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Therapeutic effect of c-Jun N-terminal kinase inhibition on pancreatic cancer

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c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase (MAPK) family, and it is reportedly involved in the development of several cancers. However, the role of JNK in pancreatic cancer has not been elucidated. We assessed the involvement of JNK in the development of pancreatic cancer and investigated the therapeutic effect of JNK inhibitors on this deadly cancer. Small interfering RNAs against JNK or the JNK inhibitor SP600125 were used to examine the role of JNK in cellular proliferation and the cell cycles of pancreatic cancer cell lines. *Ptf1a$^{cre/+}$;LSL-Kras$^{G12D/+}$;Tgfbr2$^{flox/flox}$* mice were treated with the JNK inhibitor to examine pancreatic histology and survival. The effect of JNK inhibition on tumor angiogenesis was also assessed using cell lines and murine pancreatic cancer specimens. JNK was frequently activated in human and murine pancreatic cancer *in vitro* and *in vivo*. Growth of human pancreatic cancer cell lines was suppressed by JNK inhibition through G1 arrest in the cell cycle with decreased cyclin D1 expression. In addition, oncogenic K-ras expression led to activation of JNK in pancreatic cancer cell lines. Treatment of *Ptf1a$^{cre/+}$;LSL-Kras$^{G12D/+}$;Tgfbr2$^{flox/flox}$* mice with the JNK inhibitor decreased growth of murine pancreatic cancer and prolonged survival of the mice significantly. Angiogenesis was also decreased by JNK inhibition *in vitro* and *in vivo*. In conclusion, activation of JNK promotes development of pancreatic cancer, and JNK may be a potential therapeutic target for pancreatic cancer. (*Cancer Sci* 2013; 104: 337–344)

C-Jun N-terminal kinase (JNK) is a member of the MAPK family, is activated by cytokines, growth factors and environmental stress, and controls cell proliferation, differentiation, apoptosis, and survival[1–4] through the regulation of various target molecules such as transcriptional factors c-Jun, activating transcription factor 2 (ATF2), E twenty-six-like transcription factor 1 (Elk1), and signal transducer and activator of transcription 3 (STAT 3).[1,5–7]

c-Jun N-terminal kinase has been reported to play an important role in the development of various cancers.[8–11] JNK1-deficient mice exhibited a decrease in carcinogenesis of chemically-induced gastric cancer or hepatocellular carcinoma,[12–15] and JNK2-deficient mice showed reduced formation of skin tumors.[16] Activation of JNK was reported in human pancreatic cancer specimens,[17] and growth of a pancreatic cancer cell line was reported to be inhibited by pharmacological inhibition of JNK *in vitro*.[18] However, the function of JNK in pancreatic cancer remains largely unexplored.

Ptf1a$^{cre/+}$;LSL-Kras$^{G12D/+}$;Tgfbr2$^{flox/flox}$ mice (Kras^{G12D}+Tgfbr2^{KO} mice) are pancreas-specific *Tgfbr2* knockout mice with active K-ras expression that are generated using the Cre-loxP system driven by the *Ptf1a* (*pancreatic transcription factor-1a*) promoter. These mice develop murine pancreatic intraepithelial neoplasia (mPanIN) progressively and, finally, well-differentiated pancreatic ductal adenocarcinoma (PDAC) with 100% penetrance and a median survival duration of 59 days.[19] Because the clinical and histopathological manifestations of Kras^{G12D}+Tgfbr2^{KO} mice recapitulate human PDAC, these mice are considered a good model of human PDAC and are used for the evaluation of candidate treatment drugs for PDAC.[20]

In the present study, we examined the role of JNK and the effect of JNK inhibition in pancreatic cancer using pancreatic cancer cell lines and Kras^{G12D}+Tgfbr2^{KO} mice. We found that inhibition of JNK can be a potential therapy for pancreatic cancer.

Materials and Methods

Mice. *Ptf1a$^{cre/+}$;LSL-Kras$^{G12D/+}$;Tgfbr2$^{flox/flox}$* mice (Kras^{G12D}+Tgfbr2^{KO} mice) and *Ptf1a$^{cre/+}$;LSL-Kras$^{G12D/+}$* mice (Kras^{G12D} mice) were described previously.[19–21] Genotyping of *Ptf1a$^{cre/+}$*, *LSL-Kras$^{G12D/+}$*, floxed *Tgfbr2* alleles was performed using oligonucleotide primers as described previously.[22–24] All the experimental protocols were approved by the ethics committee for animal experimentation and were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Medicine at the University of Tokyo.

RNA interference and plasmids. JNK1 small interfering RNA (siRNA) or JNK2 siRNA, cyclin D1 siRNA, KRAS siRNA, and control siRNA were purchased from Qiagen (Hilden, Germany) and transfected into the cells using RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA, USA). Wild-type Ha-ras vector and constitutively active Ha-ras (RasG12V) vector were purchased from Clontech and subcloned into pTriEx-2 (Novagen, Madison, WI, USA). Cells were seeded into 12-well plates and, after 24 h, they were transfected with 0.3 μg of expression plasmids or the control vector (pcDNA3.1) using Effectene Transfection reagent (QIAGEN).

Cell culture, cell cycle analysis, histology, immunohistochemistry, immunofluorescence, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, immunoblotting, real-time RT-PCR, cytokine array, and Enzyme-linked immunosorbent assay (ELISA). Details are described in Suppl. Doc S1.

Drug administration and experimental design in an *in vivo* model. The JNK-specific inhibitor SP600125[25] (LC Laboratories, Woburn, MA, USA) was dissolved in DMSO, and then was diluted in PBS and injected intraperitoneally into Kras^{G12D}+Tgfbr2^{KO} mice. To examine the effect of SP600125 on PDAC *in vivo*, we injected 50 mg/kg SP600125 five times per week from postnatal day 21 for 4 weeks, and then mice

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were killed. To examine survival of the mice, we injected 10 mg/kg SP600125 five times per week from postnatal day 21 until the mice became moribund. D-JNKI1 (Enzo Life Sciences, Plymouth Meeting, PA, USA), a peptide that specifically inhibits JNK, was dissolved in PBS and was injected intraperitoneally into $Kras^{G12D}$+$Tgfbr2^{KO}$ mice (5 mg/kg once in every 3 days from postnatal day 21, for 15 days).

Angiogenesis assay. Capillary formation of human umbilical vein endothelial cells (HUVECs) was assessed using the Fibrin Gel *In Vitro* Angiogenesis Assay Kit (Chemicon, Temecula, CA, USA), as described previously.[26] Details are described in Suppl. Doc S1.

