c-Fos/ERK promotes the progression from pancreatic intraepithelial neoplasia to pancreatic ductal adenocarcinoma

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Abstract. Pathogenesis of pancreatic ductal adenocarcinoma (PDAC) is thought to develop through the progression of precursor lesions, known as pancreatic intraepithelial neoplasias (PanIN). In the present study, we showed that c-Fos promoted proliferation, cell cycle and migration in pancreatic cancer cells. Caerulein was used to accelerate the pathogenesis of Pdx-cre; $Kras^{G12D}$ mice. During PanIN formation and development of PDAC, the expression of ERK and c-Fos increased concomitantly. When ERK activity was inhibited by U0126, the expression of c-Fos also decreased. Inactivation of ERK/c-Fos suppressed pancreatic lesions concurrently through proliferation, inflammation and apoptosis. Our findings suggest that the ERK/c-Fos pathway is required for PDAC initiation and progression.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths worldwide with a 5-year survival rate of less than 7%, a mortality rate that is nearly identical to the incidence rate (1,2). The main reason for the poor survival rate is that the current diagnostic methods lack the sensitivity and the specificity to identify markers of PDAC in the early stages, resulting in delayed diagnosis until the later stages of the disease. In 2015, fewer than 20% of newly diagnosed patients with pancreatic cancer were classified as having resectable cancer (3). Therefore, it is necessary to characterize PDAC's precursor lesions and the pathological mechanisms regulating the genetic progression from normal cells to PDAC (4).

Pancreatic intraepithelial neoplasia (PanIN) is the most common pancreatic precursor lesion. Based on increasing degrees of architectural and nuclear atypia, PanIN are grouped into three histological stages: PanIN-1 (PanIN-1A and PanIN-1B), PanIN-2 and PanIN-3 (5). Activating mutations in the *KRAS* oncogene are found in all three PanIN stages and over 90% of invasive PDAC (6). A genetically engineered mouse model, Pdx-cre; $Kras^{G12D}$, which uses a Pdx1 promoter to drive the expression of mutant Kras, is able to recapitulate the progression of human PanIN to PDAC successfully and serves as a convenient tool to explore the precursor lesions and the pathological mechanisms of the pancreatic cancer (7).

AP-1 component members, c-Fos and c-Jun, participate in the regulation of many processes, including proliferation, cell cycle progression, migration, differentiation, apoptosis and angiogenesis (8,9). In addition to the specific role of c-Fos as a differentiation transcription factor for osteoclasts and bone resorbing cells, c-Fos also has an important role in the tumor formation and suppression. It has been reported that c-Fos downregulation in MCF-7/ADR cells results in enhanced apoptosis and altered expression of apoptosis-associated proteins, including Bax, Bcl-2, p53 and PUMA (10). The ERK/c-Fos/MMP-7 pathway plays an important role in modulating the invasion of colon cancer cells and inhibition of this pathway holds promise as a treatment for the colon cancer metastasis (11).

In the present study, we investigated the role of AP-1 in initiating the PanIN-PDAC progression *in vivo* using Pdx-cre; $Kras^{G12D}$ mouse model. Our results indicate that the c-Fos expression, but not the c-Jun expression, increased during the development from precursor lesions to tumor. We demonstrated that ERK activates c-Fos to promote the PanIN-PDAC progression through initiation of proliferation, inflammation and apoptosis. These results will help to characterize the early prognosis of the pancreatic cancer.

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Materials and methods

Cell culture and transfection. Cell lines were obtained from the Cell Resource Center in Peking Union Medical College (PUMC; Beijing, China). Panc-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories; Thermo Fisher Scientific) with 10% fetal bovine serum (FBS) and PCT-3 cells were cultured in RPMI-1640 (HyClone Laboratories; Thermo Fisher Scientific) with 10% FBS in 5% CO_2 at 37˚C. Adherent cells were passaged every 2-3 days with 0.5 mg/ml trypsin (1:250) and 0.53 mM ethylenediaminetetraacetic acid (EDTA). siRNA oligonucleotides were designed against c-Fos as follows: GGGGCAAGGTGGA ACAGTTAT. Cells were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA).

Western blot analysis. Proteins were extracted with SDS lysis buffer [50 mM Tris-HCl (pH 6.8), 10% glycerol and 2% SDS] and quantified using the BCA protein assay reagent (Thermo Fisher Scientific). Extracts were separated on a 12% SDS-PAGE gel and electrophoretically transferred to PVDF membrane (GE Healthcare). The membrane was blocked in 5% skim milk for 1 h at room temperature and then incubated overnight with the indicated antibodies at 4˚C. Antibodies against c-Fos (1:1,000) and GAPDH (1:3,000) were purchased from Cell Signaling Technology (Danvers, MA, USA). The membrane was incubated with an anti-rabbit or an anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Chemiluminescence was detected using an ECL blot detection system (Santa Cruz Biotechnology).

