MK2461, a Multitargeted Kinase Inhibitor, Suppresses the Progression of Pancreatic Cancer by Disrupting the Interaction Between Pancreatic Cancer Cells and Stellate Cells

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Objectives: Platelet-derived growth factor receptor beta (PDGFRβ) and hepatocyte growth factor receptor (MET) expressed on pancreatic stellate cells (PSCs) are suggested as important components modulating the interactions between pancreatic cancer cells (PCCs) and PSCs. The objective of this study is to clarify the effect of MK2461, a multikinase inhibitor targeting MET and PDGFRβ, on the interaction between PCCs and PSCs.

Methods: In this study, we profiled the expression of receptor tyrosine kinases (including PDGFRβ and MET) in pancreatic cancer with quantitative targeted absolute proteomics using liquid chromatography tandem mass spectrometry. In addition, the effect of MK2461 on PCC-PSC interaction was investigated using PSCs prepared from pancreatic cancer tissues.

Results: In PSCs, PDGFRβ and MET were upregulated compared with other receptor tyrosine kinases. Conditioned medium from PSCs promoted the proliferation of PCCs, and vice versa. Moreover, MK2461 suppressed the effects of conditioned medium on PCCs and PSCs. Finally, MK2461 significantly inhibited tumor growth in mice coinjected with PCCs and PSCs.

Conclusions: The PDGFRβ and MET may play a critical role in the interaction between PCCs and PSCs, which was modulated by MK2461. Therefore, MK2461 may have therapeutic potential in the treatment of pancreatic cancer.

Key Words: pancreatic cancer, pancreatic stellate cell, MK2461, proteomics, molecular therapeutics

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Pancreatic cancer is a highly aggressive disease characterized by an extremely poor prognosis. Despite recent developments in the diagnosis and therapeutic management of pancreatic cancer, the overall 5-year survival rate is less than 5%,[1] in part because of the poor response of pancreatic cancer to most chemotherapeutic agents and radiotherapy. Therefore, improving our understanding of the development and progression of pancreatic cancer is essential.[2]

Pancreatic cancer is characterized by excessive desmoplasia, which occupies 80% of pancreatic cancer tissue.[3] However, most previous studies have focused on cancer cells themselves, and the abundant desmoplasia has been largely ignored.[4] The desmoplasia is thought to be essential for the proliferation, invasion, metastasis, and chemotherapeutic resistance of pancreatic cancer[5–10] and has been shown to be comprised primarily of pancreatic stellate cells (PSCs), which are observed in the interlobular areas and the periacinar lesions of the pancreas.[11] The PSCs are transformed from a quiescent state to myofibroblast-like cells in response to cytokines and growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF) β1, which are secreted from inflammatory cells and cancer cells. Activated PSCs are characterized by high expression of α-smooth muscle actin (SMA), and once PSCs are activated by pancreatic cancer cells (PCCs), these cells are suggested to remain in the active state via autonomous signaling loops.[4,12,13] Activated PSCs produce abundant extracellular matrix (ECM), cytokines, and growth factors, and the production of ECM contributes to excessive fibrosis thereby leading to interstitial hypertension, inefficient drug delivery,[14–16] and resistance to radiotherapy.[17] Furthermore, secreted growth factors from activated PSCs, such as PDGF and hepatocyte growth factor (HGF), promote PCC proliferation, invasion, and migration, partially through induction of the epithelial-to-mesenchymal transition.[10,18,19] In an in vivo study, PCCs subcutaneously injected with PSCs were shown to grow more rapidly than PCCs injected alone.[20] Moreover, in an orthotopic model, coinjection of PCCs with PSCs resulted in increased tumor incidence, metastasis, and tumor size.[21] In addition, PCC stimulation increases the secretion of growth factors and ECM components from PSCs.[18] Thus, reciprocal stimulation of PCCs and PSCs is essential in the progression of pancreatic cancer.

Altered expression of various receptor tyrosine kinases (RTKs) has been observed in several types of cancer, and the expression of some RTKs correlates with patient prognosis.[22–24] Growth factors mediate their effects by binding to various RTKs. Thus, profiling the expression of RTKs in both PCCs and PSCs may lead to identification of growth factors regulating the interaction between PCCs and PSCs.

MK2461 is a multikinase inhibitor that was developed as an adenosine triphosphate-competitive inhibitor of the activated HGF receptor (MET). This compound effectively inhibits constitutive or ligand-dependent phosphorylation of MET and significantly inhibits several other RTKs. Moreover, MK2461 exerts significant anti–tumor-effect activities through inhibition of MET,

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Fibroblast growth factor (FGF) receptor 2, and PDGF receptor (PDGFR) in vitro and in vivo.[25] Therefore, MK2461 may have applications as a potential anticancer agent in pancreatic cancer through disruption of RTK signaling in PCCs and PSCs.

In this study, we sought to identify novel candidate targets for improving the therapeutic management of pancreatic cancer. To this end, we measured the expression levels of 15 RTKs by quantitative targeted absolute proteomics (QTAP) and analyzed the roles of these RTKs in pancreatic cancer. In addition, we examined the effects of MK2461 on the PCC-PSC interaction.

MATERIALS AND METHODS

Cell Culture

PANC-1 cells were obtained from RIKEN BioResource Center Cell Bank (Tsukuba, Japan). Capan-2, HPAF-II, BxPC-3, SW1990, and AsPC-1 cells were purchased from American Type Culture Collection (Manassas, Va). SUIT-2, KLM-1, PK-1, and PK-8 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Nichirei Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS) with 4.5 g/L glucose, 1.5 g/L $NaHCO_3$, 70 μg/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin at 37°C in a humidified atmosphere of 5% CO_2.

Reagents and Antibodies

All of the standard peptides and stable isotope-labeled peptides used for QTAP were synthesized by Thermo Fisher Scientific (Ulm, Germany). Sequencing-grade modified trypsin (Promega, Madison, Wis) was used for trypsin digestion of the targeted proteins. MK2461 was purchased from Selleck (Houston, Tex). All other reagents were commercial products of analytical grade unless specifically described. The antibodies used in this study included anti–phospho-MET (Tyr^{1234}/Tyr^{1235}), anti-AKT, anti–phospho-AKT (Ser^{473}), anti-extracellular signal–regulated kinase 1/2 (ERK1/2), anti–phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-PDGFRβ, and antivimentin from Cell Signaling Technology (Danvers, Mass); anti-MET and anti–α-SMA from Abcam (Cambridge, Mass); anti–phospho-PDGFRβ (Tyr^{1021}) and anti–cytokeratin 19 from Santa Cruz Biotechnology (Santa Cruz, Calif); anti–Ki-67 from Nichirei Biosciences (Tokyo, Japan); and Alexa Fluor 488–conjugated anti–rabbit IgG, Alexa Fluor 555–conjugated anti–mouse IgG, horseradish peroxidase–linked anti–rabbit IgG, and horseradish peroxidase–conjugated anti–mouse IgG from Cell Signaling Technology.

Human Samples

The pancreatic tissue blocks used in this study were obtained from patients undergoing surgery at the Tohoku University Hospital. All patients were diagnosed with pancreatic cancer by biopsy before surgery. The samples were obtained in accordance with the policies and practices of the ethics committee of the Tohoku University Graduate School of Medicine, and patients provided informed consent.

Primary Culture of PSCs

The PSCs were prepared from pancreatic cancer tissues by the outgrowth method, as previously described.[20] The purity of the cells was determined by the immunofluorescence for α-SMA and vimentin as well as morphology (spindle-shaped cells with cytoplasmic extensions).[26] In this study, all of the established PSCs were used at passages 3 to 6.

