation of β-catenin and its target gene products was also noted in MiaPaCa-2 and SW1990 cells (Fig. 2C). In addition, RT-PCR analysis revealed no changes in the mRNA level of β-catenin in PAK3-knockdown AsPC-1 or MiaPa-Ca-2 cells, suggesting that this downregulation was mediated at the post-transcriptional level (Fig. 2D). The ability of PAK3 to regulate the stability of β-catenin in these cell lines was confirmed by two additional experiments. First, co-treatment of AsPC-1 cells with the proteasome inhibitor MG-132 abolished the suppressive effect of PAK3 knockdown (Fig. 2E, upper) or PAK3-DN overexpression (Fig. 2E, lower) on β-catenin expression. Second, we overexpressed HA-tagged ubiquitin (HA-Ub) via transient transfection in both AsPC-1 and MiaPaCa-2 cells to analyze the effect of PAK3 depletion on β-catenin ubiquitination via co-immunoprecipitation (co-IP). Consistent with our premise, knockdown of PAK3 was associated with increased ubiquitination of β-catenin in both cell lines (Fig. 2F).

In light of the causal relationship between Akt-GSK3β signaling and β-catenin stability, we interrogated the role of PAK3 versus PAK1 and PAK2 in regulating the phosphorylation of Akt and GSK3β. As shown, knockdown of PAK3 in three different cell lines (AsPC-1, MiaPaCa-2, and SW1990), each with two different shRNAs, resulted in concomitant decreases in the phosphorylation of Ser^{473}-Akt and Ser^{9}-GSK3β (Fig. 3A). The ability of PAK3 to regulate the Akt/GSK3β/β-catenin signaling pathway was confirmed by the opposite effects of the ectopic expression of PAK3-DN-HA versus PAK3-HA on Ser^{473}-Akt and Ser^{9}-GSK3β phosphorylation and β-catenin expression in AsPC-1 and MiaPaCa-2 cells (Fig. 3B and C). Equally important, increased β-catenin expression in PAK3-overexpressing AsPC-1 cells was accompanied by its nuclear accumulation, indicating the effect of PAK3 on the nuclear translocation of β-catenin (Supplementary Fig. S3). In contrast,