Cell proliferation assay. Cell proliferation was assessed by the CCK-8 assay (Dojindo Molecular Technologies). Cells were seeded at a density of 2,000 cells/well in 96-well plates. A total of 10 μl CCK-8 solution was added to each well containing 100 μl of culture medium and incubated for 2 h at 37˚C. Absorbance was measured at 450 nm using a multiwell spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Cell cycle assay. Cells were transfected in 6-well plates ($5x10^5$ cells/well). After 48 h, cells were harvested, washed with cold phosphate-buffered saline (PBS) and fixed in 70% ethanol overnight at 4˚C. After centrifugation at 800-1,000 rpm three times, cells were re-suspended with 500 μl PBS. Cells were stained with a solution containing 10 mg/ml RNase A, 0.1% Triton X-100 and 1 mg/ml propidium iodide. Cell cycle analysis was performed by fluorescence-activated cell sorting.

Wound-healing assay. Cells were transfected in 6-well plates ($5x10^5$ cells/well). After 24 h, a monolayer of cells was scratched by a 200 ml tip in serum-free medium. Cell migration was quantified by the area of migrated cells to the scratched cell-free zone after 24 h, and measured by ImageJ software.

Mouse model and in vivo treatment. The genetically engineered mouse stains Pdx1-cre and LSL-$Kras^{G12D}$ used in the present study were purchased from the National Cancer Institute (NCI; Rockville, MD, USA). Pdx-cre; $Kras^{G12D}$ mice were generated by crossing LSL-$Kras^{G12D}$ mice with Pdx1-cre mice. The mutant mice were genotyped by PCR using primers as followed: *lox* sense primer: AGCTAGCCACCATGGCTT GAGTAAGTCTGCA and *lox* antisense primer: CCTTTACA AGCGCACGCAGACTGTAGA; *cre* sense primer: CTGGA CTACATCTTGAGTTGC and *cre* antisense primer: GGTG TACGGTCAGTAAATTTG. Animals were housed in a clean vivarium and fed standard mouse chow. To accelerate carcinogenesis in Pdx-cre; $Kras^{G12D}$ mice, an intraperitoneal injection of caerulein (50 μg/kg/day) was administered. ERK/c-Fos signaling blockade was accomplished using the inhibitor U0126 (500 μg/kg) every two days.

Animals were housed in the Experimental Animal Center of Peking Union Medical College Hospital. They were maintained in a 12-h light/dark cycle and temperature-controlled environment with *ad libitum* access to water and food. Animals were cared for and studies were performed in accordance with the principles of the 3Rs (replacement, reduction and refinement) and guidelines of the National and Beijing Experimental Animal Welfare Ethics.

Immunohistochemical analyses. Freshly isolated biopsies of the pancreas were fixed in 10% neutral-buffered formulin (Sigma-Aldrich) for 16 h and embedded in paraffin. Next, 5-μm-thick sections were deparaffinized, rehydrated and treated in boiling sodium citrate buffer (10 mM pH 6.0) for 10 min to unmask antigens. Endogenous peroxidase activity was quenched by treating the slides with 3% hydrogen peroxide for 10 min. Sections were blocked in PBS containing 10% goat serum, 1% BSA and 0.1% Triton X-100 for 1 h at room temperature prior to being incubated overnight at 4˚C with antibodies: JNK (ab179461, Abcam, 1:100), p38 (ab170099, Abcam, 1:50), caspase-3 (ab13847, Abcam, 1:500), CK19 (10712-1-AP, Proteintech, 1:100), MMP9 (ab38898, Abcam, 1:100), ERK1/2 (#4695, Cell Signaling Technology, 1:100), amylase (12540-1-AP, Proteintech, 1:100), CD45 (ab10558, Abcam, 1:100). After incubation with secondary biotinylated antibodies (1:200; KPL), the presence of the antigens was revealed using diaminobenzadine tetrachloride (DAB; Dako) and counter stained with nuclear red or with heamatoxylin (blue). The relative quantities of IHC reaction were accessed by Image-Pro Plus 6.0.

Statistical analyses. Statistical analyzes were performed using the Student's t-test (two-tailed) in Microsoft Excel software (Microsoft, Redmond, WA, USA). The results are presented as the mean ± standard deviation of triplicates of each experiment. All experiments were performed in triplicate unless stated otherwise. $P<0.05$ were considered statistically significant.