Immunofluorescence Staining of PSC

The PSCs were fixed in 4% paraformaldehyde and blocked with 5% normal goat serum and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, Mo) in phosphate buffered saline. The PSCs were incubated with primary antibodies at 4°C overnight and were then incubated with secondary antibodies. The antibodies were diluted in phosphate buffered saline containing 1% bovine serum albumin and 0.3% Triton X-100 according to the manufacturer's instructions. The PSCs were mounted with ProLong antifade with 4′, 6-diamidino-2-phenylindole (DAPI) (Life Technologies, Waltham, Mass). Staining was observed using a confocal laser-scanning microscope (C2si, Nikon, Japan).

Preparation of Conditioned Medium

Conditioned medium (CM) was prepared to evaluate the PCC-PSC interaction.[21,27] Briefly, cells were grown to 70% to 80% confluence in DMEM containing 10% FBS. The medium was then changed to serum-free medium (SFM), which was collected after 24 to 48 hours and concentrated with a 3-kDa ultrafiltration membrane (Millipore, Billerica, Mass). Protein concentrations were determined by the Lowry method (Bio-Rad, Hercules, Calif).

The QTAP Using Liquid Chromatography Tandem Mass Spectrometry

Plasma membrane fractions were extracted, and the absolute quantity of membrane protein was quantified using a multiplex selected reaction monitoring/multiple reaction monitoring (SRM/MRM) method, as described previously.[28–30]

In the SRM/MRM analysis, the peptide for each target protein was monitored using 4 types of SRM/MRM transitions specific for that peptide. The quantitative value was calculated from the peak area ratio of the analyte and the stable isotope-labeled peptide in each SRM/MRM transition. Unless otherwise mentioned, at least 3 of 4 SRM/MRM transitions were required to be measurable for a proteotypic peptide to be judged as confirmed and for a quantitative value to be assigned. The value of the quantification limit of each protein (femtomoles per microgram of protein) was determined as described previously.[29] Peptide sequences for targeted proteins and MRM transitions (m/z values) are shown in Supplemental Table 1, http://links.lww.com/MPA/A582.

Proliferation Assay

SUIT-2 and PANC-1 cells were seeded at 3000 cells/well, and PSCs were seeded at 5000 cells/well in 96-well plates. Cells were incubated overnight in DMEM containing 10% FBS, after which the medium was changed to SFM. Then, different concentrations of CM (0, 0.1, or 0.5 mg/mL) and MK2461 (0, 0.1, 1, or 3 μM) were added, and cells were incubated for 48 hours. The SFM was added to control wells. After incubation, proliferation was measured using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Each assay was performed using triplicate wells and repeated twice. Data are shown as percentage change compared with the control.

Invasion Assay

Invasion assays were performed with 24-well BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ). SUIT-2 and PANC-1 cells (2×10^4) were resuspended in 500 μL of SFM and seeded in the upper chambers. Lower chambers contained 750 μL of CM (0, 0.1, or 0.5 mg/mL) and MK2461 (0, 0.1, or 1 μM) in SFM. Cells were incubated for 48 hours at

37°C per 5% CO_2, and cells that had invaded through the pores to the lower surface were fixed and stained with Diff-Quick reagent (Sysmex International Reagents, Kobe, Japan). Invaded cells were counted in 8 random adjacent fields using a microscope (BZ-9000; Keyence, Tokyo, Japan). Each experiment was repeated 3 times.

Migration Assay

Migration assays were performed in 24-well transwell chambers (BD Biosciences). SUIT-2 and PANC-1 cells (2×10^4) were seeded in the upper chambers of the transwells in 500 μL of SFM. Treatments were as described for the invasion assays, and analyses were performed as described for the invasion assays at 24 hours after treatment. Each experiment was repeated 3 times.

In Vivo Experiments

To evaluate the effects of MK2461 on the PCC-PSC interaction in vivo, animal experiments were performed as previously described.[17,18] All animal experiments were reviewed and approved by the Tohoku University Institutional Animal Care and Use Committee. Seven-week-old male nude mice (BALB/cAJcl-nu/nu) were obtained from CLEA Japan (Tokyo, Japan). Mice were acclimated to the animal housing facility for 1 week before studies. SUIT-2 cells and PSCs were resuspended in 100 μL of DMEM containing 20% Matrigel (BD Biosciences), and SUIT-2 cells (1×10^6) and PSCs (1×10^6) were subcutaneously coinjected into the right flanks, whereas SUIT-2 cells (1×10^6) alone were injected into the left flanks. One week later, mice were randomly divided into 2 groups (n = 7 per group) and administered either vehicle or MK2461 (20 mg/kg) twice daily for 20 days by oral gavage. MK2461 was diluted in 0.9% saline containing 30% polyethylene glycol400, 1% dimethyl sulfoxide, and 1% Tween-80. Tumor sizes were determined with calipers, and tumor volumes were calculated using the formula, $\pi/6 \times (L \times W^2)$, where L indicates the largest tumor diameter and W indicates the smallest tumor diameter. When the experiment was terminated, subcutaneous tumors were excised and weighed.

Statistical Analysis

Data are shown as the mean ± SEM. Comparisons of paired data were analyzed by 2-tailed Student's *t*-tests, and comparisons of 3 or more groups were analyzed by 1-way analysis of variance with the Tukey-Kramer multiple comparisons test. Differences with *P* values of less than 0.05 were considered significant. Statistical analyses were performed using JMP Pro 11 software (SAS Institute, Cary, NC).

RESULTS

Isolation and Identification of PSCs

The PSCs were derived from fresh human pancreatic adenocarcinoma surgical specimens by the outgrowth method, and their identities were confirmed by immunofluorescence staining for α-SMA or vimentin (Fig. 1A). The isolated cells were stained with α-SMA, a marker of activated PSCs, and expressed vimentin, a marker of mesenchymal cells, indicating that they were not PCCs. In addition, these cells exhibited a spindle-shaped morphology with cytoplasmic extensions, characteristic of myofibroblasts. The PSCs were isolated from 3 patients individually and named PSCI, PSCII, and PSCIII, respectively. Interestingly, α-SMA expression was increased in PSCs compared with TIG-1-20 cells, a normal fibroblast cell line, indicating that the isolated PSCs were in an activated state (Fig. 1B).

Role of the PCC-PSC Interaction in Cell Proliferation

To investigate the effects of PSC-CM on PCCs, proliferation assays were performed. Three conditioned media derived from PSCI, PSCII, and PSCIII promoted the proliferation of SUIT-2 and PANC-1 cells as compared with the serum-free control (Fig. 1C). For all assays, PCC proliferation was increased after exposure to PSC-CM (0.5 mg/mL) as compared with the serum-free control.

Reciprocally, the CM from both SUIT-2 and PANC-1 cells significantly increased PSCI and PSCII proliferation compared with the serum-free controls in a concentration-dependent manner (Fig. 1D).

Comparison of Cell Proliferation Induced by Paracrine and Autocrine Secretions

To elucidate whether paracrine or autocrine effects modulated PCC proliferation in our experiment, we performed proliferation assays using CM from PCCs or PSCs. SUIT-2 and PANC-2 cell proliferation rates were significantly increased in the presence of PSCI-CM as compared with SUIT-2 or PANC-1–CM at 0.5 mg/mL, respectively (Supplemental Fig. 1A, http://links.lww.com/MPA/A583). Furthermore, SUIT-2 and PANC-1–conditioned media (0.1 or 0.5 mg/mL) significantly accelerated the proliferation of PSCIs compared with PSCI-CM (Supplemental Fig. 1B, http://links.lww.com/MPA/A583). Therefore, paracrine signaling was more effective at inducing proliferation in both PCCs and PSCs.

