The RON Receptor Tyrosine Kinase Mediates Oncogenic Phenotypes in Pancreatic Cancer Cells and Is Increasingly Expressed during Pancreatic Cancer Progression

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Abstract

Pancreatic cancer is an aggressive disease characterized by rapid growth and early metastasis. The recepteur d'origine nantais (RON) receptor tyrosine kinase is overexpressed and/or constitutively active in several epithelial cancers, but its role in pancreatic cancer is unknown. In this study, we have characterized RON expression in both murine and human pancreatic cancer. Immunoblotting indicates that RON is expressed in pancreatic intraepithelial neoplasia (PanIN), primary, and metastatic cell lines both in the human and mouse. Immunostaining revealed that 93% of high-grade PanIN, 79% of primary, and 83% of metastatic lesions from human pancreatic tissue samples expressed RON, with minimal expression in normal ducts and low-grade PanIN (6% and 18%, respectively). Moreover, we show a dose-dependent effect of hepatocyte growth factor-like protein (HGFL), the RON-specific ligand, on pancreatic cancer cell migration and invasion, which was reversed by RON inhibition. Although stimulation with HGFL had no effect on proliferation, concurrent RON receptor blockade and gemcitabine treatment increased apoptosis of RON-expressing pancreatic cancer cells versus gemcitabine treatment alone. Finally, HGFL stimulation of pancreatic cancer cells resulted in increased expression of phospho-mitogen-activated protein kinase and phospho-Akt. Taken together, these findings suggest that RON receptor signaling may contribute to pancreatic carcinogenesis, and that further investigation is warranted to assess the potential of RON-directed therapies in this deadly disease. [Cancer Res 2007;67(13):6075–82]

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the United States, accounting for >32,000 deaths in 2006. Median survival for pancreatic cancer patients remains <1 year, and only 4% of patients survive 5 years following diagnosis (1). This is due, in large part, to the fact that pancreatic cancer generally metastasizes long before it can be clinically detected. Although some of the key genetic mutations present within pancreatic cancers have been identified, our understanding of signaling events that mediate progression of pancreatic cancer precursors to invasive, metastatic tumors is extremely limited (2).

Receptor tyrosine kinases (RTK) mediate numerous cellular processes known to be critical to cancer cell proliferation, invasiveness, apoptotic resistance, and metastasis (3–5). The most successful biological targeted therapies developed to date have been directed at the inhibition of RTK activity. A recent randomized trial showed improved overall survival for patients with advanced pancreatic cancer treated with an oral RTK inhibitor targeted to the epidermal growth factor receptor (EGFR) in combination with gemcitabine chemotherapy (6). The recepteur d'origine nantais (RON) receptor is an RTK with significant homology to c-Met, a potent proto-oncogene (7). The RON receptor is a heterodimeric transmembrane glycoprotein receptor comprised of disulfide-linked chains of 150 and 35 kDa (7–9). The ligand for RON is the hepatocyte growth factor-like protein (HGFL) also known as macrophage-stimulating protein (8, 10). HGFL is predominantly produced in hepatocytes and is secreted into the bloodstream in a biologically inactive form, pro-HGFL (11). Active HGFL is produced by proteolytic cleavage at extravascular sites and by proteases on the cell surface of macrophages (12, 13). Upon binding of HGFL, RON is activated via autophosphorylation within its kinase catalytic domain, resulting in a pleiotropic array of effects, including proliferation, tubular morphogenesis, angiogenesis, cellular motility, invasiveness, and resistance to anoikis (3–5). RON has been implicated in cancers of the breast, colon, and ovaries because both splice variants and receptor overexpression have been identified in these tumors (4, 14–17). Transgenic mouse models overexpressing RON in the lung result in the development of frank adenocarcinoma (18, 19). Expression of RON in the mouse mammary gland results not only in carcinoma but in the rapid development of metastases (20). Currently, the role of RON in pancreatic cancer is unknown.

In this report, we have characterized the expression of RON receptor in both human and murine pancreatic cancer cell lines, in a murine model of pancreatic cancer (21), and in human pancreatic cancer precursors, primary tumors, and metastases. These studies have revealed that RON is rarely expressed in normal pancreatic ducts or early pancreatic intraepithelial neoplasia (PanIN), the precursor lesions to invasive pancreatic cancer. In contrast, nearly all later stage PanINs, primary, and metastatic pancreatic cancers express abundant RON protein. We also show that in pancreatic cancer cells, activation of RON by HGFL induces several known oncogenic signaling pathways and enhances migration and invasion, whereas RON receptor inhibition sensitizes pancreatic cancer cells to gemcitabine-induced apoptosis. These data strongly

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suggest that RON receptor signaling contributes to pancreatic carcinogenesis, and that targeting RON may be an effective therapeutic strategy for the treatment of this deadly disease.

Materials and Methods

Cell line and maintenance. The human pancreatic cancer cell lines ASPC-1, BxPC-3, Capan-1, HPAF II, Hs766T, MiaPaCa-2, and Panc-1 were obtained from the American Type Culture Collection. The mouse pancreatic cancer cell lines 4964PDA, 4964LM, 5143PDA, 5143LM, and the mouse PanIN cell lines were derived from the PdxCre/LSL-KRAS^{G12D} and PdxCre/LSL-KRAS^{G12D} /p53$^{-/-}$ mouse models of pancreatic cancer as previously described (21, 22). ASPC-1 and BxPC-3 were maintained in RPMI. All mouse cell lines, as well as Hs766T, MiaPaCa-2, and Panc-1, were maintained in DMEM. Capan-1 was maintained in Iscove's MEM and HPAF II in MEM. All media were supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin unless otherwise noted. All cells were grown in a humidified incubator at 37°C and 5% CO_2.