Results

C-Fos promotes proliferation, cell cycle and migration in human pancreatic cancer cells. To examine the effects of c-Fos on pancreatic cancer cell growth, we first suppressed endogenous c-Fos expression in Panc-1 and PCT-3 cells by transfecting cells with siRNA against c-Fos (Fig. 1A). The results of the cell proliferation assay showed that c-Fos knockdown significantly inhibited cell growth (Fig. 1B). Flow

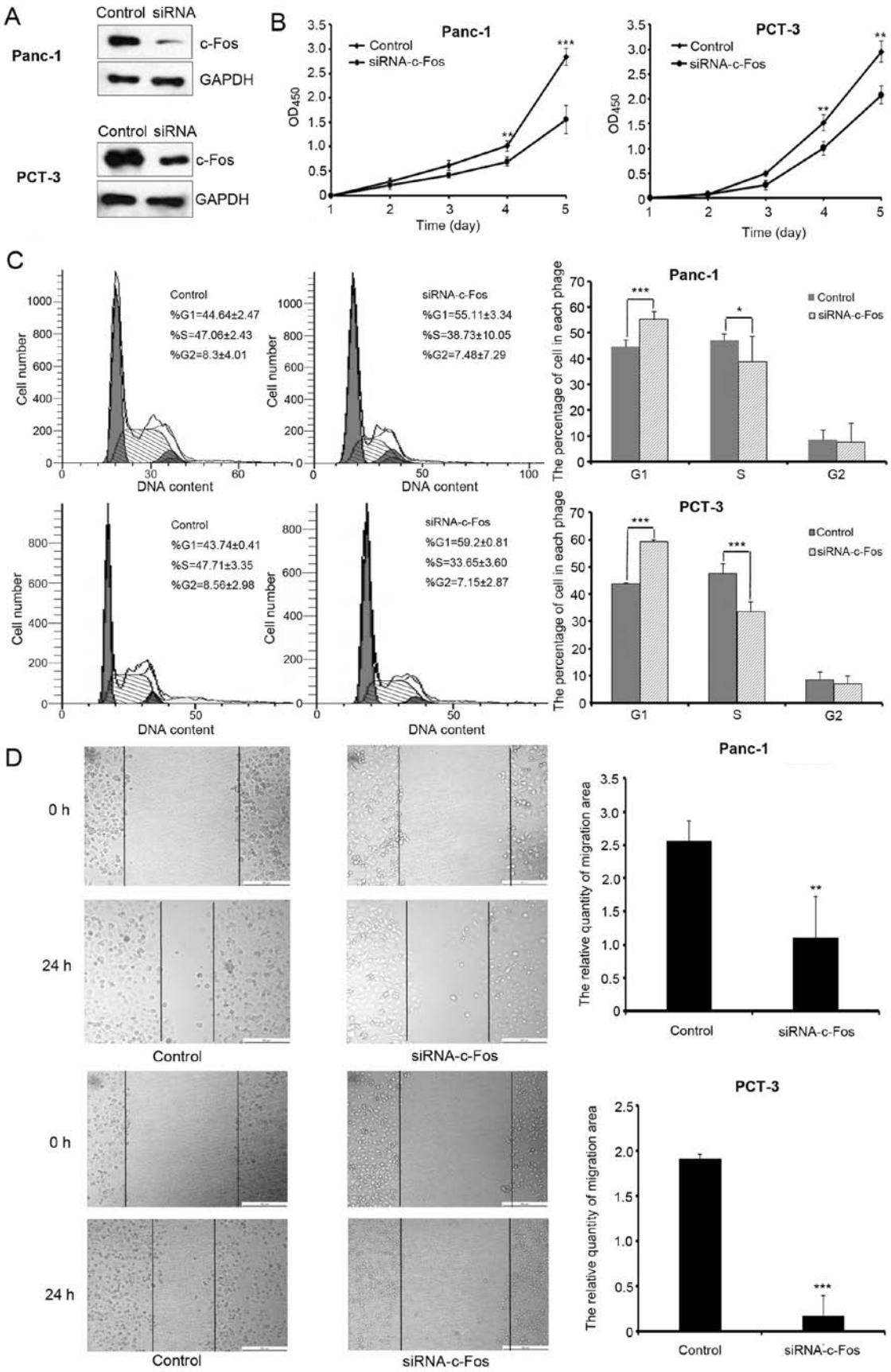

Figure 1. The effect of c-Fos on cell phenotype in human pancreatic cancer cells. (A) Knockdown of c-Fos. c-Fos siRNA or non-targeting siRNA were transfected into Panc-1 and PCT-3 cells for 48 h, and cells were extracted for western blot analysis. (B) Cell proliferation assay. c-Fos siRNA or non-targeting siRNA were transfected into Panc-1 and PCT-3 cells for 24 h, and cell survival was determined by CCK-8 assay. (C) Cell cycle assay. After transfection with c-Fos siRNA or non-targeting siRNA for 48 h, the cells were harvested for cell cycle assay using flow cytometry. (D) Wound-healing assay. After transfection with c-Fos siRNA or non-targeting siRNA for 24 h, cells were scratched with a 200 ml tip to remove the monolayer. Cell migration was quantified by the area of migrated cells to the scratched cell-free zone after 24 h. All experiments were run in triplicate, and the error bars represent the standard deviations of the triplicate samples.