Statistical analysis. The data were expressed as the mean ± SD. Differences between means were compared using Student's *t*-test; a *P*-value <0.05 was deemed to indicate statistical significance. The survival curves were plotted according to the Kaplan–Meier method and compared by the log-rank test.

Results

JNK activation in human pancreatic cancer. To analyze the role of JNK in pancreatic cancer, we first examined JNK phosphorylation in human pancreatic cancer specimens by immunohistochemical staining. As shown in Fig. 1A, we observed increased levels of phosphorylated JNK in 85/93 of the pancreatic cancer samples (91.4%), and none of the 18 normal samples showed positive staining for phosphorylated JNK. The rate of cases with phosphorylated JNK did not show a significant difference between pathological stage and histological grade (Fig. 1B). Next, we investigated phosphorylation of JNK in eight human pancreatic cancer cell lines. Phosphorylated JNK was observed in all the cell lines (Fig. 1C). These results indicate that the JNK pathway is activated in most cases of human pancreatic cancer.

The role of JNK in PDAC cell proliferation. To examine whether JNK affects tumor growth *in vitro*, we inhibited JNK in pancreatic cancer cell lines BxPC-3 and KP-4, which contain enhanced phosphorylation of JNK, using JNK1 siRNA, JNK2 siRNA, or the JNK inhibitor SP600125. Cell proliferation was significantly inhibited following JNK inhibition (Figs 2A,B and S1A,B). Next, we investigated the cell cycle status of pancreatic cancer cells by flow cytometry. Cells transfected with JNK1 or JNK2 siRNA displayed a higher proportion of cells in the G0/G1 phase, compared with control siRNA (Fig. 2C), indicating that JNK inhibition leads to G1 arrest. Then we examined the expression of genes related to cell proliferation and cell cycle regulation. The expression of cyclin D1 was decreased in cells transfected with JNK1 or JNK2 siRNA and SP600125-treated cells (Figs 2D,E and S1C,D).

We next investigated the role of Ras in JNK activation in pancreatic cancer because oncogenic K-ras mutation is known to be highly prevalent in pancreatic cancer, and Ras has been reported to cause activation of JNK.[1,27,28] We overexpressed wild-type Ras or oncogenic RasG12V in BxPC-3 and KP-4 cells using expression plasmids. The expression levels of phosphorylated JNK were higher in RasG12V cells than in wild-type Ras cells after normalization to Ras expression (Figs 2F and S1E). Extracellular signal-regulated kinase (ERK) was also activated, which was considered to be dependent on the Ras /Raf/MEK/ERK pathway. We also knocked down KRAS in

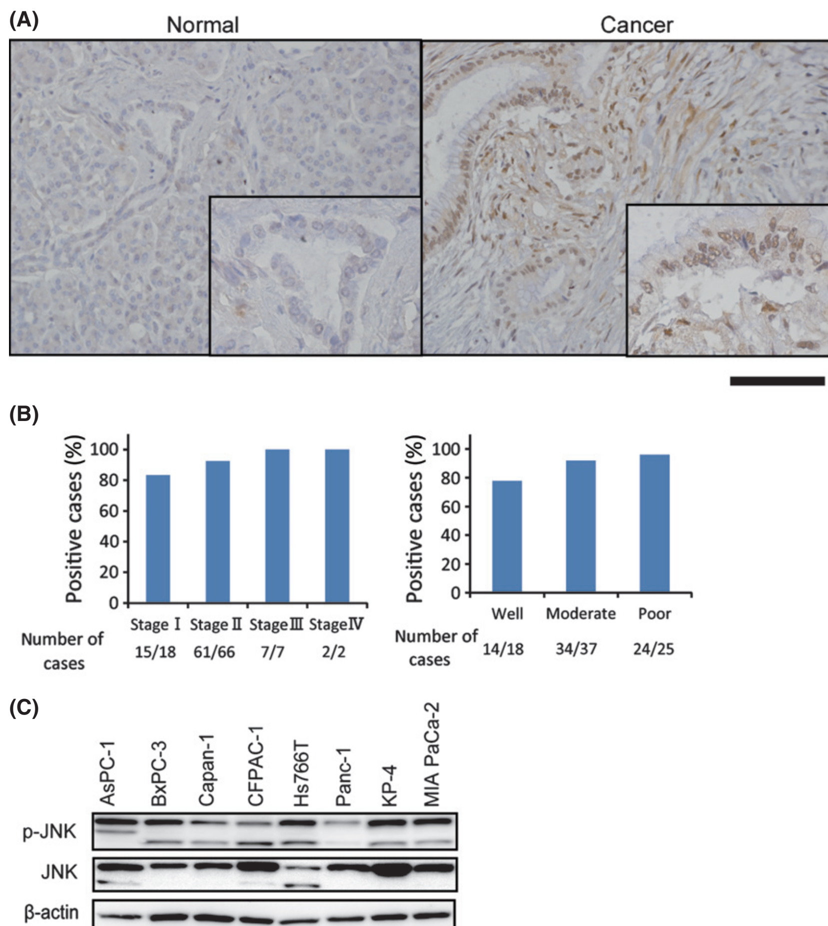

Fig. 1. Activation of c-Jun N-terminal kinase (JNK) in human pancreatic cancer. (A) Human normal pancreas (n = 18, left) and pancreatic cancer sections (n = 93, right) were stained for phosphorylated JNK, and representative images are shown (original magnification, ×200; bar, 100 μm). Insets show detailed areas containing the pancreatic duct (original magnification, ×400; bar, 50 μm). (B) The rate of positive cases for phosphorylated JNK in each clinical stage (left) and tumor differentiation (right). (C) Levels of phosphorylated and total JNK in human pancreatic cancer cell lines were determined by immunoblotting.