Cell lysates and immunoblot analysis. Cells were lysed with 2× SDS lysis buffer [125 mmol/L Tris, 20% glycerol, 4% SDS (pH, 6.8)], and protein concentrations were quantitated with a Micro BCA Protein Assay Kit (Pierce). Cell lysates prepared for the analysis of phosphorylated proteins were done with the phosphatase inhibitors, sodium orthovanadate (1 mmol/L), and sodium fluoride (50 mmol/L) in the lysis buffer. Cell lysates were analyzed on 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore) or BioTrace NT nitrocellulose membrane (Pall Corp.) for analysis of phosphorylated proteins at 4°C overnight. At this time, the membrane was blocked in blocking buffer (1× TBS + 0.05% Tween + 5% milk) for at least 1 h. The membrane was then probed with primary antibody [antibodies against phosphorylated proteins were diluted in 1× TBS + 0.05% Tween + 5% bovine serum albumin (BSA)]. Blots were visualized using horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnologies) with Supersignal WestPico Chemiluminescence Substrate (Pierce). Immunoblot membranes were probed with antibodies to either the extracellular domain of RON (β-chain), rabbit anti-RON C-20 (1:500; Santa Cruz Biotechnologies), mouse anti–phospho-Akt (1:500), rabbit anti-Akt (1:1,000), rabbit anti–phospho-Erk (1:1,000), rabbit anti-Erk (1:1,000; Cell Signaling), or rabbit anti-IκBα/β (1:250; Santa Cruz Biotechnologies). Detection of α-tubulin (1:200; Santa Cruz Biotechnologies) or β-actin (1:10,000; Sigma) served as loading controls. When appropriate, membranes were stripped with Restore Western blot Stripping Buffer (Pierce) according to the manufacturer's specifications and reprobed with primary antibody.

Immunohistochemistry. Human pancreatic tissue samples were obtained in accordance with a University of Cincinnati Institutional Review Board–approved protocol. Mouse pancreatic tissue samples were obtained from PdxCre/LSL-KRAS^{G12D} mice sacrificed at 6 months of age. Staining for RON in human and mouse pancreatic tissue samples was done essentially as previously described (23). Briefly, formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval, and inhibition of endogenous peroxidase activity was accomplished by incubating in a solution of methanol–0.9% hydrogen peroxide. Sections were incubated overnight with an antibody directed against the intracellular domain of the RON receptor (C-20) or phospho-RON (1:50 dilutions, Santa Cruz Biotechnologies). Positive RON staining was detected using 3,3′-diaminobenzidine as the substrate. Slides were counterstained with hematoxylin and permanently mounted using a xylene-based mounting medium. Tissue sections were reviewed by two pathologists (C.F.P. and P.R.) and scored according to a semiquantitative scale (0–4+ for intensity and 0–4+ for % of cells staining, where 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75%). For the purposes of reporting, tumors scored at least 2+ were considered positive for RON staining.

Cell proliferation assay. To determine the effect of RON stimulation on cell proliferation, 5×10^3 cells were plated per well in a 96-well plate in 200 μL of appropriate growth media supplemented with 1% FBS and incubated overnight. The cells were then washed twice with PBS. To determine if HGFL, independent of other serum factors, could result in increased cell proliferation, serum-free media was added with increasing amounts of HGFL (R&D Systems). The cells were incubated under these conditions for various time points (12, 24, and 48 h). Two hours before the designated time point, Alamar Blue (Biosource) was added to a final v/v concentration of 10% and allowed to incubate for 2 h. An automated fluorescent plate reader at an excitation/emission of 530/590 nm was used to measure the proliferating cell population. For the 0 h time point, Alamar Blue was added when the complete media was replaced with serum-free media. Cells stimulated with media + 10% FBS served as a positive control for proliferation, whereas media with Alamar Blue alone served as the blank, which was averaged and subtracted from the fluorescent value obtained for each well. Each experiment was done in triplicate and repeated as three independent experiments.

Cell migration and invasion assays. Cell migration and invasion assays were carried out using Costar 24-well Transwell plates with 8-μm pores (Corning Inc.). Cells were trypsinized and counted with a Vi-Cell automated cell counter (Beckman Coulter). Cells were resuspended in their appropriate growth media supplemented with 1% FBS, and 5×10^4 viable cells were plated per upper well in 200 μL media. As a chemoattractant, HGFL was suspended in the appropriate growth media for each cell line supplemented with 1% FBS at various concentrations in the bottom wells at a volume of 400 μL. Media with 1% FBS and 10% FBS were used as negative and positive controls, respectively. Cells were allowed to migrate to the HGFL for a period of 24 h, except ASPC-1 which was 48 h, at which time the media from the top and bottom wells were removed. Cells that remained in the top well were gently removed from the Transwell membrane with a foam applicator, and the cells that migrated through the membrane to the underside of the Transwell membrane were washed twice with PBS. To objectively quantify the amount of cell migration, cells were stained with the fluorescent dye, Calcein-AM (Molecular Probes). Calcein-AM, suspended in 400 μL PBS at a final concentration of 5 μmol/L, was added to each of the bottom wells and allowed to incubate for 30 min, at which time the plate was read in an automatic plate reader at an excitation/emission of 485/530 nm. The invasion assay was done in the same manner as the migration assay, except before plating of each particular cell line, 8 μg of Matrigel (BD Biosciences) diluted in PBS was plated into each of the top layers, allowed to dry, and was reconstituted with warm serum-free media for 2 h on a tabletop shaker at room temperature. This serum-free media was then carefully removed so as not to disturb the Matrigel, and the assay was done as described for the migration assay. For blocking experiments, cells in the upper wells were preincubated with goat anti-RON blocking antibody (R&D Systems) at various concentrations for 3 h before exposure to HGFL in the bottom wells for chemoattraction. Cells preincubated with 1 μg/mL of normal goat immunoglobulin G (IgG) served as control (Santa Cruz Biotechnologies). Total % cell migration or invasion was determined with the formula [(fluorescence of well of interest) − (fluorescence of baseline 5 μmol/L calcein solution)]/[(fluorescence of 50,000 cells of interest plated overnight in appropriate media with 1% FBS) − (fluorescence of baseline 5 μmol/L calcein solution)]. All assays were done in triplicate as three independent experiments.

Apoptosis assay. BxPC-3 cells were plated in a six-well plate in complete media with or without increasing amounts of an inhibitory mouse monoclonal anti-human RON antibody (R&D Systems). The cells were pretreated in this manner for 15 h at which time gemcitabine (Qventas) was added directly to the wells to achieve a final concentration of 0.1 μmol/L. Controls included cells untreated with gemcitabine and inhibitory anti-RON antibody, as well as gemcitabine-treated cells with normal mouse IgG_1. Cells were treated for 48 h, at which time the total cell population of each well, including cells floating within the media, were collected after trypsinization and gently centrifuged. The media was removed, and cells were resuspended in 1 mL of flow cytometry buffer (1× PBS + 1% BSA) with 1.5 μL/mL of the nucleic acid stain, SYTO16 (Molecular Probes), and incubated for 10 min at 37°C. After washing the cells with flow cytometry buffer, 500 μL of BD CytoFix/CytoPerm solution (BD Biosciences) was added and incubated for 10 min at room temperature. At this time, the cells were washed with 1× BD Perm/Wash buffer (BD Biosciences) and incubated in 100 μL of BD Perm/Wash buffer with 5 μg/mL rabbit anti-active caspase-3 monoclonal antibody for 20 min on ice. The cells were

