Tumor and Stem Cell Biology

Cancer Research

Silencing of RON Receptor Signaling Promotes Apoptosis and Gemcitabine Sensitivity in Pancreatic Cancers

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Abstract

The RON receptor tyrosine kinase is overexpressed in premalignant pancreatic intraepithelial neoplasia (PanIN) and in the majority of pancreatic cancers. In pancreatic cells, RON is an important K-Ras effector and RON ligand can enhance migration/invasion and apoptotic resistance. However, the pathobiological significance of RON overexpression in pancreatic cancers has yet to be fully established. In this study, we demonstrate that RON signaling mediates a unique transcriptional program that is conserved between cultured cells derived from murine PanIN or human pancreatic cancer cells grown as subcutaneous tumor xenografts. In both systems, RON signaling regulates expression of genes implicated in cancer-cell survival, including Bcl-2 and the transcription factors signal transducer and activator of transcription 3 (STAT 3) and c-Jun. shRNA-mediated silencing of RON in pancreatic cancer xenografts inhibited their growth, primarily by increasing susceptibility to apoptosis and by sensitizing them to gemcitabine treatment. Escape from RON silencing was associated with re-expression of RON and/or expression of phosphorylated forms of the related c-Met or epidermal growth factor receptors. These findings indicate that RON signaling mediates cell survival and *in vivo* resistance to gemcitabine in pancreatic cancer, and they reveal mechanisms through which pancreatic cancer cells may circumvent RON-directed therapies. *Cancer Res; 70(3); 1130–40. ©2010 AACR.*

Introduction

The median survival of pancreatic cancer patients remains less than 1 year. The disease incidence continues to increase, now making pancreatic cancer the fourth leading cause of cancer death in the United States (1). The most notable clinical features of pancreatic cancer are its propensity for early and rapid dissemination and its resistance to cytotoxic chemotherapy. It is clear that a better understanding of the biological basis of these features is desperately needed.

Our laboratory and others identified the RON tyrosine kinase receptor, a c-Met family member, as an overexpressed protein and a potential novel therapeutic target in pancreatic cancer. This finding was recently confirmed by a comprehensive analysis of the pancreatic cancer genome (2–4). RON has also been identified as a key effector of K-Ras signaling in pancreatic and lung cancer cells; it also mediates cellular migration, invasion, and apoptotic resistance in cultured pancreatic cancer cells (2, 5). These findings have raised interest in RON as a potential novel therapeutic target in pancreatic cancer. Although RON overexpression has been shown, no mutations or major splice variants of RON have been reported in pancreatic cancer specimens. It remains unclear whether the RON ligand increases proliferation in pancreatic cancer cells, as studies have reported conflicting results (2, 3). Despite these unresolved questions, several groups have reported that RON-directed therapies can reduce the growth of human pancreatic cancer xenografts (3, 6).

In the present study, we sought to investigate the relevance of RON signaling to pancreatic carcinogenesis by first characterizing the transcriptome of pancreatic cancer cells exposed to the RON ligand. Our studies revealed that RON regulates the expression of multiple genes that promote cancer cell survival, including *Bcl-2, signal transducer and activator of transcription 3* (*STAT3*), *vascular endothelial growth factor* (*VEGF*), *c-jun*, and *c-fos*. To further investigate the importance of RON signaling to pancreatic cancer cell survival, we used shRNA technology to silence RON expression in two pancreatic cancer cell lines, XPA-1 and FG. The proliferation of pancreatic cancer cells was minimally affected by loss of RON signaling, and RON-deficient cells were competent to form tumor xenografts. We show, however, that FG-derived RON-deficient tumors were significantly growth inhibited and that both FG- and XPA-derived tumors showed enhanced susceptibility to spontaneous apoptosis. In addition, XPA-1–derived RON-deficient tumors showed enhanced susceptibility to treatment with gemcitabine chemotherapy. Finally, we show that escape from RON silencing occurs in association with reexpression of the receptor and/or upregulation of the

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receptor tyrosine kinases (RTK) c-met and epidermal growth factor receptor (EGFR). These studies suggest that RON signaling contributes to pancreatic cancer cell survival and therapeutic resistance *in vivo*, and also suggest potential mechanisms of escape from RON-directed therapies.