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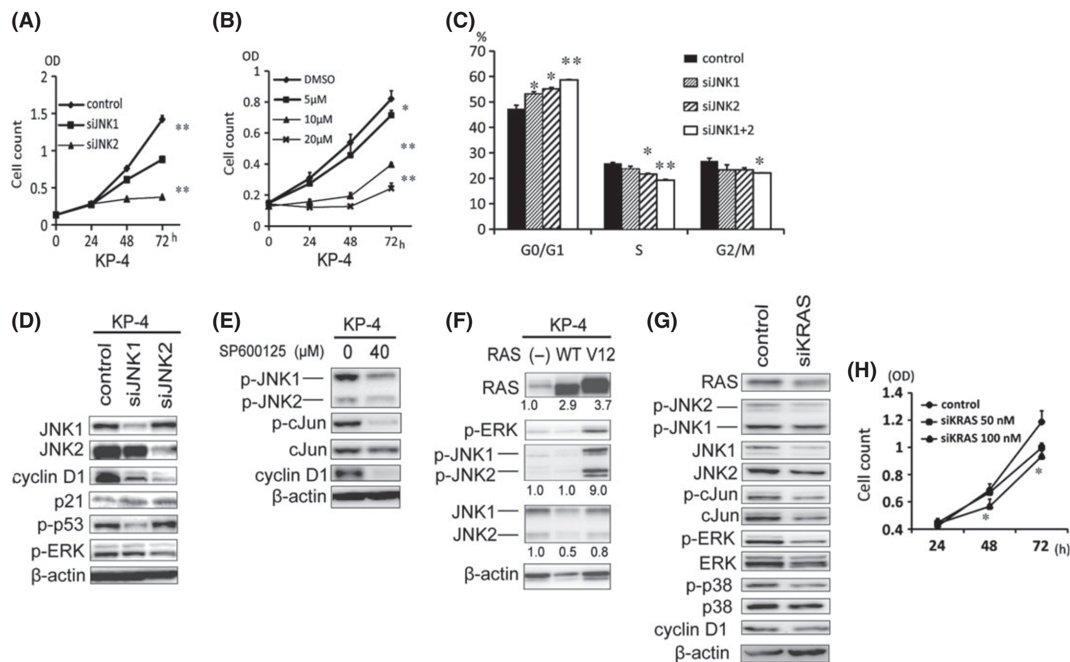

Fig. 2. Effect of c-Jun N-terminal kinase (JNK) and KRAS on the growth of pancreatic cancer cell lines. (A, B) KP-4 cells were transfected with JNK1, JNK2 or control siRNA (A), or treated with the indicated concentration of the JNK inhibitor SP600125 (B). The cell number was measured 24, 48, and 72 h after transfection or addition of SP600125. Data are plotted as means ± SD of triplicate samples (*P < 0.05, **P < 0.005 vs. control siRNA-transfected cells [A] or dimethylsulfoxide [DMSO]-treated cells [B]). Experiments were performed three times, and representative results are shown. (C) The cell cycle distribution of BxPC-3 cells transfected with JNK1, JNK2, or control siRNA. The proportion of cells in each phase of the cell cycle is shown. Data shown represent means ± SD of triplicate samples (*P < 0.05, **P < 0.005 vs. control siRNA-transfected cells). (D, E) Expression levels of indicated proteins in KP-4 cells transfected with JNK1, JNK2, or control siRNA (D) or treated with SP600125 or control vehicle (E). (F) KP-4 cells were transfected with control plasmid or an expression plasmid encoding wild-type Ras (WT) or RasG12V (V12). Immunoblotting with antibodies against the indicated proteins and the relative quantity of the blots compared with the control are shown. (G) KP-4 cells were transfected with 100 nM of control or KRAS siRNA, and then lysed after 48 h. Results of immunoblotting analysis for the indicated proteins are shown. (H) Cell numbers were measured 24, 48, and 72 h after transfection. Data are plotted as means ± SD of triplicate samples (*P < 0.05 vs. control siRNA-transfected cells).

KP-4 cells using KRAS siRNA. The level of phosphorylated JNK was decreased in KRAS knocked-down cells (Fig. 2G), and the growth of KP-4 cells was significantly inhibited by KRAS knockdown (Fig. 2H).

Cyclin D1 is a well-established cell cycle regulator of proliferation,[29] and we confirmed that growth of cell lines was inhibited by transfection of cyclin D1 siRNA (Fig. 3A). To investigate the role of Ras activation in cyclin D1 transactivation, we performed a luciferase assay using the cyclin D1 promoter-containing construct pD1luc[30] and consensus AP-1 binding site-containing AP-1luc. Overexpression of RasG12V enhanced both the cyclin D1 promoter and AP-1 transcription factor in KP-4 cells, whereas treatment with SP600125 abolished the enhanced promoter activities (Fig. 3B). We also observed that cyclin D1 mRNA was decreased by the JNK inhibitor SP600125 as well as siRNAs against JNK1 and JNK2 in KP-4 cells (Fig. 3C). These results ensured that oncogenic Ras regulates cyclin D1 transcription via JNK activation. Next, we examined the expression of cyclin D1 in human pancreatic cancer tissues. Human pancreatic cancer tissue array showed positive staining in pancreatic cancer tissue in 15/20 cases and 0/4 in normal cases (Fig. 3D). These results indicate that JNK plays an important role in the proliferation of pancreatic cancer through cyclin D1 regulation, and mutation of K-ras seems to be one of the mechanisms involved in JNK activation of pancreatic cancer.

JNK activation in murine PDAC tissue and the effect of JNK inhibition on PDAC development and survival of the mice. Although $Kras^{G12D}$+$Tgfbr2^{KO}$ mice develop PDAC, $Kras^{G12D}$ mice develop mPanIN and rarely develop pancreatic carcinoma within 1 year.[19,21] We examined JNK activation in normal, mPanIN, and PDAC tissue, obtained from wild-type, $Kras^{G12D}$, and $Kras^{G12D}$+$Tgfbr2^{KO}$ mice, respectively. Immunohistochemical staining showed increased expression of phosphorylated JNK according to the progression of PDAC, in accordance with cyclin D1 expression (Fig. 4A). A similar result was obtained in immunoblotting analysis (Fig. 4B). The levels of phosphorylated c-Jun were also elevated in accordance with histological progression; however, phosphorylated ERK was activated in mPanIN and PDAC to the same extent (Fig. 4B). These results have indicated that the JNK pathway is activated in the mouse model of PDAC with mutated K-ras and transforming growth factor (TGF)β signaling, and loss of TGFβ signaling seems to have an additional effect on JNK activity mediated by K-ras mutation *in vivo*.

To investigate the therapeutic effect of JNK inhibition *in vivo*, we treated $Kras^{G12D}$+$Tgfbr2^{KO}$ mice with JNK inhibitors. Histological analysis showed significantly decreased tumor areas in the pancreas of the mice treated with SP600125 (n = 9; 55.5 ± 20.2%) compared with the control mice (n = 8; 86.6 ± 8.9%) (Fig. 4C). We observed similar results using another JNK inhibitor, D-JNKI1, which has a mechanism of JNK inhibition distinct from that of SP600125[31] (Fig. S2A). Next, we examined whether survival is prolonged by JNK inhibitor treatment. As shown in Fig. 4D, the survival time was significantly improved in mice treated with SP600125 (median survival time, 63 days), compared with the control vehicle (57.5 days; P = 0.036, log-rank test).