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again washed with BD Perm/Wash buffer and resuspended in 100 μL BD Perm/Wash buffer with 5 μg/mL Cy5-conjugated goat anti-rabbit IgG AffiniPure F(ab)2 goat–anti-rabbit (Jackson Immuno Research) secondary antibody and incubated for 30 min on ice in the dark. The cells were washed and gently centrifuged a final time and resuspended in 1 mL fresh flow cytometry buffer for analysis using a BD-LSR (Becton Dickinson) flow cytometry machine. The instrument was aligned with Align-Flow 2.5-μm beads (Molecular Probes) for the 488- and 633-nm lasers. Data were collected and analyzed with CellQuest Pro software (Becton Dickinson). Cells were excited with the 488-nm line of an argon-ion laser and a 633-nm helium neon laser using SYTO16 and Cy5 fluorochromes. Dead cells and debris were excluded from analysis by setting the appropriate threshold trigger on forward-angle light scatter. Log fluorescence was collected for SYTO16 using a 530/30 band-pass filter and for caspase 3-Cy5 using a 670 LP (long-pass) filter. In all experiments, 1×10^4 gated events were collected. Each experiment was done in triplicate and repeated as three independent experiments.

Statistical analyses. ANOVA with Dunnett's multiple comparison test was used to compare treatment groups to the untreated control using GraphPad Prism software (GraphPad Software, Inc.). A P value of ≤ 0.05 was considered statistically significant.

Results

RON is expressed in human and murine pancreatic cancer cell lines. The expression of RON during pancreatic carcinogenesis is unknown, and thus, we first sought to evaluate this in both human pancreatic cancer cell lines and in lines derived from a KRAS-driven mouse model of pancreatic cancer. Five of seven human pancreatic cancer cell lines and all five of the murine cell lines, including those derived from PanIN cells, express RON (Fig. 1*A*). Only pro-RON (185 kDa) and the cleaved full-length β-chain (150 kDa) were visualized. Analysis of murine cell lines show that RON expression is present in a cell line derived from murine PanINs, and that this expression is maintained in cell lines derived from both murine primary pancreatic cancers and liver metastases. The human cell lines examined encompass a group with varying genetic backgrounds and degrees of differentiation, demonstrating that RON expression is not restricted to a particular genotype. Of note, the poorly differentiated cell lines MiaPaCa-2 and Panc-1 failed to express RON.

RON is expressed in high-grade PanIN and invasive pancreatic cancer in both human and mouse. To further characterize RON expression in pancreatic carcinogenesis, we examined tissues derived from *Pdx1Cre/LSL-KRAS^{G12D}* mice as well as primary and metastatic human pancreatic cancers for RON expression. These studies corroborated the findings of our immunoblots in Fig. 1*A*, revealing an extremely high incidence of RON expression. Importantly, RON expression is rarely detected in normal human or murine pancreatic ducts or PanIN 1A lesions (murine data not shown). In PanIN 1B through PanIN 3, RON expression was prominent, and this expression is maintained in primary and metastatic pancreatic cancer lesions (Fig. 1*C*). In all instances, RON expression was present at the cell membrane with occasional cytoplasmic staining. In total, 37 of 47 (79%) primary human pancreatic cancers expressed RON, whereas 20 of 24 (83%) human pancreatic cancer metastases expressed RON. A summary of the incidence of RON expression in human PanIN through metastatic pancreatic cancer is depicted in Fig. 1*B*. Finally, we investigated whether pancreatic cancers expressing RON ($n = 8$) were in fact expressing the phosphorylated, active form of the receptor. Immunostaining of human pancreatic cancers specimens with a phosphospecific RON antibody shows marked phospho-RON expression in the ductal epithelium as compared with the surrounding stroma (Fig. 1*C*). Therefore, RON is expressed highly from the early stages of pancreatic carcinogenesis through the establishment of metastatic disease and is present in its active, phosphorylated form.

Exposure to HGFL has no effect on pancreatic cancer cell proliferation. The RON receptor has been shown to mediate various oncogenic phenotypes in other cancer cell types, but its effects on pancreatic cancer cells are unknown. After demonstrating that this receptor is commonly expressed in murine and human pancreatic cancer, we next evaluated the consequences of HGFL administration on pancreatic cancer cells expressing RON receptor. We first sought to determine the effects of RON on the proliferation of human and mouse pancreatic cancer cell lines. Exposure of these cell lines to increasing amounts of HGFL showed that RON activation has no effect on the proliferation of these cells. In all cell lines tested, including murine and human, for any given time point from 0 to 48 h post-HGFL exposure, there was no statistical difference in cell proliferation despite increasing amounts of HGFL (Fig. 2). To ensure that RON is not constitutively activated via autocrine activation in these cell lines, conditioned media from serum-starved cells at 24, 48, and 72 h was tested via immunoblots for the presence of HGFL. Conditioned media from the cell lines PanIN, 5143PDA, 5143LM, BxPC-3, and MiaPaCa-2 were used, and immunoblots were probed 1:1,000 with anti-HGFL polyclonal antibody (17), and commercially available HGFL was used as a positive control. These studies show that HGFL is not produced by either PanIN cells or pancreatic cancer cells in detectable quantities to activate RON in an autocrine fashion (data not shown).

RON receptor signaling sensitizes pancreatic cancer cells to apoptosis. Activation of the RON RTK has been shown to protect murine macrophages from bacterial lipopolysaccharide-induced apoptosis (24). In addition, a murine model of mammary tumorigenesis showed that mice with RON overexpressed in mammary tissue showed decreased mammary tumor apoptosis when compared with mice with a nonfunctional RON RTK (23). Given our finding that RON does not regulate pancreatic cancer cell proliferation, we hypothesized that RON might mediate pancreatic cancer progression by enhancing apoptotic resistance. To test this hypothesis in a clinically relevant manner, RON receptor inhibition of BxPC-3 was done in the presence of the chemotherapeutic agent, gemcitabine. An active caspase-3 apoptosis assay was used to quantitate apoptosis. As illustrated in Fig. 3*D*, blockade of the RON receptor enhanced gemcitabine-induced apoptosis by as much as 32% apoptosis compared with gemcitabine alone.