Expression Levels of Membrane RTKs in Pancreatic Cancer Cell Lines, PSCs, and Pancreatic Cancer Tissues

Next, we analyzed the expression levels of 15 RTKs by QTAP in plasma membrane fractions from 10 human pancreatic cancer cell lines (Supplemental Table 2, http://links.lww.com/MPA/A584), 3 primary cultured PSCs (PSCI, PSCII, and PSCIII) (Supplemental Table 3, http://links.lww.com/MPA/A585), pancreatic cancer tissues obtained from patients 1 to 17 (PT1-17) (Supplemental Table 4, http://links.lww.com/MPA/A586), and 3 normal pancreatic tissues obtained from normal noncancerous lesions of surgical specimens from patients 1 to 3 (NT1-3) (Supplemental Table 3, http://links.lww.com/MPA/A585). The epidermal growth factor receptor (EGFR) and MET were detected in all of the pancreatic cancer cell lines, whereas EGFR, PDGFRβ, and MET were detected in the 3 PSCs. Moreover, EGFR and PDGFRβ were detected in all 17 pancreatic cancer tissues, and MET was detected in 11 pancreatic cancer tissues (64.7%). In the 3 noncancerous tissues, only EGFR was detected. Na^+/K^+ ATPase was detected in all of the samples.

EGFR expression was approximately 10- to 20-fold higher in most pancreatic cancer cell lines than in PSCs and pancreatic cancer tissues (Fig. 2A). In 8 pancreatic cancer cell lines, the expression levels of PDGFRβ were under the detection limit; however, this RTK was detected in all of the PSCs and pancreatic cancer tissues (Fig. 2A). Expression levels of PDGFRβ were equivalent between PSCs and pancreatic cancer tissues. The MET expression levels in pancreatic cancer cell lines were higher than those in PSCs and pancreatic cancer tissues (Fig. 2A).

Next, we compared the expression levels of RTKs between pancreatic cancer tissues (PT1-3) and noncancerous tissues (NT1-3). Expression levels of EGFR in pancreatic cancer tissues were 2.6- to 4.9-fold higher than those in noncancerous tissues (Fig. 2B). The expression levels of both MET and PDGFRβ in

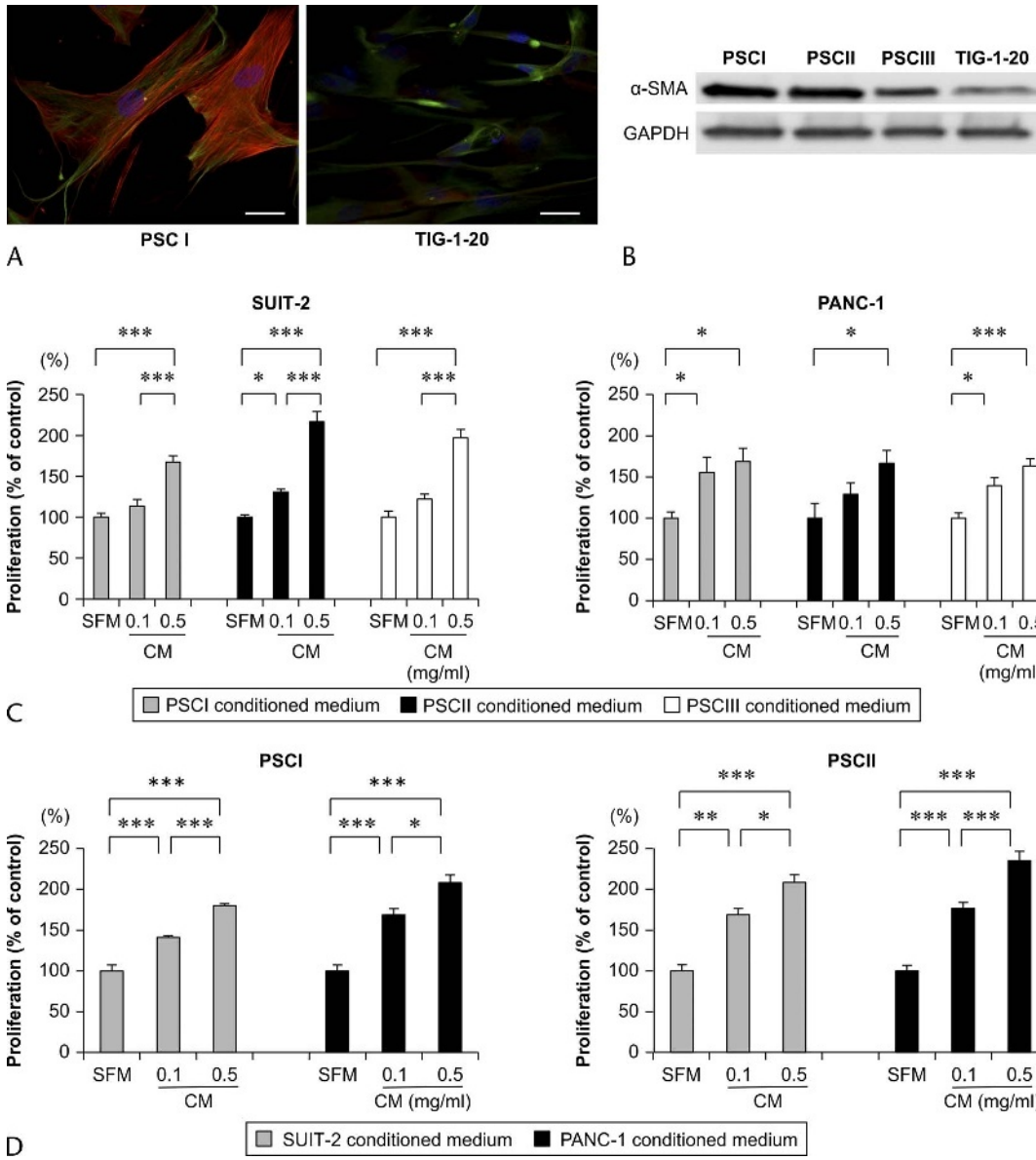

FIGURE 1. The α-SMA expression in established PSCs and effects of the PCC-PSC interaction on cell proliferation. A, Immunofluorescence staining of α-SMA and vimentin in established PSCs. Red, α-SMA; green, vimentin; and blue, DAPI. Scale bar, 100 μm. B, Protein lysates from PSCI, PSCII, or PSCIII and TIG-1-20 were analyzed for α-SMA expression by Western blotting. The GAPDH served as a loading control. C, Effects of PSCI, PSCII, and PSCIII–CM on PCC (SUIT-2 and PANC-1 cells) proliferation. Proliferation assays were performed 48 hours after adding PSC-CM. D, Effects of PCC-CM (SUIT-2 and PANC-1 cells) on PSC (PSCI and PSCII) proliferation. Proliferation assays were performed 48 hours after adding PCC-CM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus the SFM control.

noncancerous tissues were all under the detection limit, whereas both proteins were detected in pancreatic cancer tissues (Fig. 2B).

MK2461 Inhibited the Effects of PSC-CM on PCC Proliferation by Suppressing the Activation of MET and its Downstream Signaling

The RTK profiling experiments revealed that MET was expressed in both PCCs and PSCs, but not in noncancerous tissue, suggesting that inhibition of MET could effectively inhibit the interaction between PSCs and PCCs. In both SUIT-2 and PANC-1 cells, significant growth inhibition was observed at 1 μM MK2461 (Fig. 3A). In addition, 0.1 μM MK2461 significantly inhibited SUIT-2 cell proliferation induced by PSC-CM. To assess whether MK2461 actually inhibited the effects of PSC-CM, we compared the effects of MK2461 in cells treated with or without CM. In SUIT-2 and PANC-1 cells, inhibition of cell growth by 1 or 3 μM MK2461, respectively, was significantly higher in cells cultured with PSC-CM than in cells cultured in SFM (Fig. 3B). Compared with cell cultured in medium containing 10% FBS, inhibition of cell growth was also significantly higher in cells treated with PSC-CM (Fig. 3C). Furthermore, we evaluated the effects of MK2461 by Western blotting (Fig. 3D). In both SUIT-2 and PANC-1 cells, MK2461 inhibited the phosphorylation of MET, AKT, and ERK1/2 induced by PSC-CM.