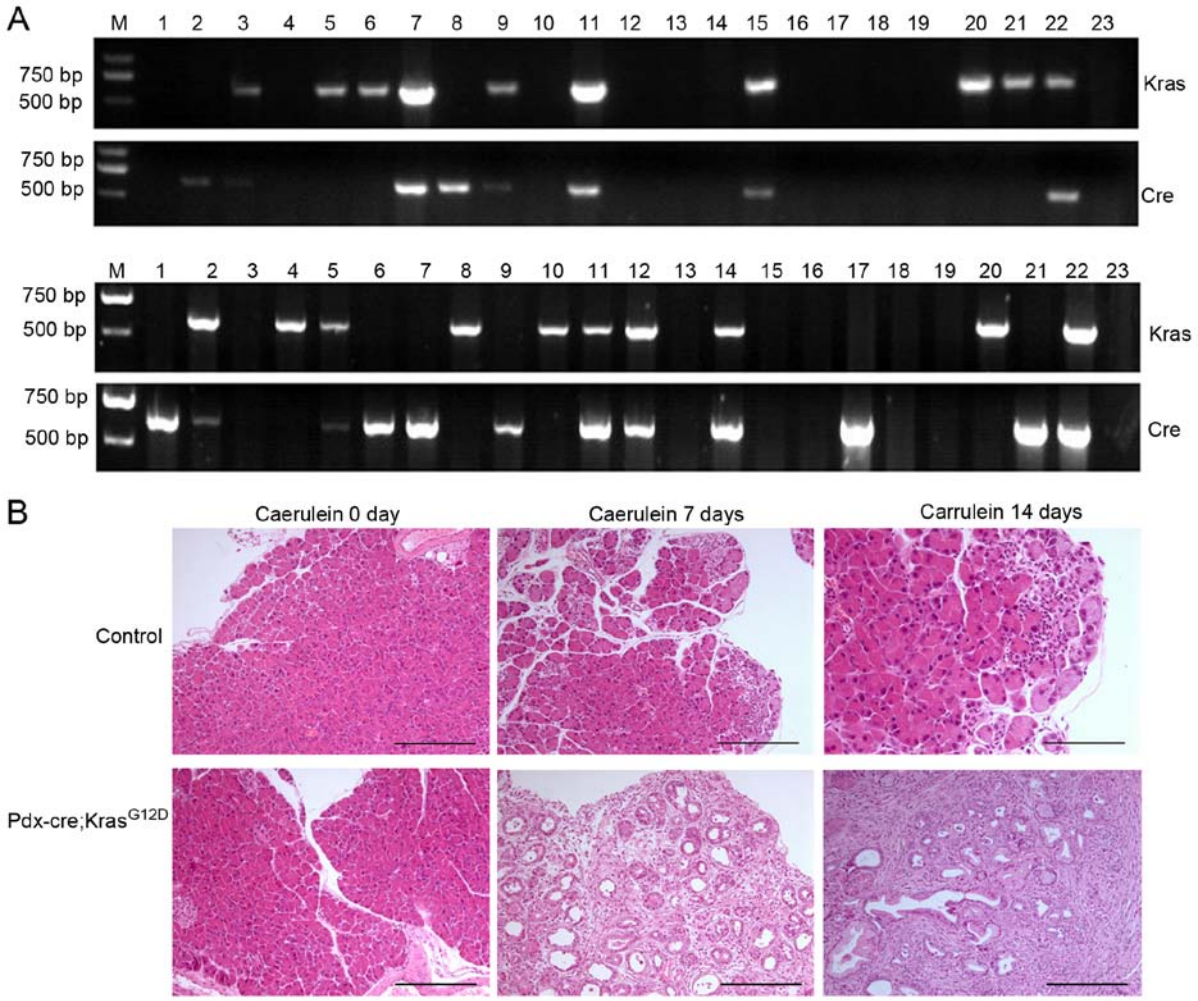

Figure 2. Characterization of the PK mouse model. (A) PCR of DNA from PK mice. DNA was isolated from mice generated by crossing LSL-Kras^{G12D} mice with Pdx1-cre mice and subjected to PCR. (B) Histological characteristics of pancreas from control or PK mice treated by caerulein for 7 days or 14 days were examined by H&E (magnification, x100).

cytometric analysis was used to test cell cycle progression. Our results show that the percentage of G1 phase cells was increased following knockdown of c-Fos expression (Fig. 1C). Wound-healing assay was performed to examine the effects of c-Fos on migration. Reduction in c-Fos levels decreased cell migration compared with the control group (Fig. 1D).