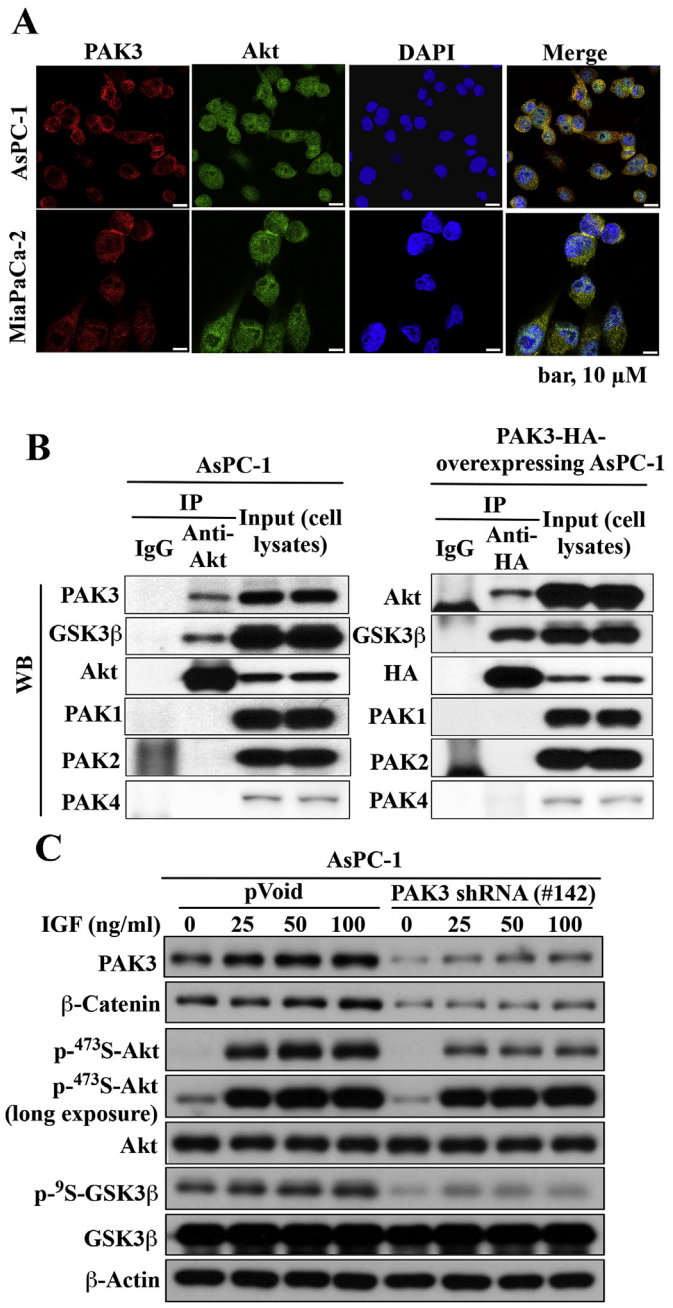

Fig. 4. Evidence that PAK3 facilitates Ser^{473}-Akt phosphorylation. (A) Immunocytochemical analysis shows the co-localization of PAK3 and Akt in AsPC-1 and MiaPaCa-2 cells. Scale bar = 10 μm. **(B)** Co-immunoprecipitation analysis demonstrates the physical association between PAK3 and Akt in AsPC-1 cells. Left, association of endogenous Akt with PAK3 and GSK3β in immunoprecipitated Akt complexes. Right, association of PAK3 with Akt and GSK3β in immunoprecipitated anti-HA complexes in PAK3-HA-overexpressing AsPC-1 cells. **(C)** Concentration-dependent effect of IGF on the expression/phosphorylation of PAK3, Ser^{473}-Akt, Akt, Ser^{9}-GSK3β, GSK3β, and β-catenin in stable control shRNA knockdown (pVOID) and stable PAK3 knockdown (#142) AsPC-1 cells.

overexpression of Flag-tagged PAK1 (PAK1-Flag) had no appreciable effect on the phosphorylation and/or expression of Akt, GSK3β, and β-catenin in AsPC-1 (Fig. 3D). The role of PAK1 in regulating Akt signaling was further refuted by lack of changes in these biomarkers in response to the pharmacological inhibition of PAK1, as manifested by reduced PAK1 phosphorylation, by the small-molecule inhibitor 2,2′-dihydroxy-1,1′-dinaphthyldisulfide (IPA-3) (Fig. 3E). In addition, we used two different shRNA to knockdown of PAK2 in three cell lines (AsPC-1, MiaPaCa-2, and SW1990). As shown, depletion of PAK2 had no appreciable effect on any of the aforementioned biomarkers in these cell lines (Supplementary Fig. S4). Together, these findings suggest the involvement of PAK3 in regulating β-catenin stability via the Akt-GSK3β signaling axis in the above three cell lines.

To illustrate the mechanistic link between PAK3 and Akt-GSK3β-β-catenin signaling, we examined the rescuing effect of overexpressing Akt or β-catenin on PAK3 knockdown-mediated suppression of tumorsphere formation in AsPC-1 and MiaPaCa-2 cells. In line with our hypothesis, overexpression of Akt or β-catenin could overcome the suppressive effect of PAK3 knockdown on the tumorsphere-forming ability of these cells (Supplementary Fig. S5).