RON signaling enhances migration and invasion in pancreatic cancer cells. Previous studies in other cell lines suggest that RON receptor signaling enhances cancer cell migration and invasiveness. We examined the effects of HGFL on both murine and human pancreatic cancer cells and found that in all RON-expressing lines, there was a dose-dependent response to HGFL in cell migration assays (Fig. 3*A*). This shows that RON signaling has the ability to induce cell migration in response to ligand.

The propensity for early invasion and metastasis is a characteristic trait of pancreatic cancer. To evaluate whether RON receptor signaling contributes to the invasive phenotype of pancreatic cancer cells, we assessed pancreatic cancer cell invasiveness through a Matrigel-coated membrane in response to HGFL. All cell lines expressing RON receptor became significantly more

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invasive after exposure to HGFL, and the degree of invasiveness increased in a dose-dependent manner (Fig. 3*B*). Even the PanIN cell line, considered not to be an invasive cell line because of its premalignant derivation, was able to invade the Matrigel basement membrane in response to HGFL administration. As expected, in the MiaPaCa-2 and Panc-1 cell lines which do not express RON, cell migration and invasiveness were unaffected by exposure to HGFL (data not shown). Importantly, the effects of HGFL were shown to

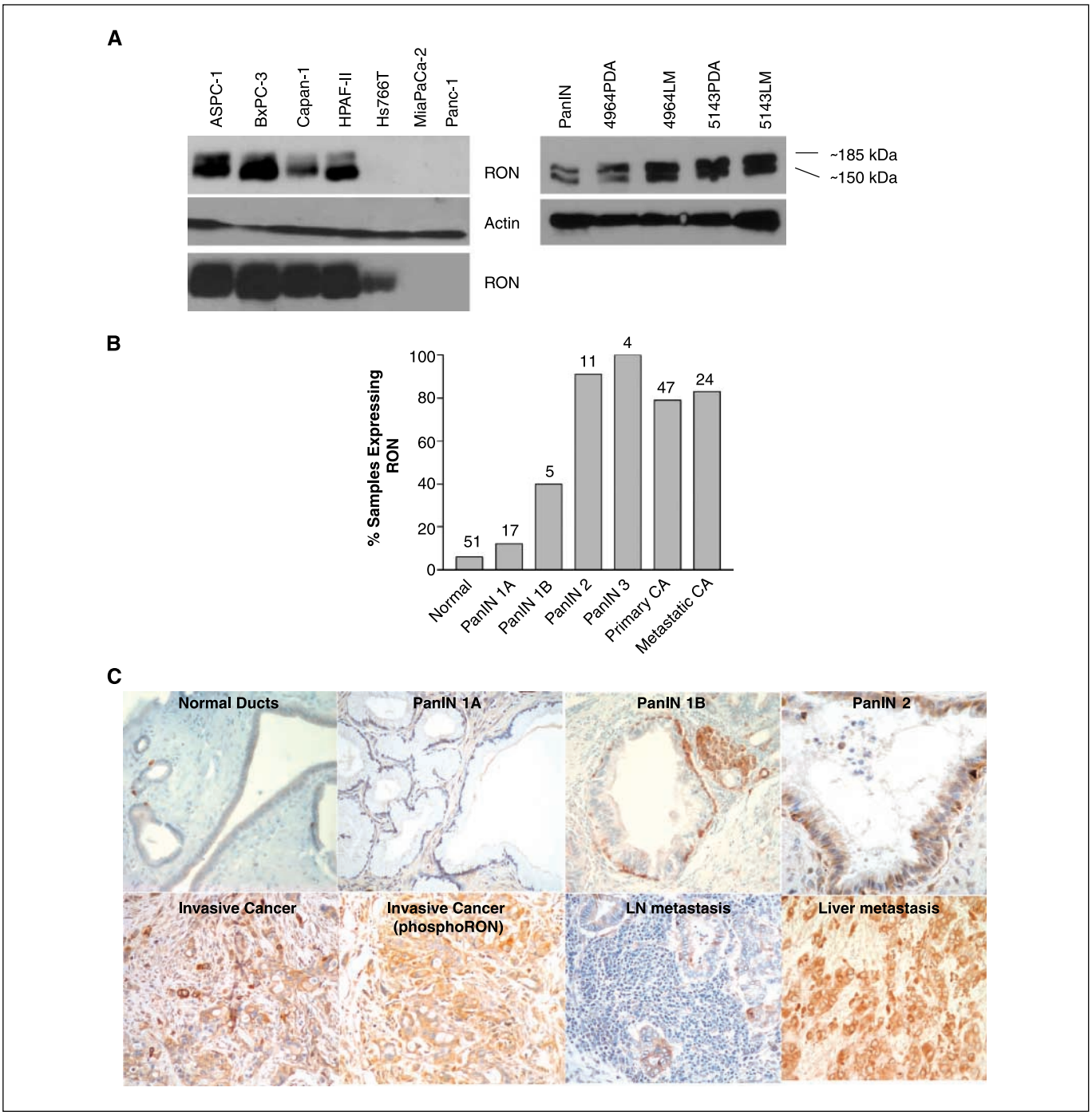

Figure 1. RON tyrosine kinase receptor is expressed in human and murine pancreatic cancer. *A,* immunoblot analysis of pancreatic cancer cell line lysate shows RON expression in five of seven human pancreatic cancer cell lines (*ASPC-1, BxPC-3, Capan-1, HPAF-II, Hs766T*) and five of five cell lines derived from PdxCre/LSL-KRAS^{G12D} mice, including primary pancreatic carcinoma (*4964PDA, 5143PDA*), liver metastatic lesions (*4964LM, 5143LM*), and PanIN lesions. Below the actin control band corresponding to the human cell lines, a longer exposure of the human cell lines immunoblot is seen to show the presence of the 150-kDa β-chain RON in Hs766T. *B,* the overall number of tissue samples expressing RON increased with severity of disease. The numbers above each bar graph represent the total number of evaluated samples possesing the indicated lesions on the X-axis. *C,* human pancreatic cancers were immunostained with a RON-specific antibody and the intense RON expression correlates with pancreatic cancer progression. Minimal expression is seen in normal pancreatic ductal epithelium with obvious expression present in PanIN 1B lesions that persists in later stage PanINs, invasive carcinoma, and in both lymph node (*LN*) and liver metastases. A series of RON-positive invasive carcinomas ($n = 8$) were also immunostained for the phosphorylated, active form of the RON receptor and showed that 100% of samples stained positive, with a single representative picture shown. This shows that total RON expression in pancreatic cancer specimens correlates with the presence of active, phosphorylated RON.