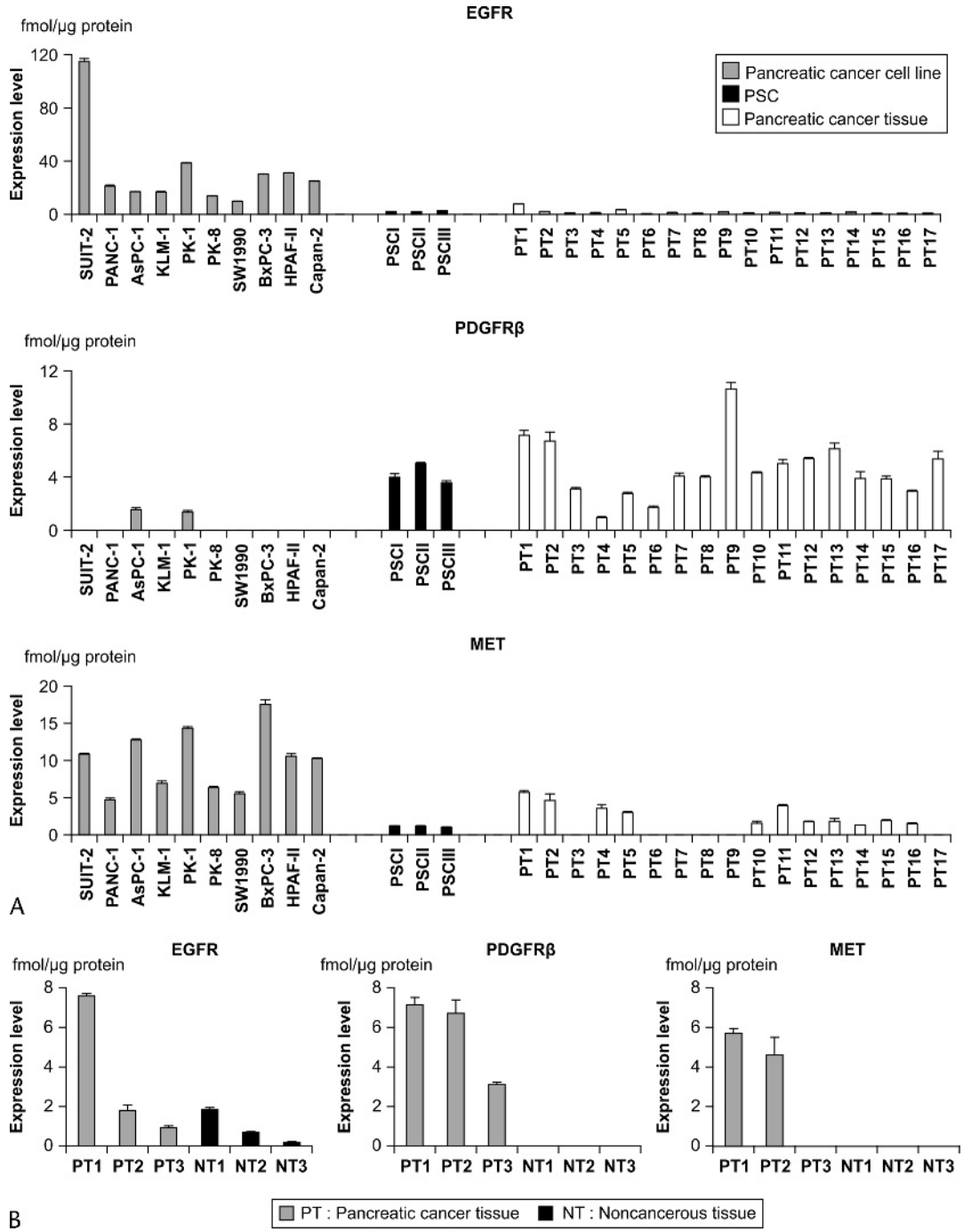

FIGURE 2. Expression levels of RTKs in plasma membrane fractions. A, Comparison of RTK (EGFR, PDGFRβ, and MET) expression levels between pancreatic cancer cell lines, PSCs, and pancreatic cancer tissues. B, Comparison of RTK (EGFR, PDGFRβ, and MET) expression levels between pancreatic cancer tissues and normal pancreatic tissues. Pancreatic cancer tissue and normal pancreatic tissue were separated from the samples given by patients 1 to 3 and used for the assay. Each bar represents the protein expression level in the plasma membrane fraction (mean ± SEM).

MK2461 Inhibited the Effects of PCC-CM on PSC Proliferation and Suppressed Activation of PDGFRβ, MET, and Downstream Signaling

Because PDGFRβ and MET were expressed in PSCs and PCC-CM–induced PSC proliferation, we next evaluated the effects of MK2461 on PSC proliferation. In both PSCIs and PSCIIs, MK2461 significantly suppressed the proliferation induced by PCC-CM when used at concentrations of 0.1 and 1 μM, respectively (Fig. 4A). Moreover, the growth of PSCIs and PSCIIs was significantly inhibited in the presence of PCC-CM compared with SFM after treatment with 0.1 or 1 μM MK2461, respectively (-Fig. 4B). Compared with cells cultured in medium containing 10% FBS, inhibition of cell growth was also significantly higher

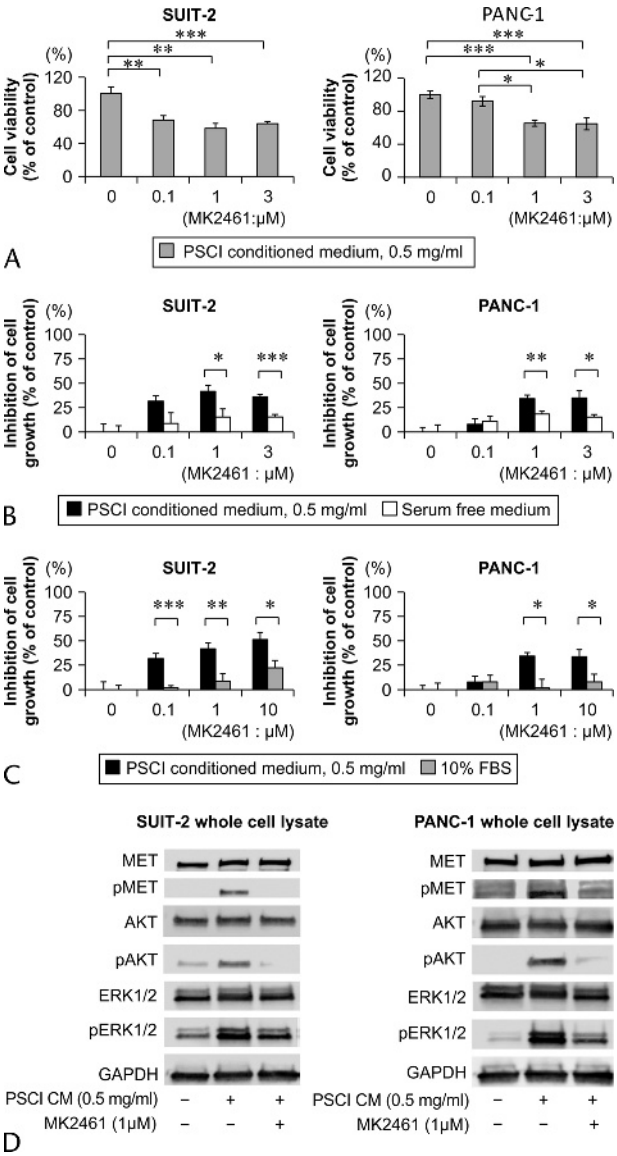

FIGURE 3. MK2461 inhibited the effects of PSC-CM on PCC proliferation and suppressed the activation of MET and downstream signaling. A, Proliferation assays were performed in SUIT-2 and PANC-1 cells 48 hours after adding PSC-CM (0.5 mg/mL) and MK2461 (0, 0.1, 1, and 3 μM). B, Comparison of PCC (SUIT-2 and PANC-1 cells) growth inhibition by MK2461 in cells treated with PSC-CM (0.5 mg/mL) or SFM. Cell growth inhibition was calculated as the percentage of absorbance differences between 0 μM and MK2461 (0.1, 1, and 3 μM). Proliferation assays were performed 48 hours after adding PSC-CM (0.5 mg/mL) and MK2461 (0, 0.1, 1, and 3 μM). C, Comparison of PCC (SUIT-2 and PANC-1 cells) growth inhibition by MK2461 in cells treated with PSC-CM (0.5 mg/mL) or 10% FBS. D, Western blotting of signaling intermediates. The PCCs (SUIT-2 and PANC-1 cells) were incubated with MK2461 (1 μM) for 4 hours, stimulated with PSC-CM (0.5 mg/mL) for 10 minutes, then lysed. The GAPDH served as the loading control. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus the untreated control.

in cells treated with PCC-CM (data not shown). In addition, phosphorylation of PDGFRβ, MET, ERK1/2, and AKT in PSCs stimulated by PCC-CM was inhibited by MK2461 (Fig. 4C).