Characterization of the mouse model. Pdx-cre; Kras^{G12D} (PK) mice are attained by crossing LSL-Kras^{G12D} mice with Pdx-cre mice. To identify transgenic mice, DNA was isolated and PCR amplification of both *lox* and *cre* genes indicated that the recombinant mouse model was established successfully (Fig. 2A). The positive rate for recombinant mice was <20%. The pancreas from PK mice is larger than their littermate controls and have more nodules, particularly those treated by caerulein for a longer period of time. Immunohistochemistry (IHC) revealed that the pancreas of PK mice treated by caerulein for 7 days developed PanIN-1 to PanIN-2 lesions, and those treated by caerulein for 14 days developed a higher degree of PanIN lesions relative to PDAC (Fig. 2B). In contrast, the pancreas from control mice developed few ductal lesions with the exception of some inflammatory infiltration after caerulein treatment for 7 or 14 days.

The expression of ERK/c-Fos increased during PDAC initiation and progression. C-Jun and c-Fos are components of the AP-1 complex, which is involved in numerous cell activities including proliferation, apoptosis, survival, tumorigenesis and tissue morphogenesis (12). To characterize the function of AP-1 in PDAC tumorigenesis, we examined the expression of both c-Jun and c-Fos in the pancreas of PK mice. Compared with the control mice, the expression level of c-Fos, but not c-Jun, was enhanced in PanIN lesions of caerulein-treated PK mice and progressively increased as pancreatic lesion stage progressed (Fig. 3A). AP-1 is reportedly involved in the downstream regulation of mitogen activated protein kinase (MAPK) (13,14); therefore, we examined the expression of ERK, JNK and p38 in PK mice. As shown in Fig. 3B, the expression of ERK is similar to c-Fos levels, whereas JNK and p38 showed little difference in expression. Therefore, the expression of ERK/c-Fos increased during PDAC initiation and progression.

ERK/c-Fos is required for PDAC initiation and progression. As ERK/c-Fos is highly expressed in pancreatic cancer, we postulated that ERK/c-Fos is required for tumorigenesis. To test this hypothesis, we treated 6-week-old PK mice with the ERK inhibitor, U0126, with or without caerulein for