In addition, in light of a recent report that PAK4 regulates NF-κB signaling, in part, by activating Akt in two pancreatic cancer cell lines (MiaPaCa-2 and T3M4) [24], we examined the effect of PAK4 knockdown on the phosphorylation and/or expression of Akt, GSK3β, β-catenin, and c-Myc in AsPC-1 and MiaPaCa-2 cells. As shown, silencing of PAK4 did not cause changes in any of these biomarkers (Fig. 3F), arguing against the involvement of PAK4 in regulating Akt-signaling in these two cell lines. This discrepancy in the function of PAK4 in mediating Akt phosphorylation between this study and the reported finding might be attributable to differences in cellular contexts under different experimental conditions, which warrants further investigations.

The causal relationship between PAK3 and Akt was suggested by the co-localization of these two kinases in AsPC-1 and MiaPaCa-2 cells via immunocytochemical analysis (Fig. 4A), which was confirmed by the following experiments. As shown, co-IP analysis using anti-Akt antibody in AsPC-1 cells showed the association of endogenous Akt with PAK3 and GSK3β, while no interactions of Akt with PAK1, PAK2, or PAK4 were noted (Fig. 4B, left). This complex formation was confirmed by using anti-HA antibody in PAK3-HA-overexpressing AsPC-1 cells (Fig. 4B, right). It is worth commenting that no cross-reactivity of anti-PAK1, anti-PAK2, or anti-PAK4 antibody with the overexpressed HA-tagged PAK3 protein was noted, indicating the specificity of these antibodies in isoform recognition. Moreover, our data show that PAK3 expression could be upregulated by insulin-like growth factor (IGF) in a dose-dependent manner in AsPC-1 cells, which was accompanied by increases in Ser^{473}-Akt and GSK3β phosphorylation and β-catenin expression (Fig. 4C). In line with our previous findings, this IGF-induced upregulation of the Akt signaling pathway could be blocked by the knockdown of PAK3 (Fig. 4C), suggesting the role of PAK3 as a downstream effector of IGF signaling in regulating Akt activation.

3.3. Role of PAK3 in regulating of CSC-like properties in pancreatic cancer cells

As the Wnt/β-catenin signaling axis plays a pivotal role in regulating CSCs [33–36], we examined the effect of PAK3 knockdown vis-à-vis that of PAK1 and PAK2 on tumorsphere formation, a surrogate measure of CSC expansion, to validate its CSC-regulatory activity [37]. In AsPC-1, MiaPaCa-2, and SW1990 cells, silencing of PAK3, but not that of PAK1 or PAK2, was effective in suppressing tumorsphere formation (Fig. 5A), suggesting the link between PAK3 and CSCs via Akt signaling. In addition, enforced expression of PAK3-HA increased the tumorsphere formation of AsPC-1 and MiaPaCa-2 cells, which, however, was not observed with PAK3-DN-HA overexpression (Fig. 5B). The ability of PAK3 to modulate the CSC population was also confirmed by the reduction in the ALDH^{+} population in PAK3-knockdown AsCP-1 cells (Fig. 5C). Mechanistically, this anti-CSC activity was associated with the suppressive effect of PAK3 knockdown on various CSC markers, including CD133, CD24, Oct4, and Nanog, in AsPC-1, MiaPaCa-2, and SW1990 (Fig. 5D).