MK2461 Inhibited the Effects of PSC-CM on PCC Invasion and Migration

Overexpression or hyperactivation of MET has been associated with increased invasiveness in several cancers.[31] Because PSC-CM activated MET (Fig. 3D), we next sought to determine whether blocking MET activation affected PCC invasion and migration. Indeed, we observed that exposure of SUIT-2 and PANC-1 cells to PSC-CM significantly enhanced the invasion and migration of these cells compared with culture under serum-free conditions in a concentration-dependent manner (Fig. 5A, B). Furthermore, inhibition of MET with increasing concentrations of MK2461 in both SUIT-2 and PANC-1 cells decreased the number of invading (Fig. 5C) or migrating (Fig. 5D) cells in a concentration-dependent manner.

MK2461 Regulated Tumor Progression In Vivo

Our results indicated that MK2461 significantly affected pancreatic cancer progression by disrupting the PCC-PSC interaction. To further characterize this process, we evaluated whether MK2461 inhibited tumor growth in a mouse xenograft model. The PSCs alone are not tumorigenic[21]; therefore, we coinjected SUIT-2 cells and PSCs into the right flanks of mice and SUIT-2 cells alone into the left flanks of mice, without using a PSC-alone control. One week after inoculation, mice were treated with MK2461 (20 mg/kg twice daily) or vehicle control for 20 days. No decreases in body weights of the mice were observed, and no treatment-related deaths were observed. In the coinjection model, MK2461 significantly inhibited tumor progression compared with vehicle, whereas in the SUIT-2–alone injection model, we did not observe any differences in tumor volumes between the MK2461-treated group and the vehicle-treated group (Fig. 6A). Consistent with this, we observed significant reductions in final tumor weights after MK2461 treatment compared with vehicle treatment for the coinjection model (vehicle, 431 ± 33.2 mg vs MK2461, 320 ± 26.3 mg; $P = 0.022$) but not for tumors containing SUIT-2 cells alone (vehicle, 259 ± 23.9 mg vs MK2461, 247 ± 12.6 mg; $P = 0.67$). Therefore, MK2461 inhibited tumor growth only in the presence of PSCs.

Because MK2461 had antitumor effects in both SUIT-2 cells and PSCs in our in vitro study (Figs. 3A and 4A), we next examined whether MK2461 inhibited the proliferation of both SUIT-2 cells and PSCs using the markers Ki-67 and cytokeratin 19 (Fig. 6B, C). In the coinjection model, the percentage of Ki-67–positive SUIT-2 cells was significantly higher in the vehicle group than in the MK2461 group, although no significant difference was observed between the vehicle group and MK2461 group in tumors arising from injection of SUIT-2 cells alone (Fig. 6D). In addition, the percentage of Ki-67–positive PSCs was significantly higher in the vehicle group than in the MK2461 group (Fig. 6E). Similarly, the number of α-SMA–positive cells per field was significantly higher in the vehicle group than in the MK2461 group (Fig. 6F).

DISCUSSION

For the development of potential new therapeutic options for the treatment of pancreatic cancer, it is important to consider the tumor microenvironment. Therefore, in this study, we assessed the interactions between PCCs and PSCs, which have been implicated in the progression to the malignant phenotype.[20,21,32] We showed that PSC-CM, which include secretions from the cells, promoted the proliferation of PCCs; conversely, CM from PCCs stimulated the proliferation of PSCs. Furthermore, we found that paracrine signaling was more effective at promoting proliferation than autocrine signaling, suggesting that components secreted from PSCs are essential to the progression of pancreatic cancer. Importantly, we also

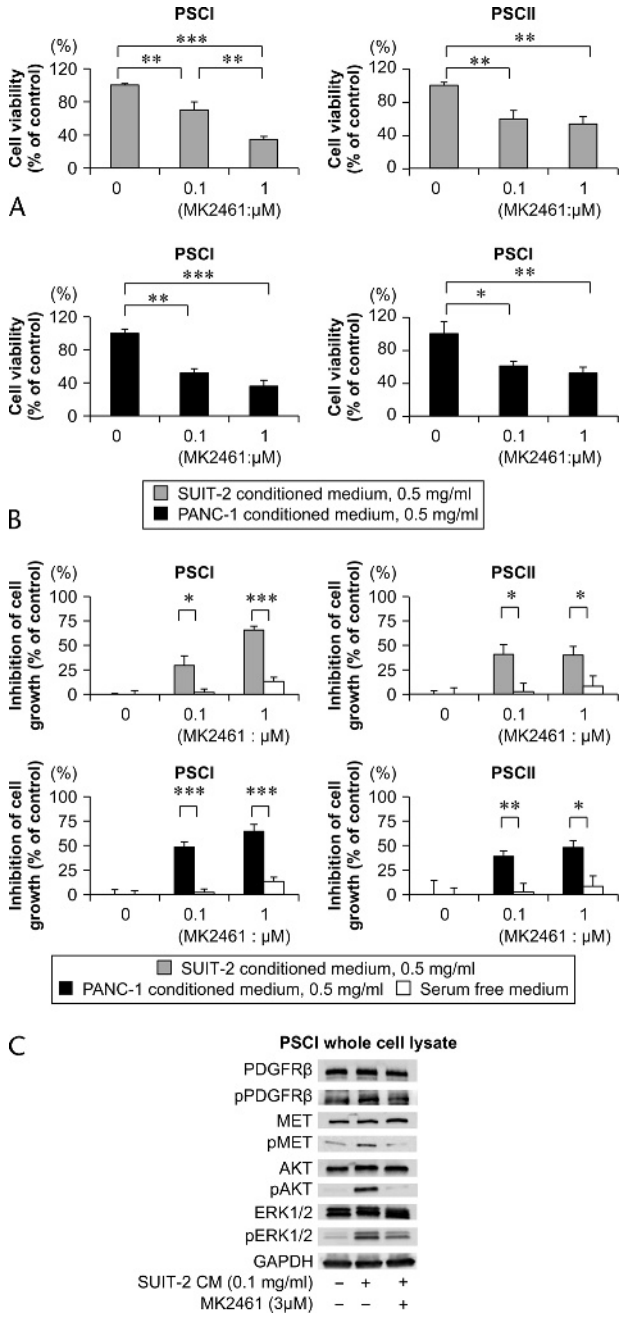

FIGURE 4. MK2461 inhibited the effects of PCC-CM on PSC proliferation and suppressed the activation of PDGFRβ, MET, and downstream signaling events. A, Proliferation assays were performed in SUIT-2 and PANC-1 cells 48 hours after adding PCC-CM (0.5 mg/mL) and MK2461 (0, 0.1, and 1 μM). B, Comparison of PSC (PSCI and PSCII) growth inhibition by MK2461 in SUIT-2 and PANC-2 cells treated for 48 hours with CM (0.5 mg/mL) or SFM. Cell growth inhibition was calculated as a percentage of absorbance differences between untreated cells and cells treated with MK2461 (0.1 and 1 μM). C, Western blotting of signaling intermediates. The PSCs were incubated with MK2461 (3 μM) for 4 hours, stimulated with SUIT-2 cell–CM (0.1 mg/mL) for 10 minutes, then lysed. The GAPDH served as the loading control. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus the untreated control.