Materials and Methods

Cell lines and maintenance. The mouse pancreatic intraepithelial neoplasia (PanIN) cell line was derived from the Pdx-1Cre/LSL-KRAS^{G12D} mouse model of pancreatic cancer (as previously described in refs. 2, 7) and was maintained in DMEM. XPA-1 cells were originally derived from a primary human pancreatic cancer xenograft established at Johns Hopkins (8, 9). The human BxPC3 cell line was obtained from the American Type Culture Collection. The XPA-1-RFP and BxPC3-RFP cell lines were constructed at AntiCancer, Inc. and kindly provided by Dr. Michael Bouvet (University of California, San Diego, La Jolla, CA) and were maintained in RPMI with 1% sodium pyruvate and 1% nonessential amino acids. The FG cell line was kindly provided by Dr. David Cheresh (University of California, San Diego) and was maintained in DMEM high-glucose medium. All media were supplemented with 1% sodium pyruvate and 1% nonessential amino acids with 10% fetal bovine serum and 1% penicillin/streptomycin unless otherwise indicated. All cells were grown in a humidified incubator at 37°C.

RON-silenced XPA-1-RFP or FG or control XPA-1-RFP cells were created through transfection with either an shRNA plasmid directed against RON (RHS1764; Open Biosystems; target sequence 5′-CGCGTAGATGGTGAATGTCATA-3′) or a control plasmid (RHS 1703; Open Biosystems) using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Stable clones were isolated, expanded, and characterized following selection in 2.5 μg/mL puromycin for 5 to 10 d for the XPA-1-RFP clones, and 1 μg/mL for 21 d for the FG clones. To label the RON-silenced FG cells with mCherry, a pCDH-MCS vector (System Biosciences) containing the *mCherry* gene at the *Nhe*1-*Bam*H1 sites was reverse transfected into the cells using Lipofectamine 2000. Following transfection, fluorescence-activated cell sorting was performed, and cells were expanded and maintained in 5 μg/mL puromycin.

Gene-chip studies. To identify genes whose expression was altered in murine PanIN cells by the RON ligand HGFL (hepatocyte growth factor-like protein), total RNAs were isolated from three independent samples of PanIN cells. At 60% to 80% confluency, PanIN cells were washed thrice and then divided into three treatment groups: (*a*) 10% serum medium, (*b*) 400 ng/mL of the RON-specific ligand HGFL (recombinant human MSP; R&D Systems) in 10% serum medium for 30 min, or (*c*) 400 ng/mL of HGFL in 10% serum medium for 12 h. Cells were then washed thrice and trypsinized. Total RNA was isolated using Trizol (Invitrogen). Total RNA was purified using RNeasy columns (Qiagen) and quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). The Affymetrix standard protocol with oligo dT primers was used to label whole RNAs. Biotinylated cRNA was purified with RNeasy columns and hybridized to Affymetrix MOE430plus2 microarrays using standard procedures (10). The 430 v.2.0 array contains 45,000 probe sets representing more than 34,000 mouse transcripts.

Bioinformatic analyses. CEL files were generated from GCOS 5.0 and subjected to Robust Multichip Average (RMA) normalization as implemented in GeneSpring 7.1. Probe sets were filtered for those whose expression exceeded RMA intensity value units greater than 6.0 in at least two replicates per condition, and for those whose expression differed between treatments on average by more than 2-fold, with a Student's *t* test false discovery rate of not more than 5%. This yielded a list of 858 probe sets that were referenced to the corresponding control values and subjected to hierarchical clustering using Pearson correlation. Clusters were evaluated for gene cofunctional relationships using the Gene Set Enrichment Analysis algorithm as implemented by the Toppgene server (11).

Quantitative reverse transcription-PCR. For quantitative reverse transcription-PCR, total RNA was purified as described above. Five hundred to 1,000 ng of RNA were converted to cDNA using random hexamers and SuperScript III (Invitrogen). Amplification was carried out with an ABI 7300 real-time PCR system (Applied Biosystems) using SYBR green. The reference gene was *β-glucuronidase*. Whenever possible, primers were designed to span an intronic sequence and were validated by PCR and gel analysis. Primer sequences for human *c-jun* (forward 5′-TCGACATGGAGTCCCAGGA-3′ and reverse 5′-GGCGATTCTCTCCAGCTTCC-3′), c-fos (forward 5′-CGGGCTTCAACGCAGACTA-3′ and reverse 5′-GGTCCGTGCAGAAGTCCTG-3′), and *RON* (forward 5′-GAGGTCAAGGATGTGCTGATTC-3′ and reverse 5′-GAATACATAGACCAGGCCCAGAATCG-3′) were designed to span an intronic sequence and were validated by PCR and gel analysis.