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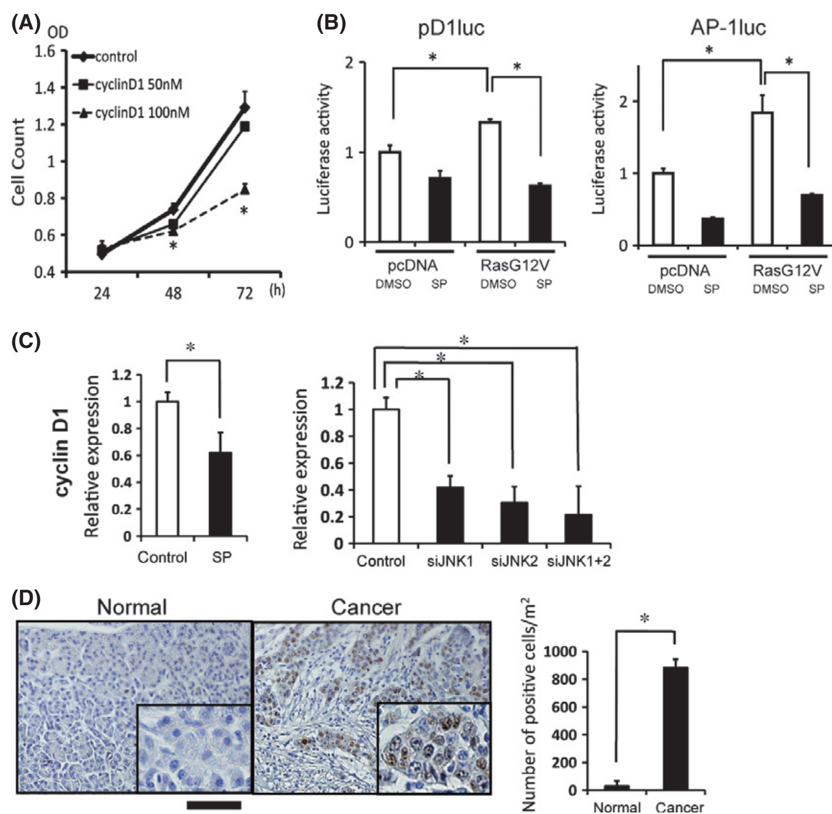

Fig. 3. Regulation of cyclin D1 by KRAS and c-Jun N-terminal kinase (JNK). (A) KP-4 cells were transfected with control siRNA and cyclin D1 siRNA. Cell numbers were measured as in Fig. 2(A). Data are plotted as means ± SD of triplicate samples (*$P < 0.05$ vs. control siRNA-transfected cells). (B) KP-4 cells were transfected with the pcDNA3.1 or RasG12V vector together with pD1luc (left) or AP-1luc (right) for 24 h. SP600125 (SP) or control vehicle (dimethylsulfoxide [DMSO]) was added to the supernatant immediately after the transfection. The ratio of reporter luciferase activity to the *Renilla* luciferase control is indicated. Results represent the means ± SD of triplicate samples (*$P < 0.05$). (C) KP-4 cells were treated with control vehicle or SP600125 (SP) (left), or transfected with control, JNK1, JNK2, or both JNK1 and 2 siRNAs (right). After 48 h, relative mRNA levels of cyclin D1 were measured by real-time reverse transcription-polymerase chain reaction (RT-PCR). Data are plotted as means ± SD of triplicate samples (*$P < 0.05$ vs. control siRNA-transfected cells). Experiments were performed three times, and representative results are shown. (D) Human normal pancreas ($n = 4$, left) and pancreatic cancer sections ($n = 20$, right) were stained for cyclin D1 and representative images are shown (original magnification ×200, bar 100 μm). Insets show detailed areas containing pancreatic duct (original magnification ×400, bar 25 μm). Results are means ± SD of six random views (*$P < 0.05$).

The PDAC specimens of the mice were immunohistochemically stained, and we observed decreased expression of phosphorylated JNK, c-Jun, cyclin D1, and PCNA in the PDAC tissues treated with SP600125 (Fig. 4E), suggesting that JNK inhibition suppressed cell proliferation. We next performed the TUNEL assay for PDAC sections. The number of TUNEL-positive cells in PDAC tissue treated with SP600125 was similar to those in control sections, showing that the anti-tumor effect of JNK inhibitors is not dependent on apoptosis (Fig. S2B). These results indicate that treatment with JNK inhibitors had therapeutic effects on PDAC in $Kras^{G12D}$+$Tgfbr2^{KO}$ mice.

The mechanism of the anti-tumor effect by JNK inhibition. To further investigate the mechanism of the anti-tumor effect of JNK inhibition, we evaluated the involvement of tumor angiogenesis. We found fewer blood vessels in PDAC specimens treated with SP600125 compared with those in control specimens (Fig. 5A). We examined the levels of cytokines in the supernatant of KP-4 cells treated with SP600125 or control vehicle using cytokine array. We found that some angiogenic cytokines, such as interleukin-8 (IL-8), growth-related oncogene α (GROα), vascular endothelial growth factor (VEGF), and monocyte chemotactic protein-1 (MCP-1),[32–35] were reduced by SP600125 treatment (Fig. S3A). We quantitated the levels of these cytokines in the supernatant of KP-4 cells treated with SP600125 or siRNA against JNK1 and 2 by ELISA, and confirmed that these cytokines are decreased in the supernatant of KP-4 cells with JNK inhibition (Figs 5B and S3B).

Next, we incubated HUVECs with the supernatant of KP-4 cells transfected with siRNAs against JNK1 and 2, and found that capillary formation by HUVECs was significantly reduced (Fig. 5C). Similar results were obtained with the supernatant of KP-4 cells treated with SP600125 (Fig. S3C). Incubating HUVECs with the supernatant of BxPC-3 cells also showed similar results (data not shown). These results indicate that JNK inhibition has anti-angiogenic effects on pancreatic cancer *in vitro* and *in vivo*, which may be associated with therapeutic effect observed *in vivo*.

CD44 is an adhesion molecule known to be upregulated in human PDACs,[36] and putative pancreatic cancer stem cells have been identified based on the expression of CD44, CD24, and epithelial specific antigen.[37] CD44-positive cells were found in PDAC of $Kras^{G12D}$+$Tgfbr2^{KO}$ mice, whereas normal acinar and ductal cells showed no CD44 staining (Fig. 5D). We observed higher expression of CD44 in PDAC tissue of $Kras^{G12D}$+$Tgfbr2^{KO}$ mice compared with mPanIN and normal pancreatic tissue (Fig. 5E). CD44-positive cells in PDAC tissue were decreased by SP600125 treatment (Fig. 5F). The KP-4 cell line showed decreased CD44 expression when treated with SP600125 (Fig. 5G). Thus, it is possible that the JNK inhibitor exerts an anti-tumor effect by decreasing CD44-positive cells.