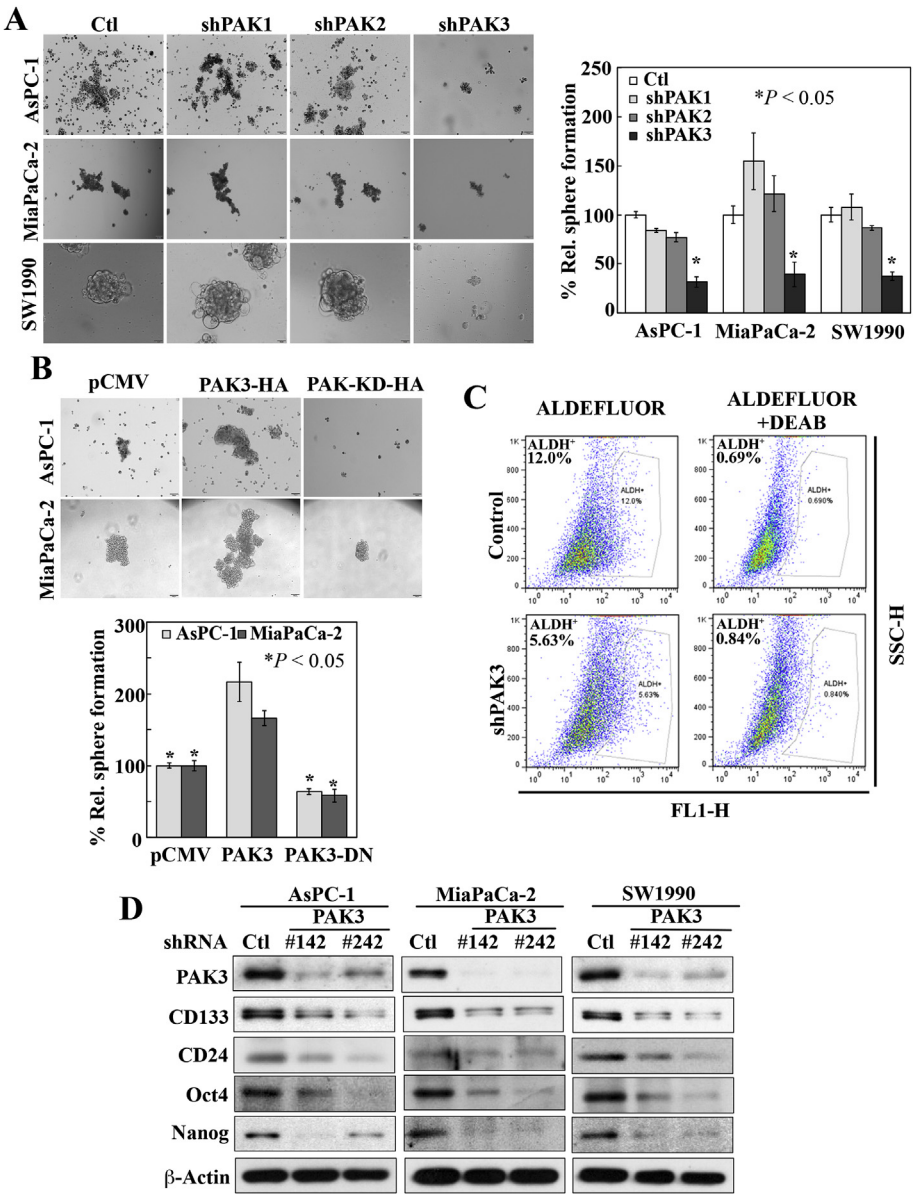

Fig. 5. PAK3 is involved in the regulation of CSC-like properties in pancreatic cancer cells. (A) Effects of stable knockdown of PAK1, PAK2, versus PAK3 on the tumorsphere formations in AsPC-1, MiaPaCa-2, and SW1990 cells. Data, means ± S.D.; $n = 3$, $^*p < 0.05$. **(B)** Effects of the ectopic expression of PAK3-HA and PAK3-DN-HA versus pCMV vector control on tumorsphere formations in AsPC-1 and MiaPaCa-2 cells. Lower, the quantitative results of tumorsphere formation. Data, means ± S.D.; $n = 3$, $^*p < 0.05$. **(C)** Flow cytometric analysis of the effect of stable PAK3 knockdown versus control cells on $ALDH^+$ cell population in AsPC-1 cells. **(D)** Effect of the stable knockdown of PAK3 versus control cells on the expression of various cancer stem cell markers, including CD133, CD24, Oct4, and Nanog, in AsPC-1, MiaPaCa-2, and SW1990 cells.