Mice and* in vivo *tumor studies. Five- to 8-wk-old athymic nude mice (National Cancer Institute-Frederick) were housed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the study was approved by the University of Cincinnati and University of California San Diego Institutional Animal Care and Use Committees. Tumor xenografts were developed from each of the three XPA-1-RFP and two FG-mCherry cell lines [untransfected parental cell line, vector control, and RON-silenced cell line (XPA-1 only)]. Two hundred microliters of RPMI for the XPA-1-RFP and 200 μL of DMEM for the FG-mCherry with 5×10^6 cells were injected s.c. into the flanks of nude mice using a 25-gauge needle. Tumor volume was measured with calipers twice weekly using the formula (length × width^2)/2. Mice were anesthetized and photon emission was measured from the tumors twice weekly using a live-animal fluorescence imager (IVIS Lumina Imaging System, Caliper, housed at UCSD) for the XPA-1-RFP mice and an OV100 (Olympus, housed at AntiCancer, Inc.) for the FG-mCherry mice. For tumor growth studies, 16 tumor xenografts were developed for each group using bilateral flank injections in eight mice. Mice were euthanized and tumors were harvested 30 d after implantation. For bromodeoxyuridine (BrdUrd) studies, mice underwent i.p. injection of 150 μg/g BrdUrd (XPA-RFP Sigma-Aldrich,

FG-mCherry, BD Biosciences) 2 h before sacrifice. For CD31 and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) studies, tumors were harvested as they became reliably palpable. For therapeutic studies using XPA-1-RFP cells, a total of 10 tumor xenografts were established in 10 mice using right-sided flank injections. When the tumor volume reached 125 mm^3, animals were randomly assigned to one of the two treatment groups: (*a*) control animals that received no additional treatment or (*b*) gemcitabine-treated animals that received 65 μg/g of drug by i.p. injection twice weekly. For mice receiving FG-mCherry cells, a total of 12 xenografts were established in six animals. They were divided into two groups, RON-silenced and parental, with both getting PBS injections twice weekly using the same volume as the XPA-RFP mice treated with gemcitabine. Mice in therapeutic studies were euthanized and tumors were harvested when the tumor volume reached 2,000 mm^3. Three XPA-RFP mice with RON-silenced tumor xenografts were euthanized and sacrificed when treatment with gemcitabine had reduced the tumor size to <40 mm^3. RON-silenced FG-mCherry mice whose tumor xenografts had not grown beyond 400 mm^3 after 8 wk were euthanized at that time.

Histology and immunohistochemistry. XPA-1-RFP xenograft tumors from nontherapeutic studies were harvested and immediately fixed in 10% formalin, were paraffin embedded, and cut into 5-μm sections. The FG-mCherry xenograft tumors were harvested and immediately embedded in optimum cutting temperature compound and cut into 10-μm sections. Sections were deparaffinized in xylene and rehydrated through a graded series of ethanol/water solutions. Samples were stained for H&E. A BrdUrd detection kit (Invitrogen) and a CD31 antibody (BD Pharmingen) were used for immunohistochemistry per the manufacturer's instructions. A TUNEL assay (Chemicon International) was performed per the manufacturer's instructions, and samples were counterstained with hematoxylin. All slides from the XPA-1-RFP xenograft tumors were prepared by the University of Cincinnati Mouse Histology Core and returned to the investigators who were blinded so that scoring was performed in an unbiased manner. Percent necrosis was determined by calculating the percentage of necrotic area relative to the total tumor area. BrdUrd incorporation was scored by counting the number of cells staining positive for BrdUrd on a ×200 high-powered field; BrdUrd-positive cells were counted in four viable random fields for each tumor specimen. For CD31 counts, three areas of peripheral tumor, devoid of necrosis and showing the highest vascularity, were identified by evaluating histologic sections at ×100 magnification. Vessels were then counted at ×200 magnification. For TUNEL scores, the Axiovision Release 4.5 software was used on the XPA-1-RFP tumors, whereas the Metamorph software was used on the FG-mCherry tumors to capture and evaluate images at ×200 magnification. The entire viable area of each tumor was measured using the Axiovision or Metamorph software, respectively. TUNEL score was calculated based on the number of TUNEL-stained cell per measured area (μm^2) of viable tumor.