Discussion

JNK1 and JNK2 may play different roles depending on the cell type and condition. For example, JNK1-deficient mice spontaneously develop intestinal tumors,[38] but loss of JNK2 shows no phenotype in the intestine.[39] In the present study, silencing JNK1 or JNK2 *in vitro* both showed an inhibitory effect on the growth of pancreatic cancer cells (Fig. 2A), indicating that JNK1 and JNK2 may be cooperatively involved in the proliferation of pancreatic cancer cell lines.

In the pancreatic cancer tissue of $Kras^{G12D}$+$Tgfbr2^{KO}$ mice, JNK1 was strongly activated, whereas JNK2 was barely activated (Fig. 4B). In human PDAC cell lines, mainly JNK2 was activated (Fig. 1C), and JNK1 was activated by K-ras induction (Fig. 2F). Conversely, histologically normal acinar cells of $Kras^{G12D}$ mice, which also contain the mutant K-ras gene knocked in by $Ptf1a^{cre}$, did not show phosphorylated JNK staining (Fig. 4A middle). Thus, K-ras mutation is

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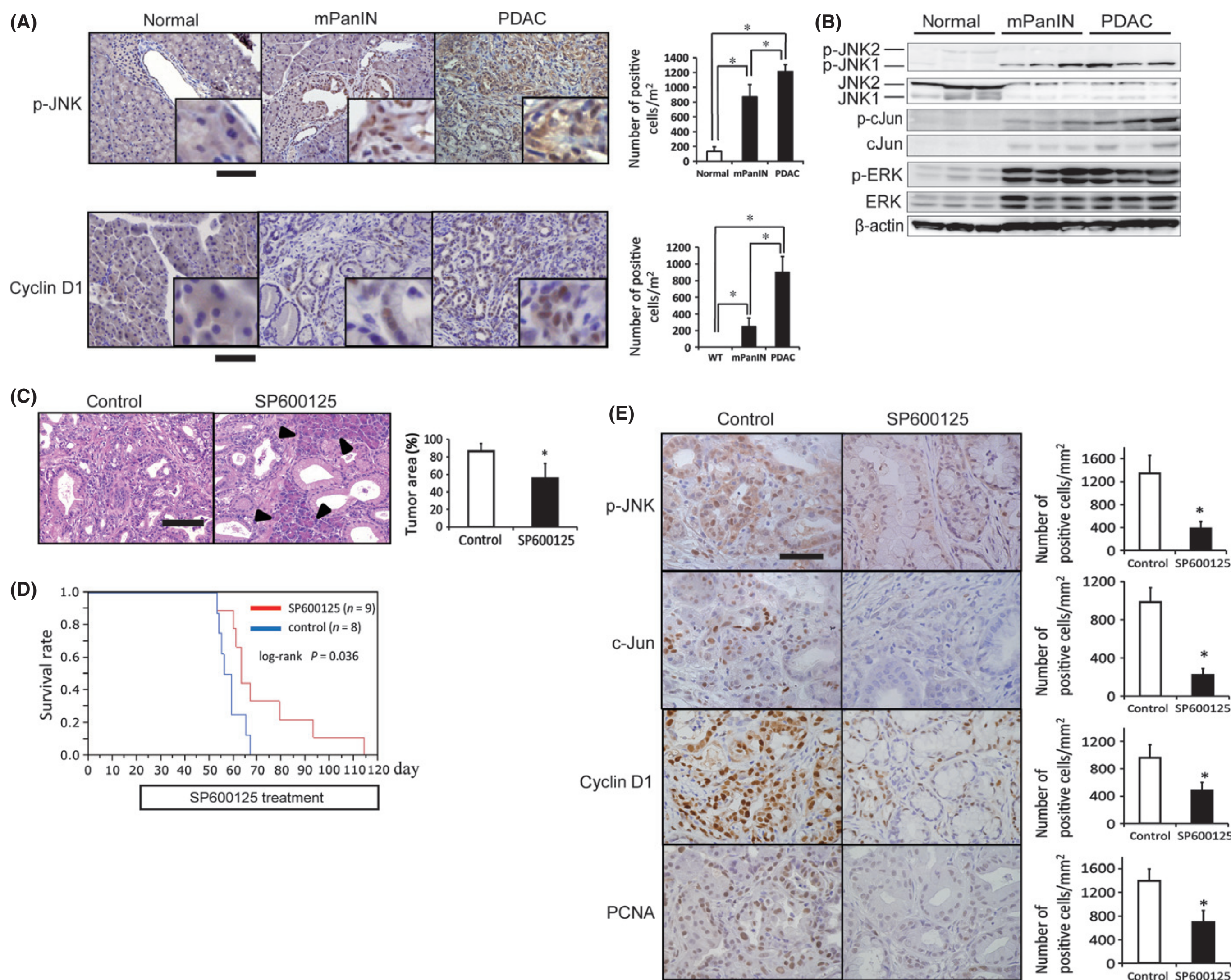

Fig. 4. The therapeutic effect of JNK inhibitors against pancreatic ductal adenocarcinoma (PDAC) *in vivo*. (A) Immunohistochemical staining for phosphorylated c-Jun N-terminal kinase (JNK) and cyclin D1 in the pancreas of wild-type (normal), $Kras^{G12D}$ (mPanIN), and $Kras^{G12D}$+$Tgfbr2^{KO}$ (PDAC) mice are shown (original magnification, ×200; bar, 100 μm). Insets are magnified images to show nuclear staining (bar, 25 μm). The numbers of cells with nuclear staining were counted and averaged (right). Results are means ± SD of six random views (*$P < 0.05$). (B) Immunoblotting analysis of the pancreatic lysate with antibodies against the indicated proteins is shown. Each lane represents an individual case. (C) Hematoxylin and eosin staining of sections of pancreas tissues from $Kras^{G12D}$+$Tgfbr2^{KO}$ mice treated with control vehicle or SP600125. Arrow heads indicate histologically normal areas (original magnification, ×200; bar, 100 μm). Tumor areas in the sections were compared (right). The data shown represent means ± SD (*$P = 0.012$). (D) Kaplan–Meier survival curves of the $Kras^{G12D}$+$Tgfbr2^{KO}$ mice treated with control vehicle ($n = 8$) or SP600125 ($n = 9$) (*$P = 0.036$, log-rank test). (E) Sections of PDAC tissues from $Kras^{G12D}$+$Tgfbr2^{KO}$ mice treated with control vehicle or SP600125 were immunostained for indicated antibodies (left) (original magnification, ×400; bar, 50 μm). The number of cells with nuclear staining with each antibody was counted and averaged (right). Results are means ± SD of three random views (*$P < 0.05$).