3.4. *In vivo efficacy of PAK3 knockdown in suppressing tumorigenicity and xenograft tumor growth in nude mice*

Pursuant to the above *in vitro* anti-CSC finding, we used an isolated stable PAK3 knockdown clone ($PAK3^{KD}$, generated from #242 shRNA) of AsPC-1 cells, to assess the *in vivo* efficacy of PAK3 inhibition in suppressing tumorigenesis and xenograft tumor growth. As shown, these $PAK3^{KD}$ cells exhibited substantially reduced, but appreciable, expression/phosphorylation of PAK3 and downstream biomarkers, including Akt, GSk3β, and β-catenin (Fig. 6A). As CSCs are characterized by their capability to form tumors from low cell numbers [38], we examined the effect of PAK3 knockdown on tumor initiation by injecting these $PAK3^{KD}$ cells at 1×10^2, 1×10^3, or 1×10^4 cells/mouse versus the same numbers of control cells subcutaneously into both flanks of the nude mice ($n = 8$ for each group). After 2–9 weeks post-injection, the tumor incidence in mice receiving $PAK3^{KD}$ xenografts is reduced by 62.5% (the 10^2 cells/mouse group) to 50% (the 10^3 and 10^4 cell/mouse groups) compared to the respective control groups, in which all mice developed tumors (Fig. 6B). In a separate experiment, the suppressive effect of PAK3 knockdown on tumor growth was evaluated by injecting mice with 1×10^6 $PAK3^{KD}$ or control cells ($n = 5$). Consistent with the results on tumor initiation, knockdown of PAK3 significantly reduced AsPC-1 xenograft tumor growth relative to the control (54% inhibition; $P < 0.05$; *t*-test) (Fig. 6C).

3.5. *Correlation of PAK3 and Ser-473 Akt phosphorylation in the course of pancreatic tumorigenesis in transgenic $KP^{fl/fl}C$ mice*

To shed light onto the clinical translation value of our finding, we conducted immunohistochemical analysis of PAK3 expression versus Ser^{473}-Akt phosphorylation in pancreatic samples from $KP^{fl/fl}C$ (*Kras*$^{+/G12D}$; *Trp53*$^{flox/flox}$; *Pdx-1-Cre*) mice at different ages (4–5 weeks, $n = 5$; 6–7 weeks, $n = 6$; over 8 weeks, $n = 6$). The $KP^{fl/fl}C$ mouse model recapitulates many aspects of human pancreatic cancer, making it one of the most relevant animal models for pancreatic cancer [39,40]. Immunohistochemistry showed age-dependent increases in the expression of cytokeratin 19 (CK19), a biomarker for lesions in the pancreas [41], indicative of the onset of tumorigenesis (Supplementary Fig. S6). This positive CK19 expression was accompanied by concomitant increases in the expression levels of PAK3 and p-Ser^{473}-Akt (Fig. 6D) ($P < 0.05$ at age over 8 weeks for both biomarkers). Although we saw a modest increase in the co-localization of PAK3 and p-Ser^{473}-Akt especially at