Immunoblotting, immunoprecipitation, and ELISA. Tumors were snap frozen before processing. They were placed on dry ice and were homogenized in radioimmunoprecipitation assay buffer (RIPA) containing complete protease inhibitors and PhosSTOP phosphatase inhibitors (Roche Applied Science). The lysates were left on ice for 30 min followed by centrifugation at 15,000 × *g* for 15 min, and then supernatants were collected. Protein concentration was determined using the Micro BCA Protein Assay kit (Pierce). Immunoblotting was performed using between 2.5 and 30 μg of lysate. Blots were analyzed on SDS-PAGE. For immunoprecipitations, 500 μg of tumor lysates were incubated with 1 μg of RON C-20 (Santa Cruz Biotechnology) for 30 min on ice followed by the addition of Protein A/G UltraLink Resin (Pierce) for 1 h at 4°C with rotation. The beads were washed two quick times followed by two 15-min washes in RIPA buffer at 4°C with rotation. After the removal of the final wash, the beads were resuspended in 1× NuPAGE LDS sample buffer (Invitrogen) containing 1× NuPAGE sample reducing agent (Invitrogen) and were incubated at 60°C for 30 min to elute the protein from the beads. Samples were analyzed by SDS-PAGE and immunoblotting. Antibodies against c-met (25H2), phospho-met (3D7-$Tyr^{1234/1235}$), Stat3 (4904), Bcl-2 (2870), p-AKT (9271), AKT (9272), p-ERK (9101), and extracellular signal-regulated kinase (ERK; 9102) were purchased from Cell Signaling Technology, Inc. Antibody against c-jun (610326) was purchased from BD Biosciences. Actin antibody was purchased from Sigma. Anti-phosphotyrosine monoclonal antibody (mAb) 4G10, anti-EGFR, and anti-phospho-EGFR (9H2-Tyr^{1173}) were purchased from Millipore. Goat anti-mouse–horseradish peroxidase (HRP; Chemicon/Millipore, Inc.) and goat anti-rabbit-HRP (Santa Cruz Biotechnology) were used as secondary antibodies at 1:5,000 dilution. The reaction was developed with Enhanced Chemiluminescence Plus reagent (GE Healthcare).

To quantify tumor VEGF expression, 200 μg of protein for each sample were diluted to a total volume of 100 μL with RIPA buffer and were analyzed with a Quantikine human VEGF immunoassay per the manufacturer's instructions (R&D Systems).

Statistical analysis. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software). One-way ANOVA or two-tailed Student's *t* tests were performed as appropriate. For all analyses, $P < 0.05$ was considered significant.

Results

RON signaling in murine PanIN cells results in large-scale alteration of gene expression patterning. Studies in numerous epithelial tumor types indicate that the activated RON receptor mediates oncogenic signaling pathways, including phosphoinositide 3-kinase (PI3K)/AKT, mitogen-activated protein kinase (MAPK), β-catenin, and others (12–14). Despite this, surprisingly little is known about what alterations in the transcriptome are mediated by RON signaling. Based on our prior studies suggesting the importance of RON in regulating pancreatic cancer cell invasion, migration, and survival, we hypothesized that RON signaling would exert potent effects on the transcriptome. Initially, we were

particularly interested in the effects of RON signaling early in pancreatic carcinogenesis. To evaluate this, we characterized the transcriptome of cells derived from murine PanIN after exposure to the RON ligand. PanIN cells were exposed to the RON-specific ligand HGFL for 30 minutes or 12 hours, and transcriptome alterations were evaluated on Affymetrix GeneChips. More than 800 differentially expressed genes were identified that followed a variety of different patterns (Fig. 1). As has been seen for the met receptor, a dichotomous pattern of gene expression appeared (15). After 30 min, early-response genes such as *egr1, egr3*, and *crp61* were upregulated. At 12 hours, the transcripts of numerous genes implicated in oncogenesis were differentially expressed. This included upregulation (3- to 10-fold) of numerous transcription factors, including *c-jun, c-fos*, and *atf-3* of the activator protein (AP-1) transcription factor complex *STAT3*; as well as genes regulating cell survival, such as *Bcl-2*; and genes regulating angiogenesis, such as *VEGF-A*. Gene-chip findings were validated using Western blot and quantitative PCR. These data suggest that through its effects on transcription, RON signaling mediates a wide array of oncogenic pathways in cells derived from pancreatic cancer precursors.