required for JNK activation but may not be sufficient for JNK activation *in vivo*. Presently, we do not have sufficient data to discuss the differences in JNK1 and JNK2 activation status between mouse and human cells or between the *in vitro* and *in vivo* model. Because JNK is known to be activated by various molecules, such as MAPK kinase 4 (MKK4) and MKK7, the difference in JNK activation between *in vitro* and *in vivo* may originate from the balance of these signals. Experiments using JNK1 or JNK2 knockout mice may help investigate the difference in JNK activation by mutant K-ras *in vitro* and *in vivo*.

c-Jun N-terminal kinase phosphorylation is stronger in PDAC than in mPanIN, whereas ERK activation was almost the same in both tissue types (Fig. 4B). This result also suggests the critical role of JNK in pancreatic carcinogenesis, consistent with the report that Ras-induced transformation of primary murine embryonic fibroblasts (MEFs) requires JNK.[40] Additionally, JNK signaling might be more important than ERK signaling in the late phase of carcinogenesis.

Cyclin D1 is one of the molecules that regulates the cell cycle and is important in many types of human cancer, including gastric cancer.[41] Cyclin D1 is overexpressed in a significant proportion of human pancreatic cancers, and elevated cyclin D1 levels correlate with abnormal growth and tumorigenicity in human pancreatic cancer.[42,43] Our results that JNK inhibition leads to decreased cyclin D1 expression and cell cycle retardation (Fig. 2C–E) are consistent with previous reports,[44–47] suggesting that the therapeutic effect of JNK inhibition is dependent on suppression of cyclin D1 expression.

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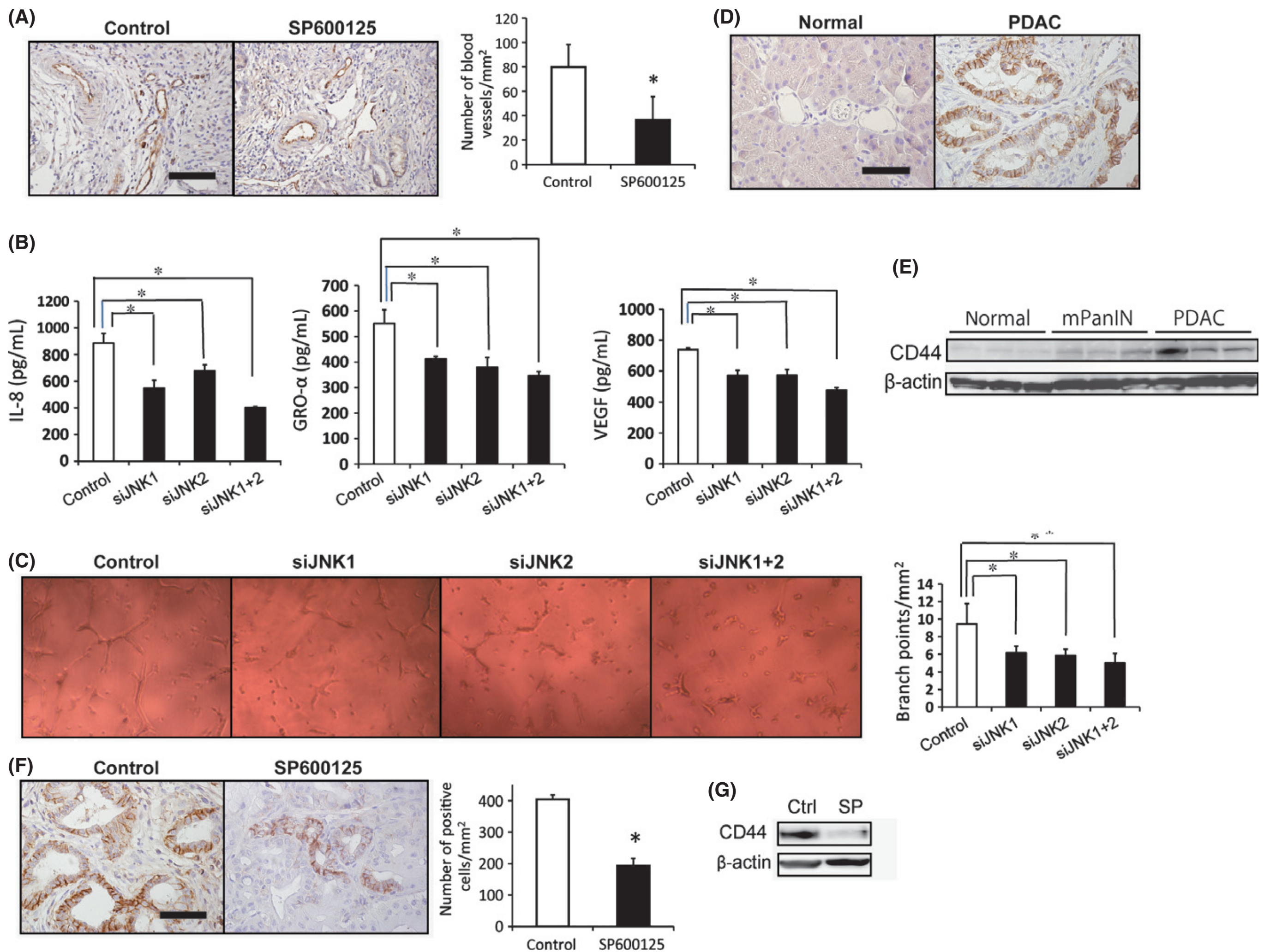