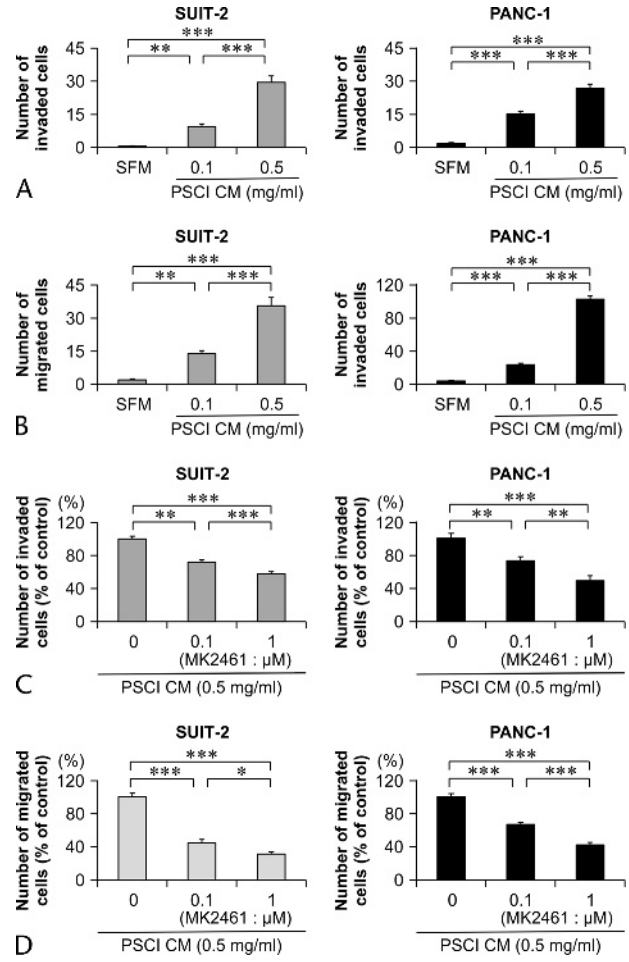

FIGURE 5. The PSC-CM promoted PCC invasion and migration in an MK2461-dependent manner. A, Invasion assays were performed in SUIT-2 and PANC-1 cells 48 hours after adding PSC-CM. $**P < 0.01$ and $***P < 0.001$ versus the SFM control. Scale bar, 50 μm. B, Migration assays were performed in SUIT-2 and PANC-1 cells 24 hours after adding PSC-CM (0.1 or 0.5 mg/mL). $**P < 0.01$ and $***P < 0.001$ versus the SFM control. Scale bar, 50 μm. C, Invasion assays were performed in SUIT-2 and PANC-1 cells 48 hours after adding PSC-CM (0.5 mg/mL) and MK2461 (0, 0.1, and 1 μM). $**P < 0.01$ and $***P < 0.001$ versus the untreated control. Scale bar, 50 μm. D, Migration assays were performed in SUIT-2 and PANC-1 cells 24 hours after adding PSC-CM (0.5 mg/mL) and MK2461 (0, 0.1, and 1 μM). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus the untreated control. Scale bar, 50 μm.

found that this effect was controlled by MK2461, a multitargeted kinase inhibitor, suggesting that MK2461 may represent a novel therapeutic agent for the treatment of pancreatic cancer.

Previous studies have shown that PSCs exhibit increased secretion of growth factors, such as PDGF, HGF, connective tissue growth factor, and FGF, as well as ECM components, including collagen type I and fibronectin, through the PCC-PSC interaction, resulting in enhancement of PCC proliferation.[18] On the other hand, PCCs secrete growth factors such as PDGF, FGF, and TGFβ1 to promote PSC proliferation and to stimulate the secretion of additional growth factors and ECM components from PSCs.[10,20] Therefore, we hypothesized that inhibition of RTK signaling could suppress the progression of pancreatic cancer.

Using QTAP by liquid chromatography tandem mass spectrometry, we revealed the expression levels of 15 RTKs among