RON signaling regulates pancreatic cancer cell survival in vivo. In previous work, we found that RON signaling activates the MAPK and PI3K/AKT signaling pathways and promotes apoptotic resistance in pancreatic cancer cells, including the murine PanIN cell line (2). Given our current findings that RON signaling regulates the expression of genes that promote cancer cell survival *in vitro*, we sought to determine if RON signaling was important for the regulation of survival pathways *in vivo*. For these studies, we used the human pancreatic cancer cell lines XPA-1 and FG and stably transfected them with shRNAs specific to the RON receptor transcript or a nonsense control. FG cells are mutant for both KRAS and P53, whereas XPA-1 cells, which were derived from a primary human pancreatic cancer xenograft, are wild-type for KRAS and have mutant P53. These cell lines were chosen as they have a varied genetic background, yet each overexpresses the RON receptor. Interestingly, we were unsuccessful in our attempt to develop RON-deficient BxPC3 cell lines, suggesting that RON may be a critical regulator

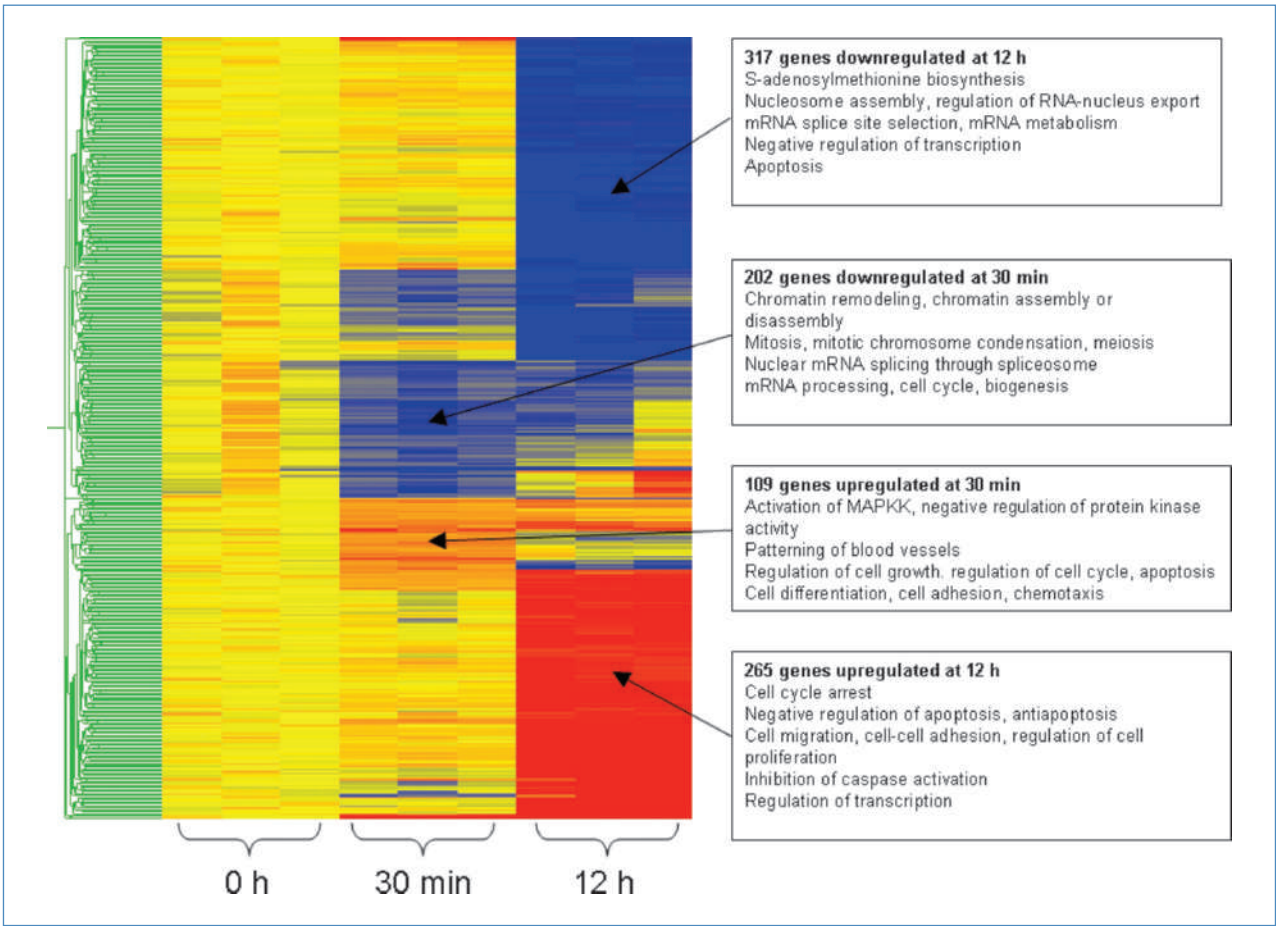