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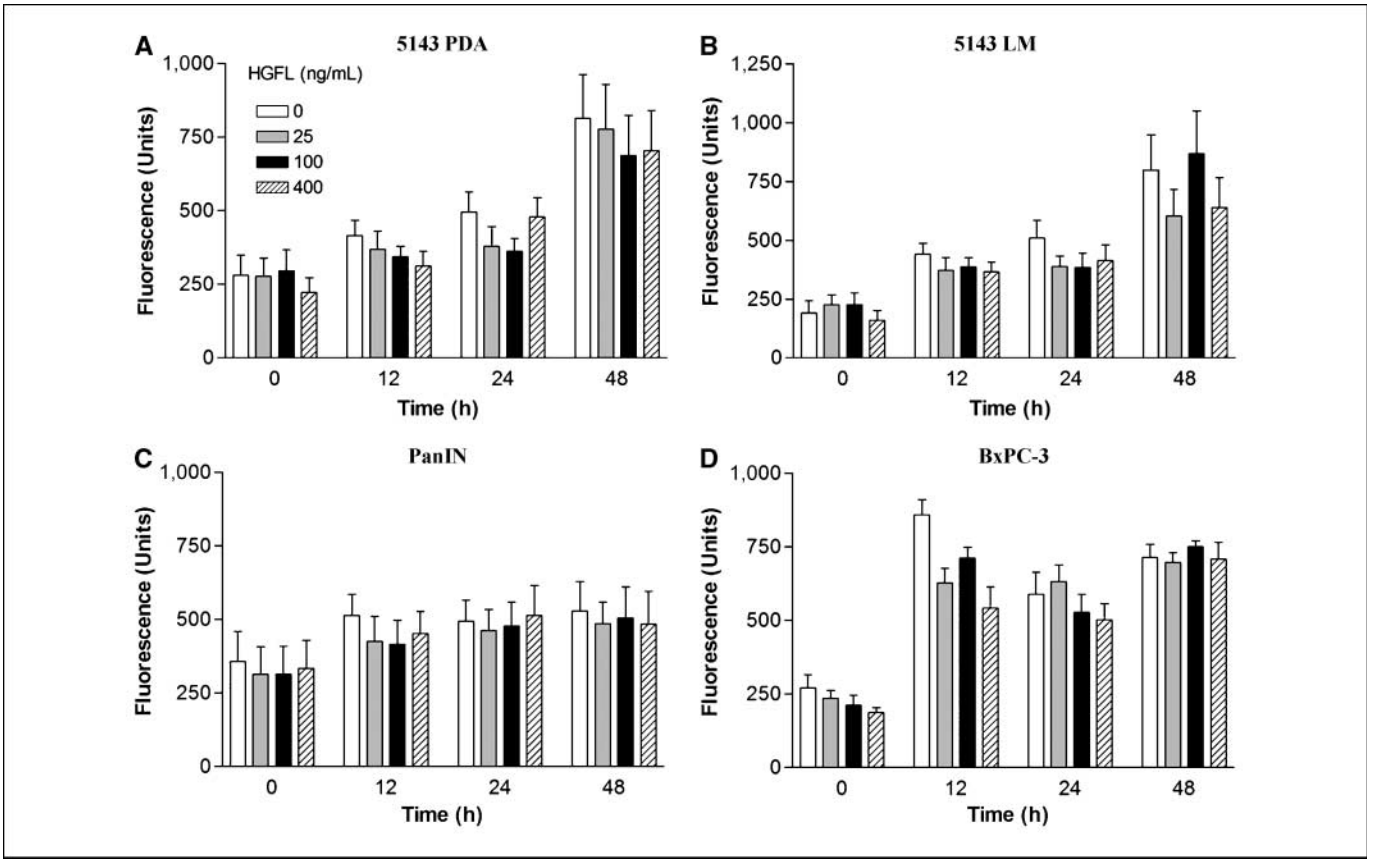

Figure 2. RON activation has no effect on proliferation in murine and human pancreatic cancer cell lines. Alamar Blue proliferation assays were done in 5143PDA (*A*), 5143LM (*B*), PanIN (*C*), and BxPC-3 cells (*D*). Baseline fluorescent measurements corresponding to cell proliferation were acquired at 0 h, followed by 12, 24, and 48 h post-HGFL administration. No statistical difference in proliferation was seen in any cell line from 0 to 48 h post-HGFL. $P > 0.05$ for all time points compared with untreated control.

be specific to RON receptor because preincubation with an anti-RON blocking antibody prevented HGFL-induced migration (Fig. 3*C*).

RON signaling activates known oncogenic pathways in pancreatic cancer cells. The mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K)/Akt, and nuclear factor-κB (NFκB) pathways are critical regulators of oncogenic phenotypes, including apoptotic resistance, invasion, and migration. Each of these pathways has been separately implicated in the pathogenesis of pancreatic cancer (25–28) and as a downstream target of RON signaling (23, 29, 30). To interrogate these signaling pathways as potential downstream targets of RON activation in pancreatic cancer, we analyzed pancreatic cancer cell lysates from cell lines treated with 400 ng/mL of HGFL for 1 h. Immunoblot analysis showed an up-regulation of the phosphorylated, active forms of Akt and extracellular signal-regulated kinase 1/2 (ERK1/2). As an indicator of NFκB activity, interrogation of IκBα/β expression was examined but showed no change following HGFL exposure (Fig. 4*A–C*). Thus, ligand binding to RON receptor in pancreatic cancer cells results in the activation of several important oncogenic signaling pathways.

Discussion

Pancreatic cancer is one of the most lethal cancers in humans, with the incidence of the disease closely matching the death rate. Currently, only 15% to 20% of pancreatic cancer patients present with clinically operable disease, and despite modern surgical techniques and adjuvant chemo- and radiation therapies, cure of even this subgroup of patients is rare. To develop more successful treatments for pancreatic cancer, it is essential that we gain an increased understanding of those molecular events that underlie the most characteristic features of the disease, its impressive resistance to chemotherapy, and propensity for early invasion and metastasis. RTKs regulate multiple cellular processes critical to tumor development and maintenance. Targeting RTK function using small molecule inhibitors and monoclonal antibodies has evolved as the most effective biological therapy for cancer to date. In this study, we have examined the expression and function of the RTK, RON in pancreatic cancer. Our studies show that RON is expressed in human pancreatic cancers and in those arising in a relevant murine model driven by KRAS. The frequency of RON expression increases as the disease progresses from PanIN to invasive cancer to metastatic disease. Treatment of pancreatic cancer cell lines with the RON-specific ligand, HGFL, resulted in a dose dependent increase in cell migration and invasion, which was reversed with RON receptor inhibition. RON also seems to mediate apoptotic resistance in pancreatic cancer cells as blockade of the RON receptor enhanced chemosensitivity to gemcitabine, the current standard chemotherapy for pancreatic cancer patients.