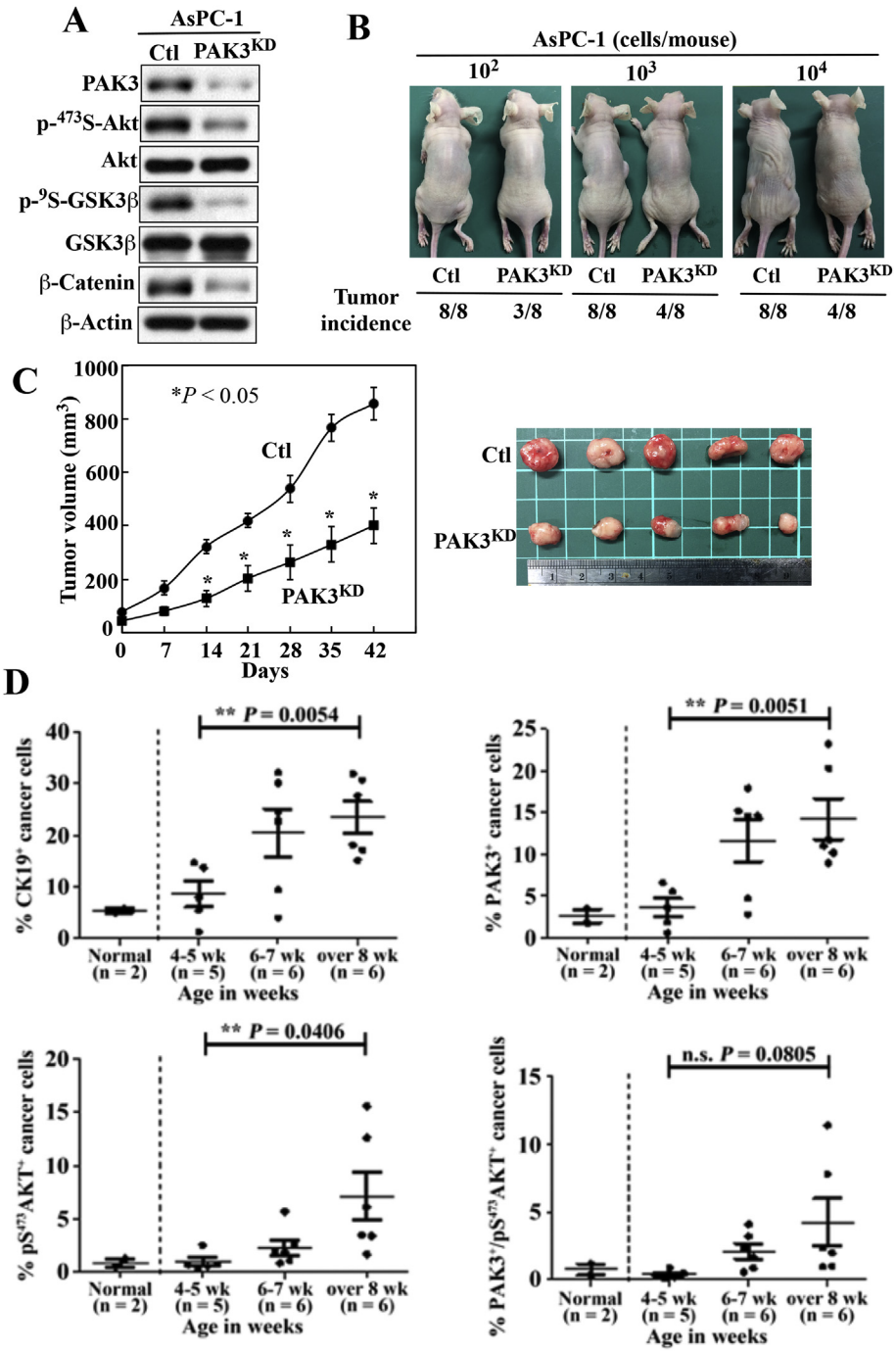

Fig. 6. ***In vivo*** **efficacy of stable PAK3 knockdown in suppressing AsPC-1 tumorigenicity and xenograft tumor growth in nude mice. (A)** Expression/phosphorylation levels of PAK3, Akt, GSK3β, and β-catenin in control knockdown versus $PAK3^{KD}$ AsPC-1 cells. **(B)** Comparison of the tumor incidence between $PAK3^{KD}$ and control AsPC-1 cells. Different amounts of cells (10^2, 10^3, and 10^4 cells per mouse) were subcutaneously injected into both flanks of the mice. **(C)** Suppressive effect of PAK3 knockdown on subcutaneous AsPC-1 xenograft tumor growth (means ± S.E.M., $n = 5$). **(D)** Immunohistochemical analysis of the expression levels of CK19, PAK3, and p-Ser^{473}-Akt in pancreatic samples from $KP^{fl/fl}C$ mice of three different age groups (4–5 weeks, $n = 5$; 6–7 weeks, $n = 6$; over 8 weeks, $n = 6$) relative to normal wild-type mice ($n = 2$).

the over-8-weeks group, this increase, however, was not statistically significant (Fig. 6D) ($P = 0.0805$), which might be due to the involvement of different Akt upstream kinases in pancreatic cancer (please see Discussion section).