Figure 1. RON receptor activation results in 858 differentially expressed genes after 30 min and 12 h. Affymetrix GeneChip analyses were performed using PanIN cells treated with 400 ng/mL of HGFL for 0 min (untreated), 30 min, or 12 h. Three hundred eleven genes were differentially expressed at 30 min, whereas 582 were altered at 12 h. *Red areas,* genes that were upregulated; *blue areas,* genes that were downregulated.

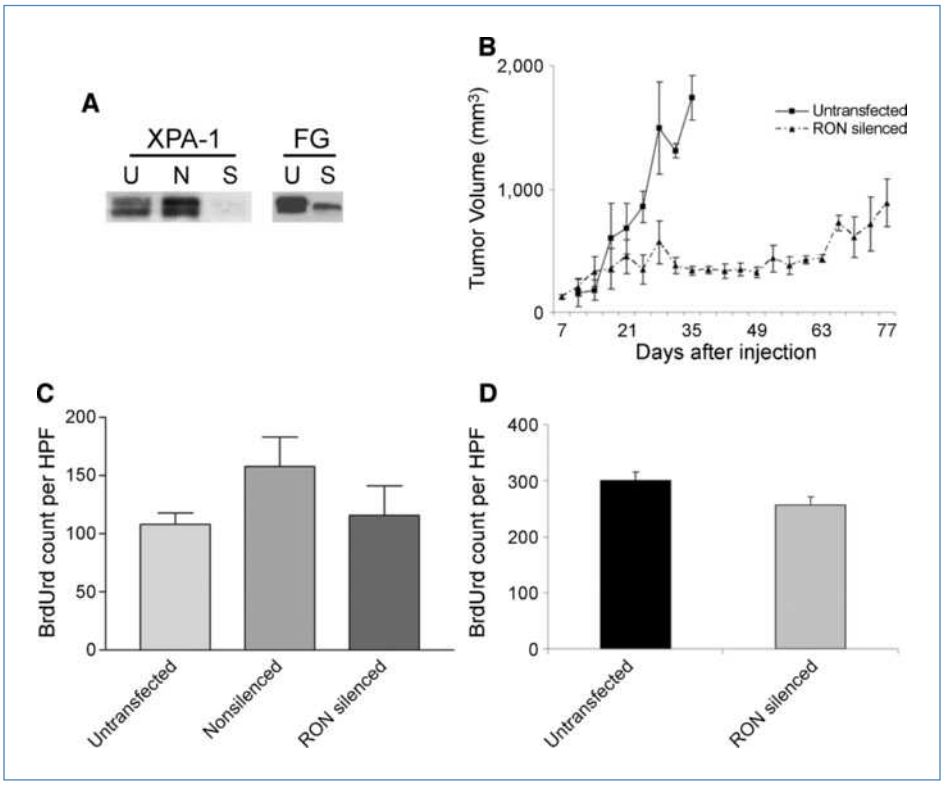

Figure 2. RON downregulation suppresses tumor growth in human xenograft tumors. A, XPA-1– and FG-derived tumor xenografts were screened for RON expression by immunoblotting. *U,* untransfected parental cell line; *N,* nonsilencing shRNA vector–transfected cell line; *S,* RON-silenced cell line. B, growth of FG-derived tumor xenografts. Tumors were measured twice weekly by using calipers, and growth was plotted. C (XPA) and D, (FG) mice were injected with BrdUrd 2 h before sacrifice. Sectioned tumors were stained and scored for BrdUrd incorporation. No statistical difference was seen in the latency, growth, tumor volume, or proliferation in the RON-silenced tumors relative to controls; $P > 0.05$ for both XPA-1 and FG.