Several RTKs have been implicated in pancreatic carcinogenesis. Overexpression of EGFR and HER2/neu have been reported in up

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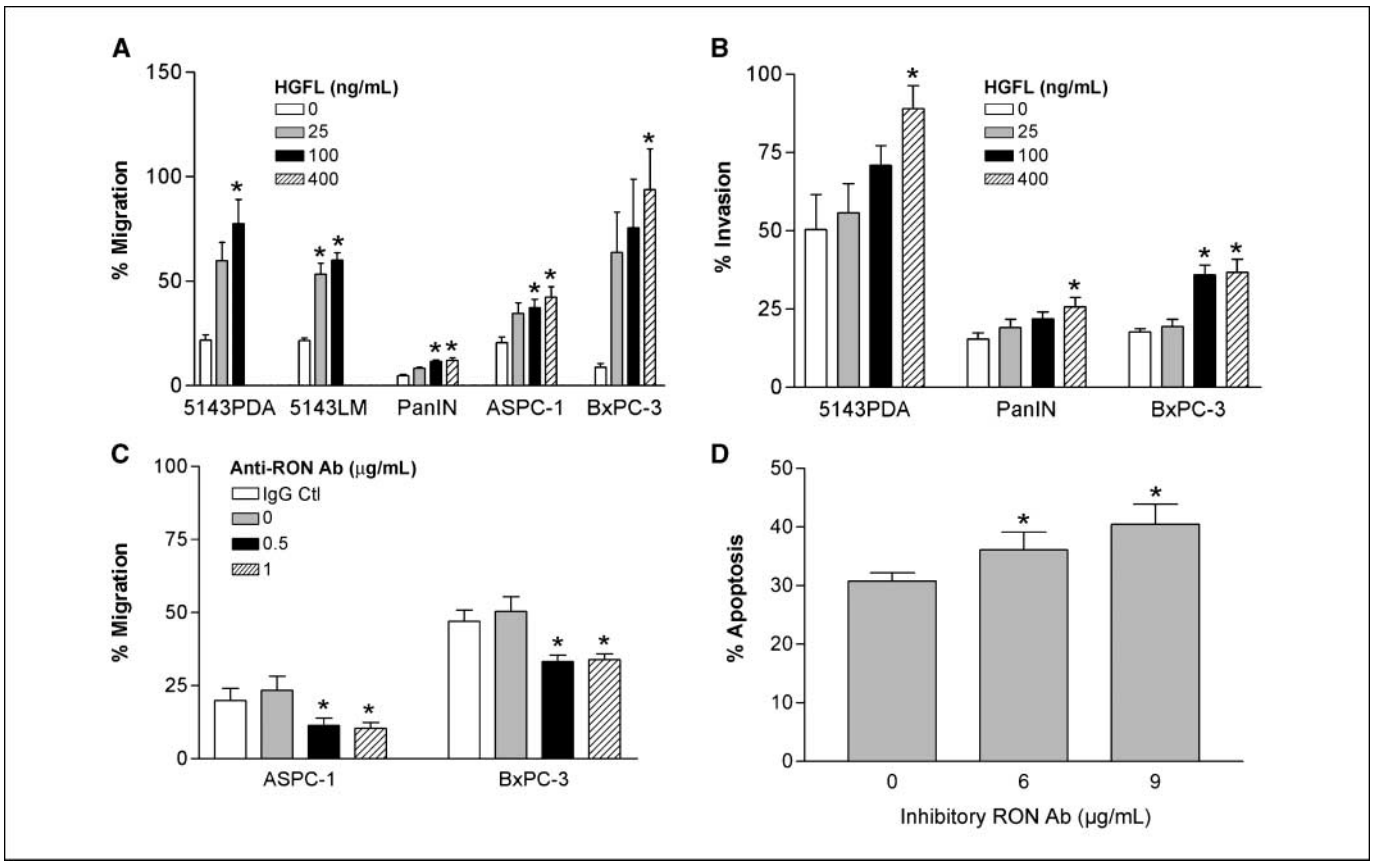

Figure 3. RON activation increases migration, invasiveness, and apoptotic resistance in pancreatic cancer cells. *A,* pancreatic cancer cells showed a dose-dependent increase in migration. *B,* the ability to invade a Matrigel basement membrane in response to the RON ligand, HGFL. This ability to migrate in response to 100 ng/mL of HGFL was abrogated by preincubation with inhibitory RON antibody (*C*). *D,* incubating BxPC-3 cells with inhibitory RON antibody before treatment with 0.1 μmol/L gemcitabine resulted in a 32% increase in apoptosis. *, $P < 0.05$.

to 90% and 83% of human pancreatic cancers, respectively (31–35). A study by Day et al. (36) showed an increase in HER-2/*neu* expression both in PanIN lesions and pancreatic cancer. Transgenic mice overexpressing EGFR ligands such as transforming growth factor-α give rise to pancreatic neoplasia, whereas pancreatic cancers initiated by KRAS in the mouse overexpress both EGFR and HER2 (22, 37). Perhaps most importantly, a recent phase III trial showed a modest but significant improvement in overall survival for pancreatic cancer patients treated with gemcitabine and a combination of the EGFR small molecule inhibitor, Erlotinib, compared with gemcitabine alone (6). Again, whereas this study provided proof of principle that targeting RTK activity can be an