Fig. 5. Mechanism of the anti-tumor effect of c-Jun N-terminal kinase (JNK) inhibition on pancreatic cancer. (A) Pancreatic ductal adenocarcinoma (PDAC) sections of $Kras^{G12D}$+$Tgfbr2^{KO}$ mice treated with control vehicle or SP600125 were immunohistochemically stained for vWF (left) (original magnification ×200; bar, 100 μm). The number of blood vessels in PDAC sections was counted (right). Three random views were chosen in each case, and the numbers of blood vessels were counted and averaged. Results are means ± SD of triplicate samples (*$P < 0.05$). (B) Enzyme linked immunosorbent assays of interleukin-8 (IL-8), growth-related oncogene α (GROα), and vascular endothelial growth factor (VEGF) in the supernatants of KP-4 cells transfected with control, JNK1, JNK2, or JNK1 and 2 siRNAs (100 nM, 48 h). Results are means ± SD of triplicate samples (*$P < 0.05$). (C) Capillary formation of human umbilical vein endothelial cells (HUVECs) in the supernatant of KP-4 cells transfected with control, JNK1, JNK2, or JNK1 and 2 siRNAs. The numbers of branch points were counted. Results are means ± SD of triplicate samples (*$P < 0.05$). Representative images are shown at the left. (D) Pancreatic tissues of wild-type (normal) or $Kras^{G12D}$+$Tgfbr2^{KO}$ (PDAC) mice were immunohistochemically stained for CD44 (original magnification ×400; bar, 50 μm). (E) Immunoblotting analysis of pancreatic lysate with antibodies against CD44. Each lane represents an individual case. (F) Pancreatic ductal adenocarcinoma sections of $Kras^{G12D}$+$Tgfbr2^{KO}$ mice treated with control vehicle or SP600125 were immunohistochemically stained for CD44 (left) (original magnification ×400; bar, 50 μm). The number of CD44 positive cells was counted (right). Three random views were chosen in each case, and the numbers of cells were counted and averaged. Results are means ± SD of triplicate samples (*$P < 0.05$). (G) The expression level of CD44 in KP-4 cells 24 h after treatment with SP600125 (Ctrl: control vehicle, SP: SP600125).

c-Jun N-terminal kinase-targeted cancer therapy has been investigated mainly *in vitro* using antisense oligonucleotides and pharmacological inhibitors.[9,18,48] Regarding pancreatic cancer, SP600125 inhibits the growth of the pancreatic cancer cell line MIAPaCa2.[18] We demonstrated that JNK inhibition suppressed progression of PDAC and prolonged the survival of mice with PDAC (Fig. 4A–D). As the result of TUNEL staining showed that apoptosis was not increased in the tumors of SP600125-treated mice (Fig. S2B), the anti-tumor effect by JNK inhibition is not dependent on apoptosis in our *in vivo* model.

The growth and progression of solid tumors are affected by angiogenesis,[49] and angiogenesis has been reported to have a positive correlation with disease progression of pancreatic cancer.[50,51] Furthermore, JNK and p38 are essential for VEGF mRNA stabilization *in vitro*.[52] In the present study, we observed decreased angiogenesis by treatment with SP600125 *in vitro* and *in vivo*. Therefore, suppression of vascularization is probably one of the mechanisms of the anti-tumor effect by the JNK inhibitor *in vivo*. Conversely, stromal cells surrounding tumors can secrete angiogenic cytokines. We found that some stromal cells are positive for phosphorylated JNK in immunohistochemical staining (Figs 1A and 4A). The effect of the JNK inhibitor on fibroblasts or macrophages in the PDAC remains to be investigated.

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We also found that the JNK inhibitor decreased CD44 expression in PDAC of $Kras^{G12D}$+$Tgfbr2^{KO}$ mice. The CD44 expression level is associated with poor prognosis and gemcitabine resistance in pancreatic cancer.[53] Our results indicate that JNK inhibition may affect CD44-positive cells (Fig. 5D–G). Because CD44-positive pancreatic cancer cells were previously shown to increase when treated with gemcitabine,[53] a combination of JNK inhibition with gemcitabine treatment may be more effective, by overcoming gemcitabine resistance.

In summary, we showed that JNK has a cancer-promoting effect in pancreatic cancer, and inhibiting JNK leads to growth inhibition, cell cycle arrest, and decreased angiogenesis, resulting in prolonged survival. Thus, JNK is a potential therapeutic target for pancreatic cancer.

Acknowledgments

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Disclosure Statement

All authors declare that they have no conflict of interest.