of cell survival or proliferation in this line. In contrast, RON-deficient XPA-1 and FG cells were viable. Immunoblot analysis showed that RON expression was reduced by >95% in shRON-XPA-1 cells relative to controls and ~80% in FG cells (data not shown). Both shRON-XPA-1 and shRON-FG cells were competent to develop subcutaneous tumor xenografts. For all animal studies, we used XPA-1 cells transduced with an RFP-expressing retrovirus and FG cells transduced with mCherry to allow for noninvasive imaging. We developed tumor xenografts by injecting 5×10^6 RON-silenced or RON-expressing cells into the subcutaneous flank of nude mice. After documenting decreased RON protein expression in our xenografts (Fig. 2A), we next examined their growth characteristics. RON-deficient FG cells immediately showed growth inhibition compared with controls (Fig. 2B). RON-deficient XPA-1 tumors initially appeared to grow at a similar rate to the controls. Although it was slightly reduced in RON-deficient FG cells, BrdUrd incorporation was not statistically different in RON-deficient cells versus parental cells in either XPA-1 or FG (Fig. 2C and D, respectively). When tumors were excised, we noted significantly more necrosis in RON-deficient tumors, particularly in the XPA-1–derived subset. We quantified necrosis after H&E staining and observed an 85% increase in the area of necrosis within RON-deficient tumors compared with controls ($P = 0.001$; Fig. 3A). Evaluation of photon emission from RON-deficient FG and XPA-1 xenografts showed a 3-fold relative decrease ($P = 0.002$) in emission from RON-silenced tumors compared with RON-expressing controls, suggesting that a significantly less viable tumor was present within the RON-deficient tumors (Fig. 3B). These data are consistent with earlier *in vitro* findings that alterations in RON signaling had no effect on the proliferation of RON-expressing pancreatic cancer cell lines (2). Together, these data show that although RON signaling may not significantly influence the proliferation of the pancreatic xenografts, it may be essential for cell survival within the tumors themselves.

Microvessel density is enhanced in RON-deficient pancreatic cancer xenografts. The pathologic finding of tumor necrosis may be attributed to rapid tumor growth that exceeds the capacity of tumor blood supply (i.e., a failure of angiogenesis) or an increased susceptibility to cell death. Effective antiangiogenic therapies may result in increased tumor necrosis and the associated decrease in tumor microvessel counts (16, 17). Our gene-chip studies showed an upregulation in the transcription of VEGF-A after RON activation. Therefore, we hypothesized that the increased necrosis seen in RON-silenced tumor xenografts may be attributable to a failure of angiogenesis secondary to the loss of tumor-derived VEGF. We therefore examined VEGF production and tumor microvessel counts in pancreatic cancer xenografts derived from RON-silenced and control cells. Tumors were excised and analyzed soon after becoming readily palpable. VEGF ELISA performed on tumor lysates revealed no difference in VEGF levels in RON-silenced tumors relative to RON-expressing controls (data not shown). Curiously, CD31 staining performed on nonnecrotic areas of the tumor revealed that microvessel density was increased by 75% in shRON-FG tumors, and by 31% in shRON-XPA tumors (data not shown). These data suggest that the effects of RON

signaling on neovascularization are complex but that inhibition of angiogenesis is not likely to be the primary cause for the decreased growth and necrosis observed in RON-silenced tumors.

Ron silencing results in increased susceptibility to apoptosis in pancreatic cancer xenografts. Given these findings, we hypothesized that the diminished growth and necrosis observed in RON-deficient tumors may be attributable to increased susceptibility to apoptosis (secondary necrosis). Our earlier *in vitro* studies showed that RON may play a role in apoptotic resistance (2). To investigate the possibility that a differential rate of apoptosis was occurring in the absence of RON, we performed a TUNEL assay on shRON-XPA-1, shRON-FG, and control tumor tissues. When examining only the cellular areas of the tumors, we noted a 43% and 74% increase in the number of apoptotic cells in the RON-silenced XPA-1– and FG-derived tumors, respectively, relative to RON-expressing controls ($P < 0.05$ for each; Fig. 4A). This finding suggests that an increase in apoptosis was primarily responsible for the decreased growth seen in RON-silenced tumors.

To explore the molecular pathways responsible for the reduced cell survival in RON-silenced tumors, we used our PanIN cell microarray data as a basis to examine potential RON-mediated regulators of apoptosis (Fig. 1). Proteins involved in the regulation of apoptosis include bcl-2, c-jun, c-fos, and STAT3 (18–20). Each of these was significantly upregulated on the microarray after RON activation in PanIN cells. C-jun and STAT3 have been identified as potential downstream targets of RON activation in tumor types other than pancreatic cancer (12). Bcl-2, a potent prosurvival regulator of apoptosis, has not been previously identified as a target of RON signaling, although its role in pancreatic cancer has been described (19, 20). Additionally, PI3K and MAPK are well-known signaling pathways activated by RON in many cancers, including mouse PanIN and human pancreatic cancer cells (2, 3). Immunoblots revealed decreases in the expression of