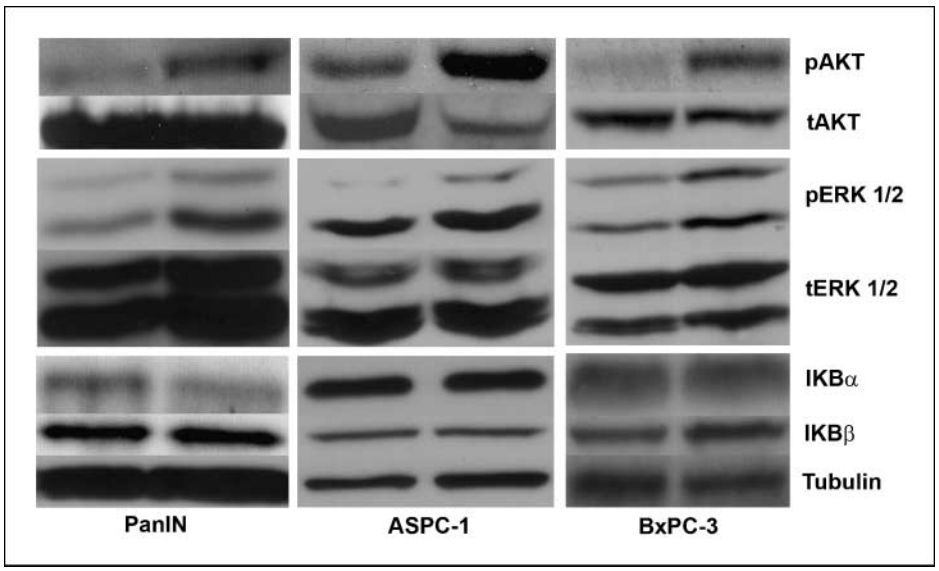

Figure 4. RON activation in pancreatic cancer cells results in the up-regulation of known oncogenic signaling pathways. Immunoblot analysis for total and phosph-Akt, phospho-MAPK, and IκB-α and IκB-β following exposure to HGFL in the murine PanIN cell line and in the human pancreatic cancer cell lines ASPC-1 and BxPC-3. A clear up-regulation of the active, phosphorylated forms of Akt and ERK are shown in all cell lines after 1 h of RON activation. However, no differences in IκB levels were detected post-HGFL compared with control.

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effective strategy in the treatment of pancreatic cancer, the demonstrable benefit to patients was small. This is not surprising in that pancreatic cancer cells are marked by profound chromosomal instability and a number of genetic alterations such that oncogenic pathways critical to tumor growth and maintenance are activated via multiple effectors. Given that downstream targets of EGFR are also activated by other RTKs, it is logical to hypothesize that combined inhibition of multiple RTKs may have synergistic activity. In support of this concept, our group has previously shown crosstalk between EGFR and RON, and a recent publication showed decreased tumor growth of pancreatic cancer cell xenografts when the RON and EGFR receptors were inhibited (38, 39). It is therefore possible to envision targeting RON signaling as part of a multipronged approach to pancreatic cancer therapy.

Interestingly, RON does not seem to mediate proliferation in PanIN or pancreatic cancer cells, but does impart resistance to apoptosis, a central feature of the malignant cell. RON signaling may thus prevent PanIN cells from undergoing apoptosis and allow for the accumulation of genetic mutations necessary for their transformation to frank malignancy. Following exposure to HGFL, we observed up-regulation of the phosphorylated, active forms of Akt and ERK1/2. It is likely that the activation of PI3K/Akt and MAPK signaling contribute to RON-mediated apoptotic resistance, although this also remains to be directly tested and is a subject of our ongoing work. Given that both the PI3K/Akt and MAPK pathways are activated directly by mutant KRAS as well, it is actually somewhat surprising that RON inhibition alone resulted in a moderate increase in pancreatic cancer cell apoptosis and suggests that RON may activate numerous pathways involved in apoptotic resistance. In support of this possibility, RON overexpression has been show to initiate tumor formation in the mammary gland and lung in the mouse (18–20, 23). It will be necessary to directly test whether RON overexpression can initiate and/or accelerate pancreatic carcinogenesis in murine models.

Pancreatic cancer is an aggressive disease characterized by early invasion and metastasis. In the present study, we have shown that the incidence of RON expression increases with the progression of pancreatic cancer precursors toward invasive and metastatic cancer, suggesting that RON-related signaling may help to drive this process. We consistently observed that HGFL induced substantial increases in migration and invasion in both human and mouse pancreatic cancer cell lines, as well as in cells derived from murine PanIN lesions. It is intriguing to postulate that given the high RON expression, we have observed in PanIN lesions, circulating HGFL, or that present in the hepatic stroma, may activate or accelerate the aggressive metastatic phenotype that is so characteristic of pancreatic cancer. Given that we did not find HGFL produced in significant quantities by the pancreatic cancer cell lines, this external HGFL concentration gradient could become even more important in directing cell invasion and motility. RON expression has been linked to aggressive metastatic behavior in breast cancer where RON expression was an independent predictor of decreased survival and early relapses in node-negative patients (40).

Another question raised by our study relates to the mechanism of RON receptor overexpression in pancreatic cancer. RON splice variants have been shown in colon cancer, but not in other tumor types where transcriptional mechanisms seem to be more significant. Given that mutant KRAS is the earliest known genetic alteration in pancreatic cancer, we hypothesize that at least some regulators of RON expression are downstream of KRAS. This is strongly supported by the high level of RON expression in our murine PanIN cells, which harbor only mutant KRAS and no tumor suppressor mutations characteristic of pancreatic cancers. The mechanism of RON overexpression in human pancreatic cancers is likely more complex and also awaits further studies.

In summary, our study has shown for the first time that the RON receptor is highly expressed in pancreatic cancers and suggests that it may contribute to biological aggressiveness so characteristic of this disease. Further investigations are therefore required to determine if RON signaling regulates pancreatic cancer initiation and/or progression and if targeting the RON receptor alone or in combination with other agents will prove to be an effective therapy for pancreatic cancer.