4. Discussion

Accumulating evidence has revealed the oncogenic function of the PAK family members, especially PAK1 and PAK4, in promoting cell proliferation, survival, motility, and angiogenesis in several types of cancer cells, in part, through the upregulation of Akt signaling [42]. Previous studies have associated PAK3 mutations with mental retardation [43–45], and have suggested its involvement in the maintenance of glucose homeostasis in adult mice [46]. However, the role of PAK3 in regulating cancer cell functions remains unclear. The present study obtained the first evidence that PAK3 plays a role in promoting cancer cell proliferation and CSCs expansion, at least in part, by regulating the Akt-GSk3β-β-catenin signaling pathway in pancreatic cancer cell lines (AsPC-1, MiaPaCa-2, and SW1990). These findings underlie the impetus of PAK3 as a key effector in governing the malignant phenotype of pancreatic cancer cells, which might foster a new strategy for pancreatic cancer therapy. Although PAK1 and PAK4 have been reported to act as a Ser^{473}-Akt kinase in different cell systems, genetic knockdown and/or pharmacological inhibition of either PAK isoform had no appreciable effect on Ser^{473}-Akt phosphorylation or β-catenin expression in the present study (Fig. 3). Based on these findings, we hypothesized that different upstream kinases might be involved in different pancreatic cancer cell lines or under different cellular

contexts. This premise was supported by several lines of evidence. For example, in contrast to AsPC-1 and other cell lines examined (Fig. 3), knockdown of PAK3 in Panc-1 cells did not cause changes in the level of Ser^{473}-Akt phosphorylation or β-catenin expression (Supplementary Fig. S7A, left panel). Moreover, in line with the reports that the mTORC2 complex facilitates Ser^{473}-Akt phosphorylation [47,48], treatment of Panc-1 cells with the mTORC2 inhibitor KU-63794 led to parallel decreases in the phosphorylation levels of Ser^{473}-Akt and the mTORC2 substrate p70S6K (Supplementary Fig. S7A, right panel), while AsPC-1, MiaPaCa-2, and SW1990 cells were insensitive to the drug effect on Akt phosphorylation (Supplementary Fig. S7B). Together, these findings suggested the complexity in the network of upstream kinases in regulating Ser^{473}-Akt in different pancreatic cancer cell lines, i.e., while PAK3 acted as a major upstream kinase of Akt in AsPC-1, MiaPaCa-2, and SW1990 cells, mTORC2 was responsible for the Ser^{473}-Akt kinase in Panc-1 cells. In principle, mutations in the PBS/AID regulatory region or catalytic kinase domain can functionally alter the activities of PAKs. Although there is little clinical evidence for cancer cells harboring mutations in PAK genes, a mutation found in the kinase domain of PAK4 (E329K) led to increases in kinase activity and promoting malignancy phenotypes [49]. Moreover, a number of mutations in PBD domain or catalytic domain of PAK1 have been shown to active or impair its kinase activity [50,51], which warrants further investigation.

In summary, we report a novel function of PAK3 in regulating the Akt/GSK3β/β-catenin pathway in several pancreatic cancer cells examined. Together with data from other laboratories, this finding underlies the intricate role of different PAK family members in regulating cancer cell functions in a cell line/context-specific manner. However, there is insufficient evidence to support the clinical relevance of PAK3 or any other PAK family member in pancreatic tumorigenesis. It warrants clarification whether PAKs or mTORC2 plays a more dominant role in regulating Akt signaling in human pancreatic tumors, which is currently underway.

Conflicts of interest

All of the authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.04.026.

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