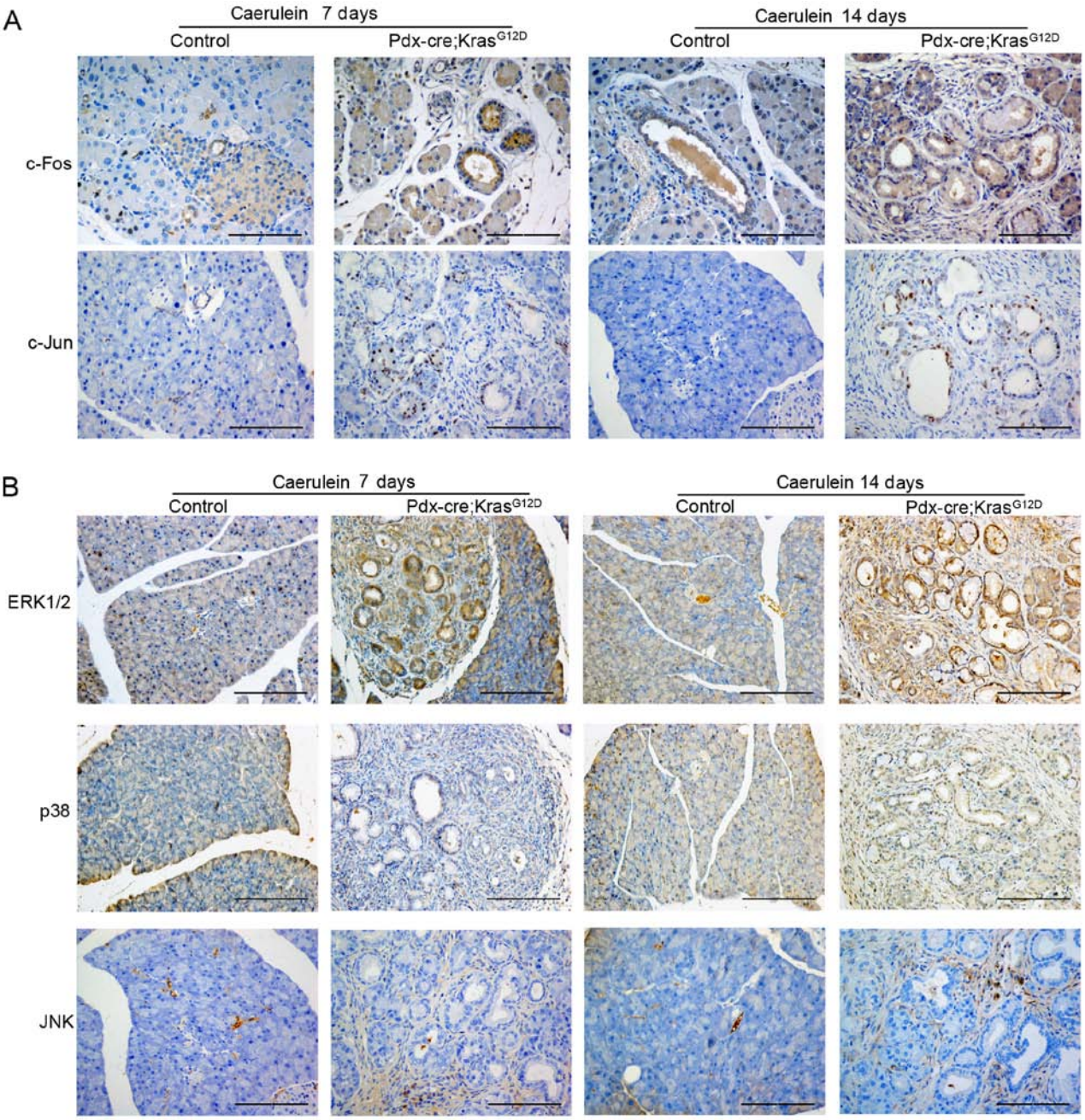

Figure 3. MAPK and AP-1 pathways in PK mice. The expression of c-Fos and c-Jun (A), ERK, JNK and p38 (B) in the pancreas from control or PK mice treated by caerulein for 7 days or 14 days was determined using IHC (magnification, x200).

15 days. U0126 reduced the expression of ERK and c-Fos and suppressed pancreatic ductal lesions (Fig. 4A). Furthermore, after 15 days of U0126+caerulein injection, the exocrine compartment of PK mice was partially replaced by fibrosis and ductal structures, as evident by the expression level of acinar marker amylase and duct marker cytokeratin 19 (CK19) (Fig. 4B). In contrast, the pancreas of PK mice treated with only caerulein displayed markedly more CK 19-positive duct structures and fewer amylase-positive parenchyma.

Suppression of ERK/c-Fos attenuates inflammation and proliferation, but promotes apoptosis. To determine the role of ERK/c-Fos in PanIN formation and development of PDAC, we tested additional markers of tumor phenotype. Levels of proliferation marker Ki-67 and inflammatory marker CD45 expression were substantially higher in PanIN and PDAC from PK mice compared to control mice, whereas the apoptosis marker, caspase-3, exhibited the opposite results (Fig. 5). Immunohistochemistry data also suggested that U0126 inhibits Ki-67 and CD45 expression and increased the levels of caspase-3. Therefore, the suppression of ERK/c-Fos attenuates inflammation and proliferation but promotes apoptosis to repress PanIN and PDAC progression.

Discussion

PDAC remains a highly lethal disease (15,16). The posterior location of pancreas, which is in close proximity to duodenum,