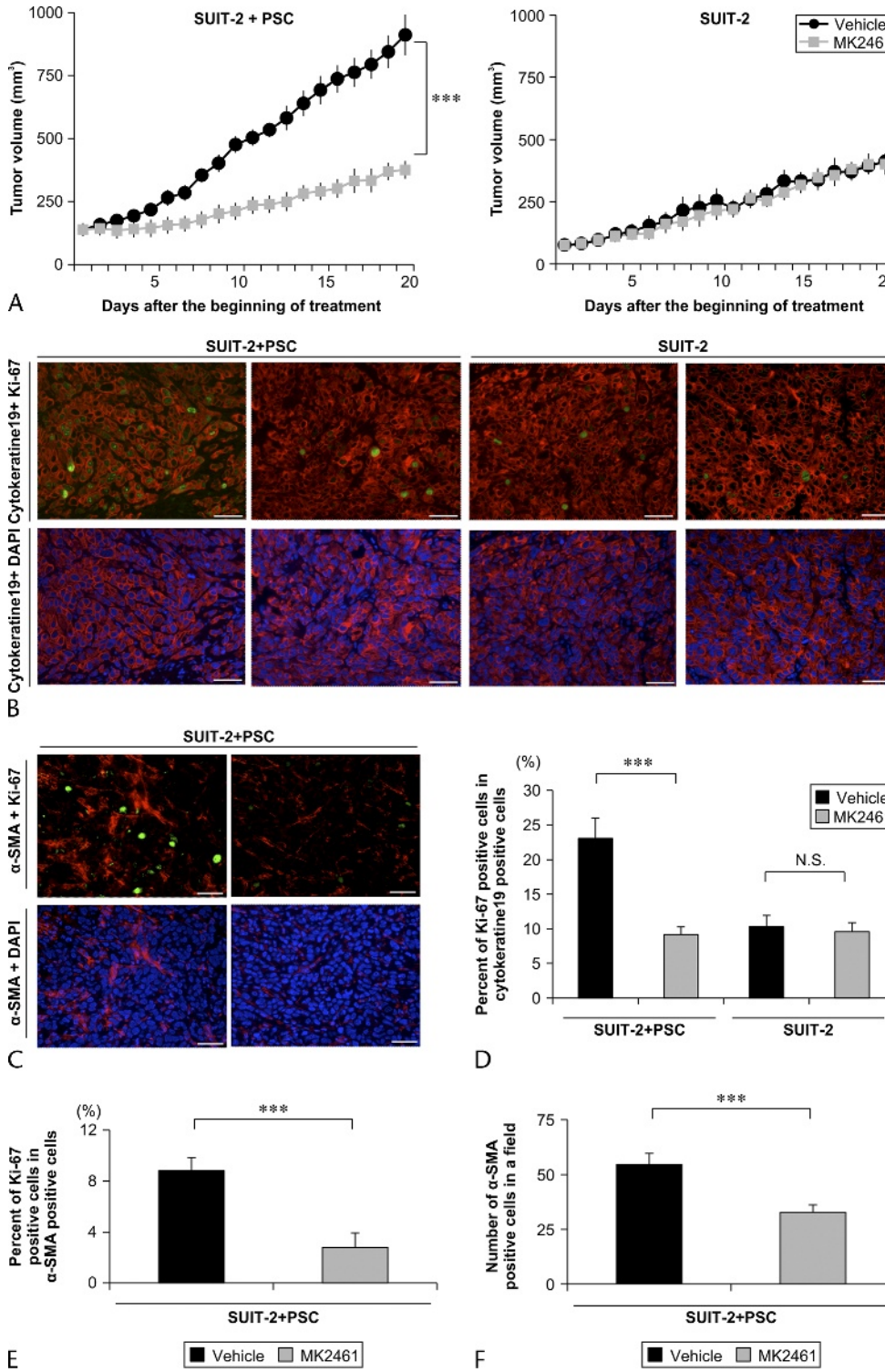

FIGURE 6. MK2461 regulated tumor progression in vivo. The effects of MK2461 on tumor progression were assessed using a xenograft model. SUIT-2 cells and PSCs were subcutaneously coinjected into the right flanks of mice, whereas SUIT-2 cells were injected alone into the left flanks of mice. One week later, mice were administered vehicle or MK2461 (20 mg/kg) twice daily for 20 days by oral gavage. A, Coinjection model. SUIT-2 injection alone. B, Immunofluorescence staining for cytokeratin 19 + Ki-67 (top panel) and cytokeratin 19 + DAPI (bottom panel). Red, cytokeratin 19; green, Ki-67; and blue, DAPI. Scale bar, 50 μm. C, Immunofluorescence staining for α-SMA + Ki-67 (top panel) and α-SMA + DAPI (bottom panel). Red, α-SMA; green, Ki-67; and blue, DAPI. Scale bar, 50 μm. D, The percentage of Ki-67–positive cells in cytokeratin 19–positive cells. The stained cells were counted in 3 random fields. E, The percentage of Ki-67–positive cells in α-SMA–positive cells. The stained cells were counted in 3 random fields. F, The number of α-SMA–positive cells in each field. The stained cells were counted in 3 random fields. ***$P < 0.001$.

10 pancreatic cancer cell lines, 3 primary cultured PSCs, and 17 human pancreatic cancer tissues. We used purified plasma membrane fractions; therefore, the results accurately reflected the RTK expression levels presented on the cellular membrane. Importantly, our data demonstrated that EGFR, MET, and PDGFRβ were expressed in pancreatic cancer cell lines, PSCs, and/or pancreatic tissues. Thus, our data supported that these RTKs likely played an important role in the PCC-PSC interaction.

The expression levels of RTKs in pancreatic cancer tissues were almost equivalent to those of PSCs, consistent with the observation that 80% of pancreatic tissue consists of PSCs. Previous studies have shown that the expression of PDGFRβ in PSCs correlates with the prognosis of patients with pancreatic cancer.[33,34] In our study, all of the established PSCs expressed PDGFRβ. Moreover, previous studies have shown that PCCs express MET, and that MET expression is correlated with prognosis.[35–37] The HGF secretion from PSCs has been shown to accelerate the progression of pancreatic cancer by increasing cell proliferation, invasion, and migration.[38–40] Our RTK profiling showed that MET was expressed not only in PCCs but also in PSCs. Interestingly, despite the high expression of MET in PCC, SUIT-2 cells alone are only weakly tumorigenic and essentially insensitive to MET inhibition in the absence of PSCs or PSC-CM. This suggests that MET is inactive in PCCs and requires PSCs for activation. Hence, the HGF/MET pathway is thought to be important in mediating the interaction between PCCs and PSCs.

The PSCs are considered a potential target for pancreatic cancer therapy, and PSC proliferation induced by components secreted from PCCs is significantly inhibited by PDGF-neutralizing antibodies.[20] Therefore, targeting MET and PDGFRβ should be more effective for the inhibition of PSC proliferation. Previous studies have also shown that inhibition of HGF/MET signaling contributes to the regulation of PCC progression in vitro and in vivo.[41] Therefore, in this study, we focused on the effects of MK2461, a multikinase inhibitor targeting MET and PDGFRβ, on the interaction between PSCs and PCCs. We did not use EGFR inhibitors because, although EGFR was detected in both pancreatic cancer cell lines and PSCs, EGF is not thought to be secreted from PSCs, and the role of EGF/EGFR signaling is supposed to be small in the PSC-PCC interaction. In our pilot study, gefitinib, an EGFR inhibitor, did not suppress SUIT-2 or PSC proliferation after treatment with CM. In addition, in a clinical trial examining the efficacy of an EGFR inhibitor in the treatment of pancreatic cancer, no clinically significant effects were observed.[42]

In our study, we found that MK2461 significantly inhibited PCC proliferation induced by PSC-CM. MK2461 also significantly inhibited PCC proliferation in the presence of PSC-CM compared with SFM and medium containing 10% FBS. These data indicated that MK2461 required the addition of PSC-CM to exert its inhibitory functions. Moreover, MET and downstream signaling components, such as ERK1/2 and AKT, were activated by PSC-CM, and the phosphorylation of these enzymes was suppressed by MK2461 treatment. Consistent with this, MK2461 significantly inhibited PSC proliferation in the presence of PCC-CM compared with SFM. The MET, PDGFRβ, ERK1/2, and AKT were activated by PCC-CM but inactivated by treatment with MK2461. Because MET was activated by PCC-CM, these data suggested that HGF was secreted from both PCCs and PSCs and contributed to autocrine signaling.

An important observation in our model was that MK2461 significantly inhibited both PCC and PSC proliferation, disrupting the PCC-PSC interaction. Previous studies have focused on the inhibition of cell growth for either PCCs or PSCs. In the studies focusing on the tumor microenvironment, inhibiting PSC proliferation and suppressing the secretion of growth factors and other components from PSCs have been shown to result in inhibition of pancreatic cancer progression or enhancement of chemotherapeutic effects.[15,16,43] In our study, MK2461 was found to contribute to the reduction in PSC secretion by inhibiting PSC proliferation. Furthermore, MK2461 also inhibited the activation of MET, which was highly expressed in PCCs and was activated by PSC-CM. Therefore, MK2461 was thought to exert substantial inhibitory effects on pancreatic cancer progression.

Both PDGF and HGF have been reported to promote cancer cell invasion and migration,[39,44] and previous studies have indicated that PSC-CM promotes PCC invasion and migration,[18,21,32] which was confirmed by the results of our study. We also revealed that MK2461 inhibited the effects of PSC-CM on PCC invasion and migration. Therefore, future studies should assess whether MK2461 reduces the incidence of metastasis using orthotopic xenograft models.

In our in vivo study, we revealed that MK2461 significantly inhibited tumor growth in coinjection models, and that the effects of MK2461 were not observed in tumors derived from SUIT-2 cells alone. Surprisingly, tumor volumes in the MK2461 group in the coinjection model were equivalent to those in the vehicle group in the tumors derived from injection of SUIT-2 cells alone; therefore, these findings suggested that MK2461 strongly inhibited tumor growth by disrupting the PCC-PSC interaction. Targeting HGF/MET signaling with a monovalent monoclonal antibody against MET was reported to inhibit HGF paracrine-driven pancreatic tumor growth in a xenograft model.[41] However, although SUIT-2 cells secreted HGF, MK2461 did not suppress tumor progression in our mouse model in which SUIT-2 cells were injected alone. These data were consistent with our results showing that MK2461 did not significantly suppress the proliferation of SUIT-2 and PANC-1 cells treated with 10% FBS compared with PSC-CM in an in vitro study. In addition, these data also suggested that the paracrine pathway was essential to the growth of PCCs, consistent with the results of our in vitro study.

Immunofluorescence staining for Ki-67 showed that MK2461 inhibited both PCC and PSC proliferation in coinjection models but not in tumors derived from injection of SUIT-2 cells alone. These results confirmed the reduction in tumor volume in the coinjection model. In addition, our results suggested that MK2461 inhibited PSC proliferation more strongly than PCC proliferation. Moreover, the number of α-SMA–positive cells in the MK2461 group was less than 30% that of the vehicle group, whereas the number of cytokeratin 19–positive cells was equivalent between the 2 groups (data not shown). This indicated that the area occupied by PSCs in tumors was reduced, consistent with the results of immunofluorescence staining for Ki-67.

Clinically, our data may have implications in the field of personalized medicine. Indeed, we showed that MET exhibited differential expression in pancreatic cancer tissues, suggesting that MK2461 may be more effective in some patients (ie, those expressing high levels of MET) than in others. These conjectures will need to be explored further in additional studies.