References

1 Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 2000; **103**: 239–52.
2 Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Genet Dev* 2002; **12**: 14–21.
3 Chen YR, Meyer CF, Tan TH. Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. *J Biol Chem* 1996; **271**: 631–4.
4 Guo YL, Baysal K, Kang B, Yang LJ, Williamson JR. Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. *J Biol Chem* 1998; **273**: 4027–34.
5 Liu J, Lin A. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res* 2005; **15**: 36–42.
6 Whitmarsh AJ, Davis RJ. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med (Berl)* 1996; **74**: 589–607.
7 Miyazaki T, Bub JD, Iwamoto Y. c-Jun NH(2)-terminal kinase mediates leptin-stimulated androgen-independent prostate cancer cell proliferation via signal transducer and activator of transcription 3 and Akt . *Biochim Biophys Acta* 2008; **1782**: 593–604.
8 Sancho R, Nateri AS, de Vinuesa AG *et al.* JNK signalling modulates intestinal homeostasis and tumourigenesis in mice. *EMBO J* 2009; **28**: 1843–54.
9 Bost F, McKay R, Bost M, Potapova O, Dean NM, Mercola D. The Jun kinase 2 isoform is preferentially required for epidermal growth factor-induced transformation of human A549 lung carcinoma cells. *Mol Cell Biol* 1999; **19**: 1938–49.
10 Cui J, Han SY, Wang C *et al.* c-Jun NH(2)-terminal kinase 2alpha2 promotes the tumorigenicity of human glioblastoma cells. *Cancer Res* 2006; **66**: 10024–31.
11 Yang YM, Bost F, Charbono W *et al.* C-Jun NH(2)-terminal kinase mediates proliferation and tumor growth of human prostate carcinoma. *Clin Cancer Res* 2003; **9**: 391–401.
12 Shibata W, Maeda S, Hikiba Y *et al.* c-Jun NH2-terminal kinase 1 is a critical regulator for the development of gastric cancer in mice. *Cancer Res* 2008; **68**: 5031–9.
13 Sakurai T, Maeda S, Chang L, Karin M. Loss of hepatic NF-kappa B activity enhances chemical hepatocarcinogenesis through sustained c-Jun N-terminal kinase 1 activation . *Proc Natl Acad Sci USA* 2006; **103**: 10544–51.
14 Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF. Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. *J Clin Invest* 2008; **118**: 3943–53.
15 Chen F, Castranova V. Beyond apoptosis of JNK1 in liver cancer. *Cell Cycle* 2009; **8**: 1145–7.
16 Chen N, Nomura M, She QB *et al.* Suppression of skin tumorigenesis in c-Jun NH(2)-terminal kinase-2-deficient mice. *Cancer Res* 2001; **61**: 3908–12.
17 Tessari G, Ferrara C, Poletti A *et al.* The expression of proto-oncogene c-jun in human pancreatic cancer. *Anticancer Res* 1999; **19**: 863–7.
18 Ennis BW, Fultz KE, Smith KA *et al.* Inhibition of tumor growth, angiogenesis, and tumor cell proliferation by a small molecule inhibitor of c-Jun N-terminal kinase. *J Pharmacol Exp Ther* 2005; **313**: 325–32.
19 Ijichi H, Chytil A, Gorska AE *et al.* Aggressive pancreatic ductal adenocarcinoma in mice caused by pancreas-specific blockade of transforming growth factor-beta signaling in cooperation with active Kras expression. *Genes Dev* 2006; **20**: 3147–60.
20 Ijichi H, Chytil A, Gorska AE *et al.* Inhibiting Cxcr2 disrupts tumor-stromal interactions and improves survival in a mouse model of pancreatic ductal adenocarcinoma. *J Clin Invest* 2011; **121**: 4106–17.
21 Hingorani SR, Petricoin EF, Maitra A *et al.* Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003; **4**: 437–50.
22 Chytil A, Magnuson MA, Wright CV, Moses HL. Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. *Genesis* 2002; **32**: 73–5.
23 Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet* 2002; **32**: 128–34.
24 Jackson EL, Willis N, Mercer K *et al.* Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev* 2001; **15**: 3243–8.
25 Bennett BL, Sasaki DT, Murray BW *et al.* SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci USA* 2001; **98**: 13681–6.
26 Sakamoto K, Maeda S, Hikiba Y *et al.* Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. *Clin Cancer Res* 2009; **15**: 2248–58.
27 Minden A, Lin A, McMahon M *et al.* Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* 1994; **266**: 1719–23.
28 Olson MF, Ashworth A, Hall A. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* 1995; **269**: 1270–2.
29 Sherr CJ. D-type cyclins. *Trends Biochem Sci* 1995; **20**: 187–90.
30 Müller H, Lukas J, Schneider A *et al.* Cyclin D1 expression is regulated by the retinoblastoma protein. *Proc Natl Acad Sci USA* 1994; **91**: 2945–9.
31 Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF. Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes* 2001; **50**: 77–82.
32 Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* 2004; **6**: 447–58.
33 Luan J, Shattuck-Brandt R, Haghnegahdar H *et al.* Mechanism and biological significance of constitutive expression of MGSA/GRO chemokines in malignant melanoma tumor progression. *J Leukoc Biol* 1997; **62**: 588–97.
34 Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989; **246**: 1306–9.
35 Koga M, Kai H, Egami K *et al.* Mutant MCP-1 therapy inhibits tumor angiogenesis and growth of malignant melanoma in mice. *Biochem Biophys Res Commun* 2008; **365**: 279–84.
36 Castellà EM, Ariza A, Ojanguren I *et al.* Differential expression of CD44v6 in adenocarcinoma of the pancreas: an immunohistochemical study. *Virchows Arch* 1996; **429**: 191–5.
37 Li C, Heidt DG, Dalerba P *et al.* Identification of pancreatic cancer stem cells. *Cancer Res* 2007; **67**: 1030–7.
38 Tong C, Yin Z, Song Z, *et al.*, c-Jun NH2-terminal kinase 1 plays a critical role in intestinal homeostasis. *Am J Pathol* 2007; **171**: 297–303.
39 Bi X, Pohl NM, Yin Z, Yang W. Loss of JNK2 increases intestinal tumor susceptibility in Apc1638 + /- mice with dietary modulation. *Carcinogenesis* 2011; **32**: 584–8.
40 Cellurale C, Sabio G, Kennedy NJ *et al.* Requirement of c-Jun NH(2)-terminal kinase for Ras-initiated tumor formation. *Mol Cell Biol* 2011; **31**: 1565–76.
41 Hayakawa Y, Hirata Y, Nakagawa H *et al.* Apoptosis signal-regulating kinase 1 and cyclin D1 compose a positive feedback loop contributing to tumor growth in gastric cancer. *Proc Natl Acad Sci USA* 2011; **108**: 780–5.
42 Gansauge S, Gansauge F, Ramadani M *et al.* Overexpression of cyclin D1 in human pancreatic carcinoma is associated with poor prognosis. *Cancer Res* 1997; **57**: 1634–7.

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43 Kornmann M, Arber N, Korc M. Inhibition of basal and mitogen-stimulated pancreatic cancer cell growth by cyclin D1 antisense is associated with loss of tumorigenicity and potentiation of cytotoxicity to cisplatinum. *J Clin Invest* 1998; **101**: 344–52.
44 Albanese C, Johnson J, Watanabe G *et al.* Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J Biol Chem* 1995; **270**: 23589–97.
45 Shaulian E, Karin M. AP-1 in cell proliferation and survival. *Oncogene* 2001; **20**: 2390–400.
46 Herber B, Truss M, Beato M, Müller R. Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene* 1994; **9**: 2105–7.
47 Bakiri L, Lallemand D, Bossy-Wetzel E, Yaniv M. Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *EMBO J* 2000; **19**: 2056–68.
48 Potapova O, Gorospe M, Dougherty RH, Dean NM, Gaarde WA, Holbrook NJ. Inhibition of c-Jun N-terminal kinase 2 expression suppresses growth and induces apoptosis of human tumor cells in a p53-dependent manner. *Mol Cell Biol* 2000; **20**: 1713–22.
49 Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995; **1**: 27–31.
50 Itakura J, Ishiwata T, Friess H *et al.* Enhanced expression of vascular endothelial growth factor in human pancreatic cancer correlates with local disease progression. *Clin Cancer Res* 1997; **3**: 1309–16.
51 Ikeda N, Adachi M, Taki T *et al.* Prognostic significance of angiogenesis in human pancreatic cancer. *Br J Cancer* 1999; **79**: 1553–63.
52 Pagès G, Berra E, Milanini J, Levy AP, Pouysségur J. Stress-activated protein kinases (JNK and p38/HOG) are essential for vascular endothelial growth factor mRNA stability. *J Biol Chem* 2000; **275**: 26484–91.
53 Hong SP, Wen J, Bang S, Park S, Song SY. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. *Int J Cancer* 2009; **125**: 2323–31.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Effect of JNK inhibition and KRAS expression on pancreatic cancer cell line BxPC-3.

Fig S2. Effect of JNK inhibitors against PDAC *in vivo.*

Fig S3. Effect of JNK inhibition on angiogenic cytokines of KP-4 cells.

Doc S1. Materials and methods.

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