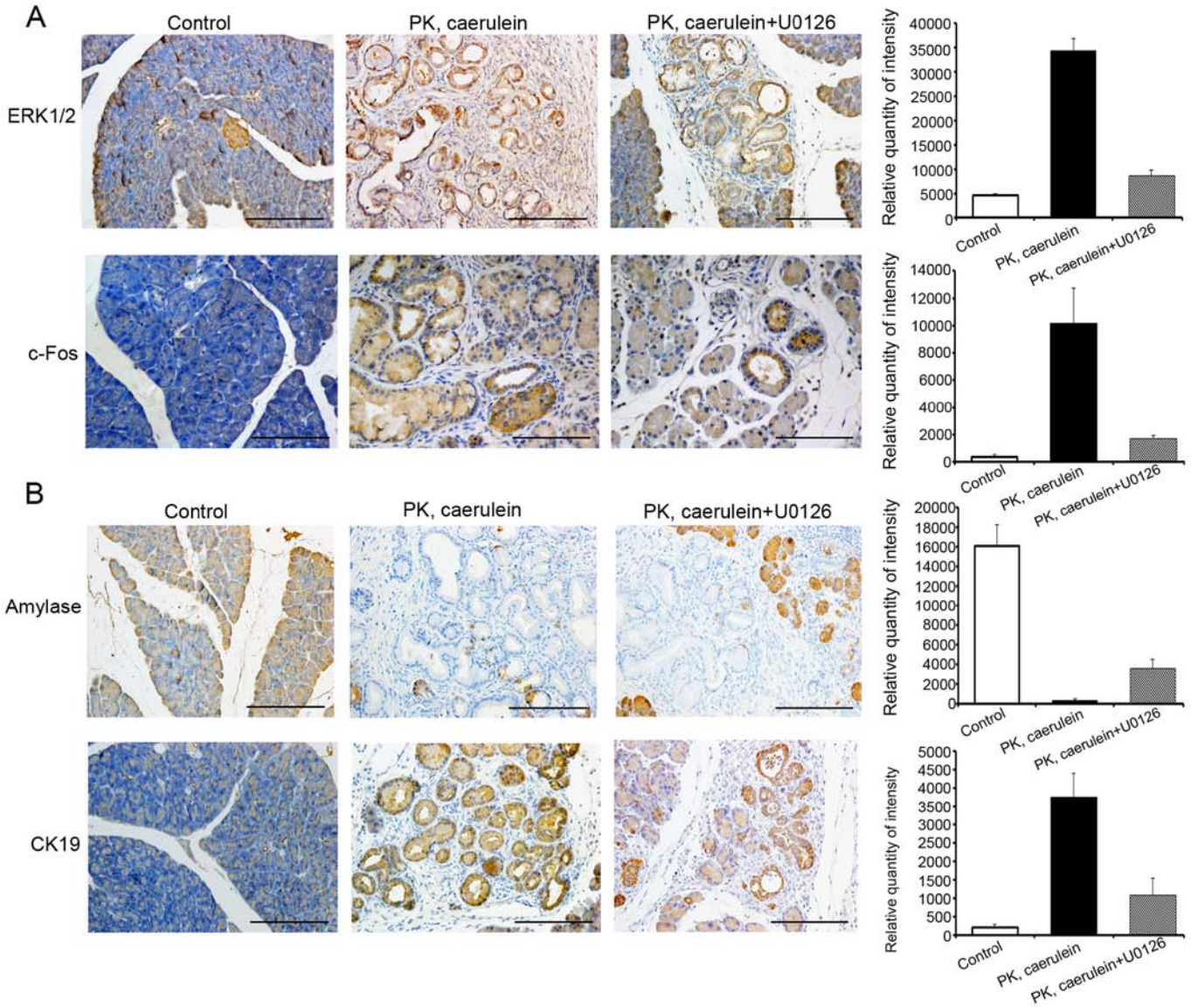

Figure 4. The effect of U0126 on PanIN-PDAC development. After 15 days of treatment with U0126+caerulein or caerulein only, the pancreas from mice were subjected to IHC (magnification, x200) to test the expression of c-Fos and c-Jun (A), amylase and CK19 (B).

common bile duct, celiac plexus, superior mesenteric artery (SMA), and portal vein, contributes to late diagnosis, as well as the bothersome symptoms of obstruction of biliary drainage, including infection, pain, chemotherapy resistance and unresectable pancreatic cancer (17-20). A genetically engineered mouse model is an appropriate tool to investigate the pathological mechanisms and early diagnosis of pancreatic cancer.

The Pdx-cre; $Kras^{G12D}$ (PK) mouse model, which targets pancreas-specific expression of mutated Kras, recapitulates the human PanIN-to-PDAC sequence (7). PanIN lesions begin to appear at approximately 2 months, and high-grade PanINs are observed at 5 months. The progression from PanIN to invasive and metastatic PDAC occurs over several months, which is not conducive for a research study. Chronic pancreatitis has been identified as risk factor for PDAC development in humans and has been shown to significantly accelerate PanIN and PDAC development in Kras-driven mouse models (21,22). Acute pancreatitis can progress to chronic pancreatitis in human patients under certain conditions (23). Several studies have also shown that acute pancreatitis markedly accelerates PanIN and PDAC development in Kras-driven mouse models (24). In response to acute pancreatitis induced by the cholecystokinin analog caerulein, acini transiently dedifferentiated into duct-like structures. Mutant Kras compromises the ability of acinar cells to regenerate following acute pancreatitis and locks damaged cells in a persistently dedifferentiated ductal state that can rapidly give rise to PanINs (25,26). Thus, caerulein-induced pancreatitis provides a permissive environment for Kras-driven neoplasia. In the present study, the pancreas of PK mice treated by caerulein for 7 days developed PanIN-1 to PanIN-2 lesions, and PK mice treated by caerulein for 14 days developed a higher degree of PanIN lesions relative to PDAC. This improved PK mouse model allowed us to study PanIN to PDAC development on an accelerated timeline.

MAPKs are a family of serine-threonine protein kinases involved in many cellular processes including cell proliferation,