In conclusion, the profiling of 15 RTKs showed that PDGFRβ and MET were highly expressed in PCCs and PSCs. Moreover, MK2461 treatment effectively inhibited tumor progression in pancreatic cancer by disrupting the PCC-PSC interaction in vitro and in vivo. Therefore, MK2461 may represent a novel chemotherapeutic agent for the treatment of pancreatic cancer, particularly in patients with high tumoral expression of MET.

REFERENCES

1. Hidalgo M. Pancreatic cancer. *N Engl J Med*. 2010;362:1605–1617.
2. Li D, Xie K, Wolff R, et al. Pancreatic cancer. *Lancet*. 2004;363:1049–1057.

3. Erkan M, Reiser-Erkan C, Michalski CW, et al. Tumor microenvironment and progression of pancreatic cancer. *Exp Oncol*. 2010;32:128–131.
4. Masamune A, Watanabe T, Kikuta K, et al. Roles of pancreatic stellate cells in pancreatic inflammation and fibrosis. *Clin Gastroenterol Hepatol*. 2009;7(11 Suppl):S48–S54.
5. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature*. 2001;411:375–379.
6. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006;6:392–401.
7. Mueller MM, Fusenig NE. Friends or foes—bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer*. 2004;4:839–849.
8. Fidler IJ. The organ microenvironment and cancer metastasis. *Differentiation*. 2002;70:498–505.
9. Apte MV, Wilson JS, Lugea A, et al. A starring role for stellate cells in the pancreatic cancer microenvironment. *Gastroenterology*. 2013;144:1210–1219.
10. Mahadevan D, Von Hoff DD. Tumor-stroma interactions in pancreatic ductal adenocarcinoma. *Mol Cancer Ther*. 2007;6:1186–1197.
11. Erkan M, Adler G, Apte MV, et al. StellaTUM: current consensus and discussion on pancreatic stellate cell research. *Gut*. 2012;61:172–178.
12. Apte MV, Park S, Phillips PA, et al. Desmoplastic reaction in pancreatic cancer: role of pancreatic stellate cells. *Pancreas*. 2004;29:179–187.
13. Erkan M, Kleeff J, Gorbachevski A, et al. Periostin creates a tumor-supportive microenvironment in the pancreas by sustaining fibrogenic stellate cell activity. *Gastroenterology*. 2007;132:1447–1464.
14. Pietras K, Ostman A, Sjöquist M, et al. Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. *Cancer Res*. 2001;61:2929–2934.
15. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 2009;324:1457–1461.
16. Jacobetz MA, Chan DS, Neesse A, et al. Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer. *Gut*. 2013;62:112–120.
17. Mantoni TS, Lunardi S, Al-Assar O, et al. Pancreatic stellate cells radioprotect pancreatic cancer cells through β1-integrin signaling. *Cancer Res*. 2011;71:3453–3458.
18. Kozono S, Ohuchida K, Eguchi D, et al. Pirfenidone inhibits pancreatic cancer desmoplasia by regulating stellate cells. *Cancer Res*. 2013;73:2345–2356.
19. Kikuta K, Masamune A, Watanabe T, et al. Pancreatic stellate cells promote epithelial-mesenchymal transition in pancreatic cancer cells. *Biochem Biophys Res Commun*. 2010;403:380–384.
20. Bachem MG, Schünemann M, Ramadani M, et al. Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology*. 2005;128:907–921.
21. Hwang RF, Moore T, Arumugam T, et al. Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer Res*. 2008;68:918–926.
22. Smith RA, Tang J, Tudur-Smith C, et al. Meta-analysis of immunohistochemical prognostic markers in resected pancreatic cancer. *Br J Cancer*. 2011;104:1440–1451.
23. Kurokawa Y, Matsuura N, Kawabata R, et al. Prognostic impact of major receptor tyrosine kinase expression in gastric cancer. *Ann Surg Oncol*. 2014;21(Suppl 4):S584–S590.
24. Yamauchi M, Yamaguchi R, Nakata A, et al. Epidermal growth factor receptor tyrosine kinase defines critical prognostic genes of stage I lung adenocarcinoma. *PLoS One*. 2012;7:e43923.
25. Pan BS, Chan GK, Chenard M, et al. MK-2461, a novel multitargeted kinase inhibitor, preferentially inhibits the activated c-Met receptor. *Cancer Res*. 2010;70:1524–1533.
26. Ikenaga N, Ohuchida K, Mizumoto K, et al. CD10+ pancreatic stellate cells enhance the progression of pancreatic cancer. *Gastroenterology*. 2010;139:1041–1051, 1051.e1-e8.
27. Wehr AY, Furth EE, Sangar V, et al. Analysis of the human pancreatic stellate cell secreted proteome. *Pancreas*. 2011;40:557–566.
28. Obuchi W, Ohtsuki S, Uchida Y, et al. Identification of transporters associated with Etoposide sensitivity of stomach cancer cell lines and methotrexate sensitivity of breast cancer cell lines by quantitative targeted absolute proteomics. *Mol Pharmacol*. 2013;83:490–500.
29. Kamiie J, Ohtsuki S, Iwase R, et al. Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharm Res*. 2008;25:1469–1483.
30. Uchida Y, Tachikawa M, Obuchi W, et al. A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood-brain barrier in ddY, FVB, and C57BL/6J mice. *Fluids Barriers CNS*. 2013;10:21.
31. Boccaccio C, Comoglio PM. Invasive growth: a MET-driven genetic programme for cancer and stem cells. *Nat Rev Cancer*. 2006;6:637–645.
32. Vonlaufen A, Joshi S, Qu C, et al. Pancreatic stellate cells: partners in crime with pancreatic cancer cells. *Cancer Res*. 2008;68:2085–2093.
33. Yuzawa S, Kano MR, Einama T, et al. PDGFRβ expression in tumor stroma of pancreatic adenocarcinoma as a reliable prognostic marker. *Med Oncol*. 2012;29:2824–2830.
34. Haber PS, Keogh GW, Apte MV, et al. Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis. *Am J Pathol*. 1999;155:1087–1095.
35. Ebert M, Yokoyama M, Friess H, et al. Coexpression of the c-met proto-oncogene and hepatocyte growth factor in human pancreatic cancer. *Cancer Res*. 1994;54:5775–5778.
36. Zhu GH, Huang C, Qiu ZJ, et al. Expression and prognostic significance of CD151, c-Met, and integrin alpha3/alpha6 in pancreatic ductal adenocarcinoma. *Dig Dis Sci*. 2011;56:1090–1098.
37. Ide T, Kitajima Y, Miyoshi A, et al. The hypoxic environment in tumor-stromal cells accelerates pancreatic cancer progression via the activation of paracrine hepatocyte growth factor/c-Met signaling. *Ann Surg Oncol*. 2007;14:2600–2607.
38. Nakamura T, Nishizawa T, Hagiya M, et al. Molecular cloning and expression of human hepatocyte growth factor. *Nature*. 1989;342:440–443.
39. Peruzzi B, Bottaro DP. Targeting the c-Met signaling pathway in cancer. *Clin Cancer Res*. 2006;12:3657–3660.
40. Cheng N, Chytil A, Shyr Y, et al. Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion. *Mol Cancer Res*. 2008;6:1521–1533.
41. Jin H, Yang R, Zheng Z, et al. MetMAb, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. *Cancer Res*. 2008;68:4360–4368.
42. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007;25:1960–1966.
43. Hwang RF, Moore TT, Hattersley MM, et al. Inhibition of the hedgehog pathway targets the tumor-associated stroma in pancreatic cancer. *Mol Cancer Res*. 2012;10:1147–1157.
44. Wang Z, Ahmad A, Li Y, et al. Emerging roles of PDGF-D signaling pathway in tumor development and progression. *Biochim Biophys Acta*. 2010;1806:122–130.