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References

1. American Cancer Society. Cancer Facts and Figures 2006. Atlanta: American Cancer Society; 2006.
2. Sohn TA, Yeo CJ. The molecular genetics of pancreatic ductal carcinoma: a review. Surg Oncol 2000;9:95–101.
3. Iwama A, Yamaguchi N, Suda T. STK/RON receptor tyrosine kinase mediates both apoptotic and growth signals via the multifunctional docking site conserved among the HGF receptor family. EMBO J 1996;15:5866–75.
4. Peace BE, Hughes MJ, Degen SJ, Waltz SE. Point mutations and overexpression of RON induce transformation, tumor formation, and metastasis. Oncogene 2001;20:6142–51.
5. Wang MH, Dlugosz AA, Sun Y, Suda T, Skeel A, Leonard EJ. Macrophage-stimulating protein induces proliferation and migration of murine keratinocytes. Exp Cell Res 1996;226:39–46.
6. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared to gemcitabine alone in patients with advanced pancreatic cancer. A phase III trial of the National Cancer Institute of Canada Clinical Trials Group [NCIC-CTG]. ASCO Meeting Abstracts 2005;23:1.
7. Ronsin C, Muscatelli F, Mattei MG, Breathnach R. A novel putative receptor protein tyrosine kinase of the met family. Oncogene 1993;5:1195–202.
8. Wang MH, Ronsin C, Gesnel MC, et al. Identification of the RON gene product as the receptor for the human macrophage stimulating protein. Science 1994;266:117–9.
9. Carafoli F, Chirgadze DY, Blundell TL, Gherardi E. Crystal structure of the β-chain of human hepatocyte growth factor-like/macrophage stimulating protein. FEBS J 2005;272:5799–807.
10. Gaudino G, Follenzi A, Naldini L, et al. RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. EMBO J 1994;13:3524–32.
11. Bezerra JA, Witte DP, Aronow BJ, Degen SJ. Hepatocyte-specific expression of the mouse hepatocyte growth factor-like protein. Hepatology 1993;18:394–9.
12. Nanney LB, Skeel A, Luan J, et al. Proteolytic cleavage and activation of pro-macrophage–stimulating protein and upregulation of its receptor in tissue injury. J Invest Dermatol 1998;111:573–81.
13. Wang MH, Skeel A, Leonard EJ. Proteolytic cleavage and activation of pro-macrophage–stimulating protein by resident peritoneal macrophage membrane proteases. J Clin Invest 1996;97:720–7.
14. Collesi C, Santoro MM, Gaudino G, Comoglio PM. A splicing variant of the RON transcript induces constitutive tyrosine kinase activity and an invasive phenotype. Mol Cell Biol 1996;16:5518–26.
15. Wang MH, Kurtz AL, Chen Y. Identification of a novel splicing product of the RON receptor tyrosine kinase in human colorectal carcinoma cells. Carcinogenesis 2000;21:1507–12.
16. Okino T, Egami H, Ohmachi H, et al. Presence of RON receptor tyrosine kinase and its splicing variant in malignant and non-malignant human colonic mucosa. Int J Oncol 1999;15:709–14.
17. Waltz SE, McDowell SA, Muraoka RS, et al. Functional characterization of domains contained in hepatocyte growth factor-like protein. J Biol Chem 1997;272:30526–37.
18. Chen YQ, Zhou YQ, Fisher JH, Wang MH. Targeted expression of the receptor tyrosine kinase RON in distal

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lung epithelial cells results in multiple tumor formation: Oncogenic potential of RON *in vivo*. Oncogene 2002;21: 6382–6.
19. Chen YQ, Zhou YQ, Fu LH, Wang D, Wang MH. Multiple pulmonary adenomas in the lung of transgenic mice overexpressing the RON receptor tyrosine kinase. recepteur d'origine nantais. Carcinogenesis 2002;23: 1811–9.
20. Zinser GM, Leonis MA, Toney K, et al. Mammary-specific RON receptor overexpression induces highly metastatic mammary tumors associated with β-catenin activation. Cancer Res 2006;66:11967–74.
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. Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KRASG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 2005;7:469–83.
23. Peace BE, Toney-Earley K, Collins MH, Waltz SE. RON receptor signaling augments mammary tumor formation and metastasis in a murine model of breast cancer. Cancer Res 2005;65:1285–93.
24. Chen YQ, Zhou YQ, Wang MH. Activation of the RON receptor tyrosine kinase protects murine macrophages from apoptotic death induced by bacterial lipopolysaccharide. J Leukoc Biol 2002;71:359–66.
25. Tanno S, Tanno S, Mitsuuchi Y, Altomare DA, Xiao GH, Testa JR. AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells. Cancer Res 2001;61:589–93.
26. Fahy BN, Schlieman M, Virudachalam S, Bold RJ. AKT inhibition is associated with chemosensitisation in the pancreatic cancer cell line MIA-PaCa-2. Br J Cancer 2003;89:391–7.
27. Fahy BN, Schlieman MG, Virudachalam S, Bold RJ. Inhibition of AKT abrogates chemotherapy-induced NF-κB survival mechanisms: implications for therapy in pancreatic cancer. J Am Coll Surg 2004;198:591–9.
28. Ng SSW, Tsao MS, Chow S, Hedley DW. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res 2000;60:5451–5.
29. Danilkovitch-Miagkova A. Oncogenic signaling pathways activated by RON receptor tyrosine kinase. Curr Cancer Drug Targets 2003;3:31–40.
30. Danilkovitch A, Leonard EJ. Kinases involved in MSP/RON signaling. J Leukoc Biol 1999;65:345–8.
31. Yamanaka Y, Friess H, Kobrin MS, Buchler M, Beger HG, Korc M. Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness. Anticancer Res 1993;13:565–9.
32. Xiong HQ, Rosenberg A, LoBuglio A, et al. Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, in combination with gemcitabine for advanced pancreatic cancer: a multicenter phase II trial. J Clin Oncol 2004;22:2610–6.
33. Potti A, Ganti AK, Tendulkar K, et al. HER-2/neu and CD117 (C-kit) overexpression in hepatocellular and pancreatic carcinoma. Anticancer Res 2003;23:2671–4.
34. Dergham ST, Dugan MC, Arlauskas P, et al. Relationship of family cancer history to the expression of p53, p21WAF-1, HER-2/neu, and K-ras mutation in pancreatic adenocarcinoma. Int J Pancreatol 1997;21:225–34.
35. Dugan MC, Dergham ST, Kucway R, et al. HER-2/neu expression in pancreatic adenocarcinoma: relation to tumor differentiation and survival. Pancreas 1997;14: 229–36.
36. Day JD, Digiuseppe JA, Yeo C, et al. Immunohistochemical evaluation of HER-2/neu expression in pancreatic adenocarcinoma and pancreatic intraepithelial neoplasms. Hum Pathol 1996;27:119–24.
37. Wagner M, Greten FR, Weber CK, et al. A murine tumor progression model for pancreatic cancer recapitulating the genetic alterations of the human disease. Genes Dev 2001;15:286–93.
38. Peace BE, Hill KJ, Degen SJ, Waltz SE. Cross-talk between the receptor tyrosine kinases RON and epidermal growth factor receptor. Exp Cell Res 2003;289:317–25.
39. O'Toole JM, Rabenau KE, Burns K, et al. Therapeutic implications of a human neutralizing antibody to the macrophage-stimulating protein receptor tyrosine kinase (RON), a c-MET family member. Cancer Res 2006; 66:9162–70.
40. Lee WY, Chen HH, Chow NH, Su WC, Lin PW, Guo HR. Prognostic significance of co-expression of RON and MET receptors in node-negative breast cancer patients. Clin Cancer Res 2005;11:2222–8.

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The RON Receptor Tyrosine Kinase Mediates Oncogenic Phenotypes in Pancreatic Cancer Cells and Is Increasingly Expressed during Pancreatic Cancer Progression

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