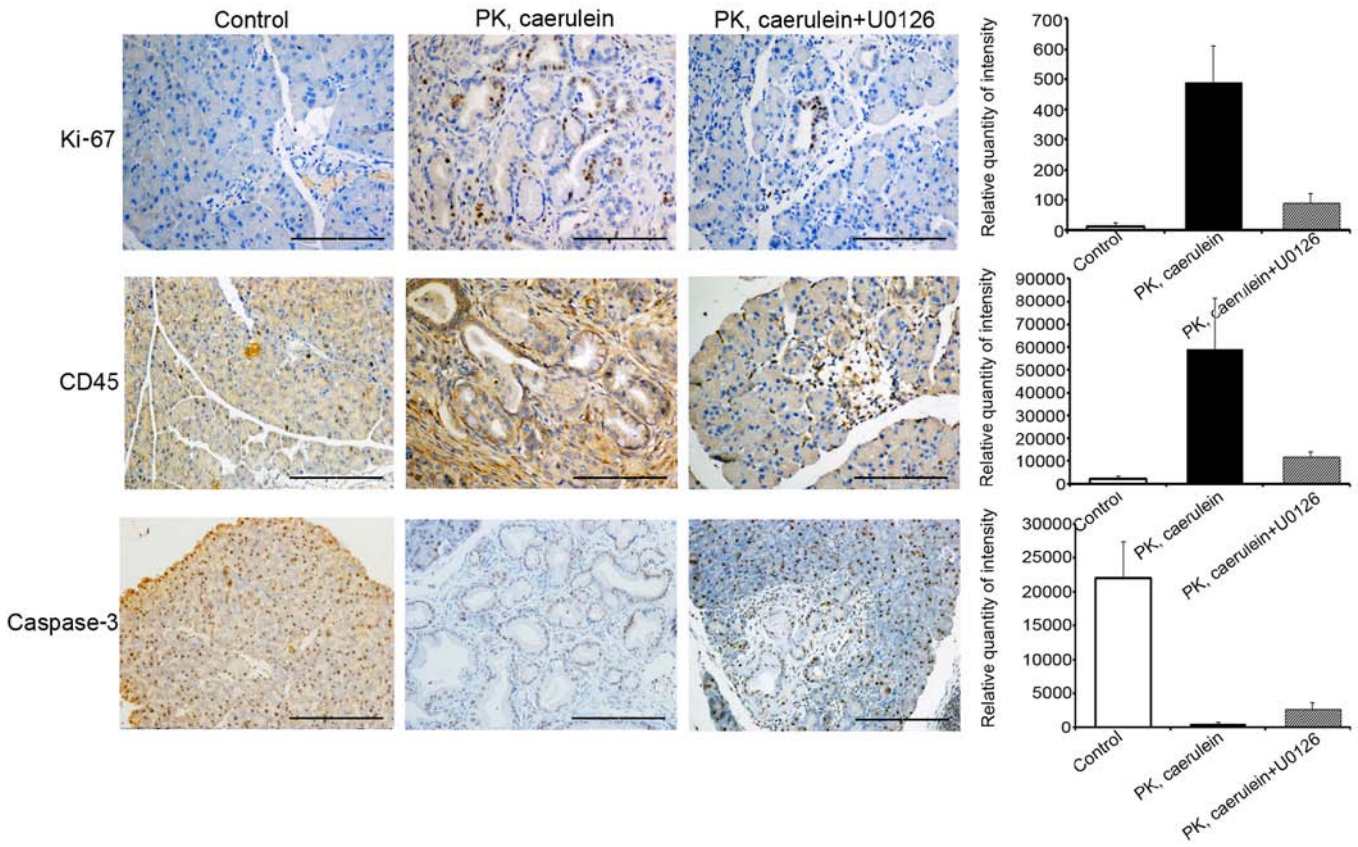

Figure 5. The effect of U0126 on proliferation, inflammation and apoptosis in PK mice. Mice were injected with U0126+caerulein or caerulein only, for 15 days and IHC (magnification, x200) was used to examine the level of Ki-67, CD45 and caspase-3.

differentiation, inflammation and cell death (27). Activation of several MAPKs, including ERK, p38 and c-Jun N-terminal kinase (JNK) are able to stimulate AP-1 (28). The results in PK mice suggested that the expression of ERK and c-Fos increased during PanIN formation and progression to PDAC, whereas the level of p38, JNK and c-Jun remained unchanged. In addition to the *Kras* gene, inactivation of other genes are involved in the pathophysiology of PDAC, including *INK4A*, *TP53*, *SMAD4* or *BRCA2* (29). Additionally, several signaling pathways may positively or negatively modulate the PanIN and PDAC development, including TGFα, Hedgehog, Notch, EGFR and STAT (30-32). Here we provided evidence that the ERK/c-Fos pathway is essential for Kras-driven PDAC. Furthermore, our experiment showed that the ERK/c-Fos inhibitor U0126 suppressed acinar-ductal metaplasia, proliferation and inflammation and promoted apoptosis, leading to inhibition of pancreatic ductal lesions.

In summary, our results demonstrate that c-Fos induced cell growth, cell cycle and migration in the the pancreatic cancer cells. The *in vivo* experiments revealed that the expression of c-Fos, and the upstream transcription factor ERK, increased during PanIN formation. Additionally, the ERK/c-Fos inhibitor, U0126, suppressed the PanIN/PDAC progression initiation through proliferation, inflammation and apoptosis. Our findings suggest that the ERK/c-Fos pathway is required for PanIN formation and progression to PDAC, which will help to characterize the early prognosis of pancreatic cancer.

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