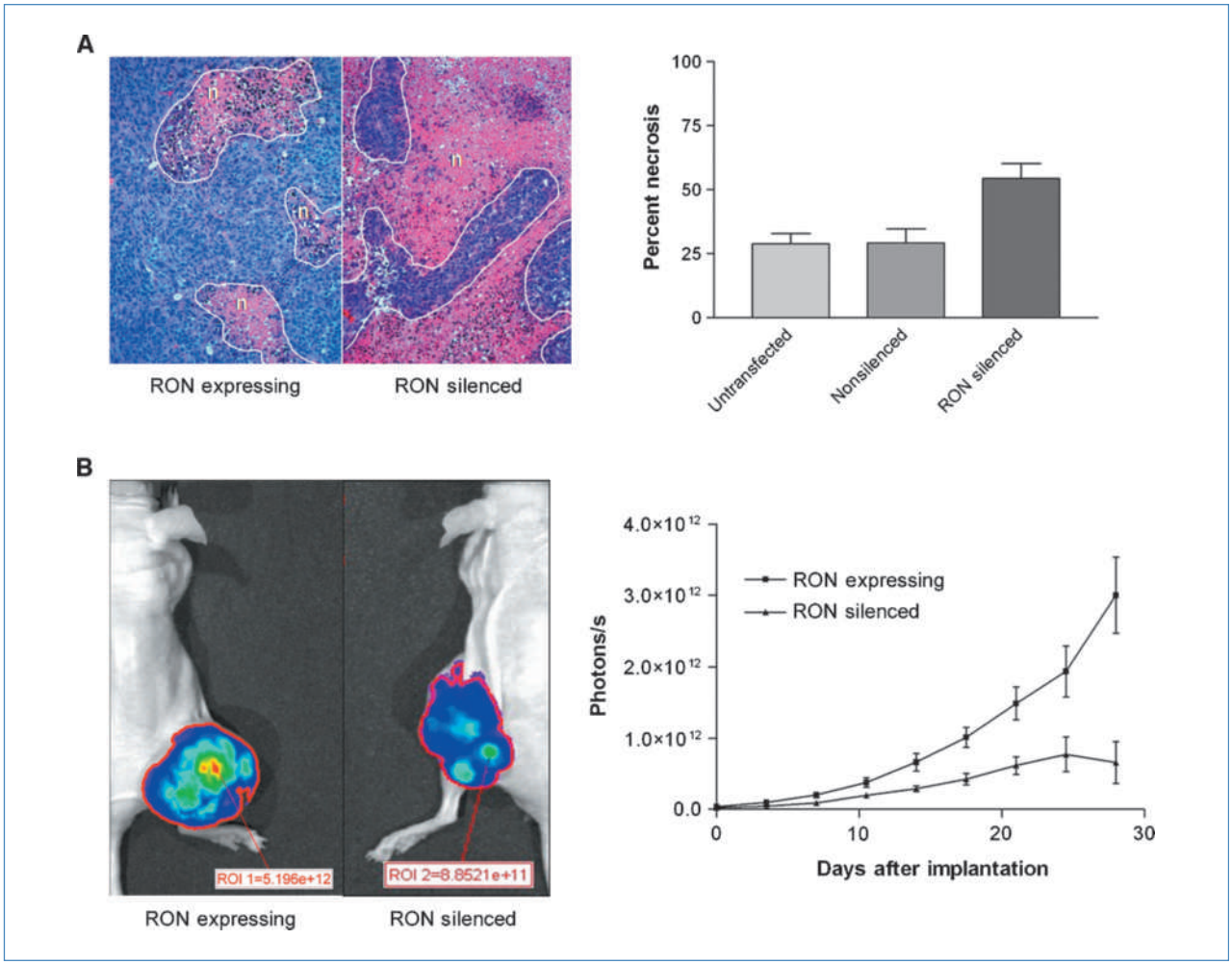

Figure 3. Downregulation of RON results in increased necrosis in pancreatic xenografts. A, H&E staining revealed an 85% increase in necrosis in RON-silenced tumors. *n,* necrotic areas of tumor ($P = 0.001$). B, viable RFP-labeled XPA-1 cells were detected within the tumor xenografts using the IVIS lumina live-animal imager to measure photon emission twice weekly. There was a 3-fold reduction in photon emission from RON-silenced tumors after 30 d of growth ($P = 0.002$). *ROI,* region of interest.

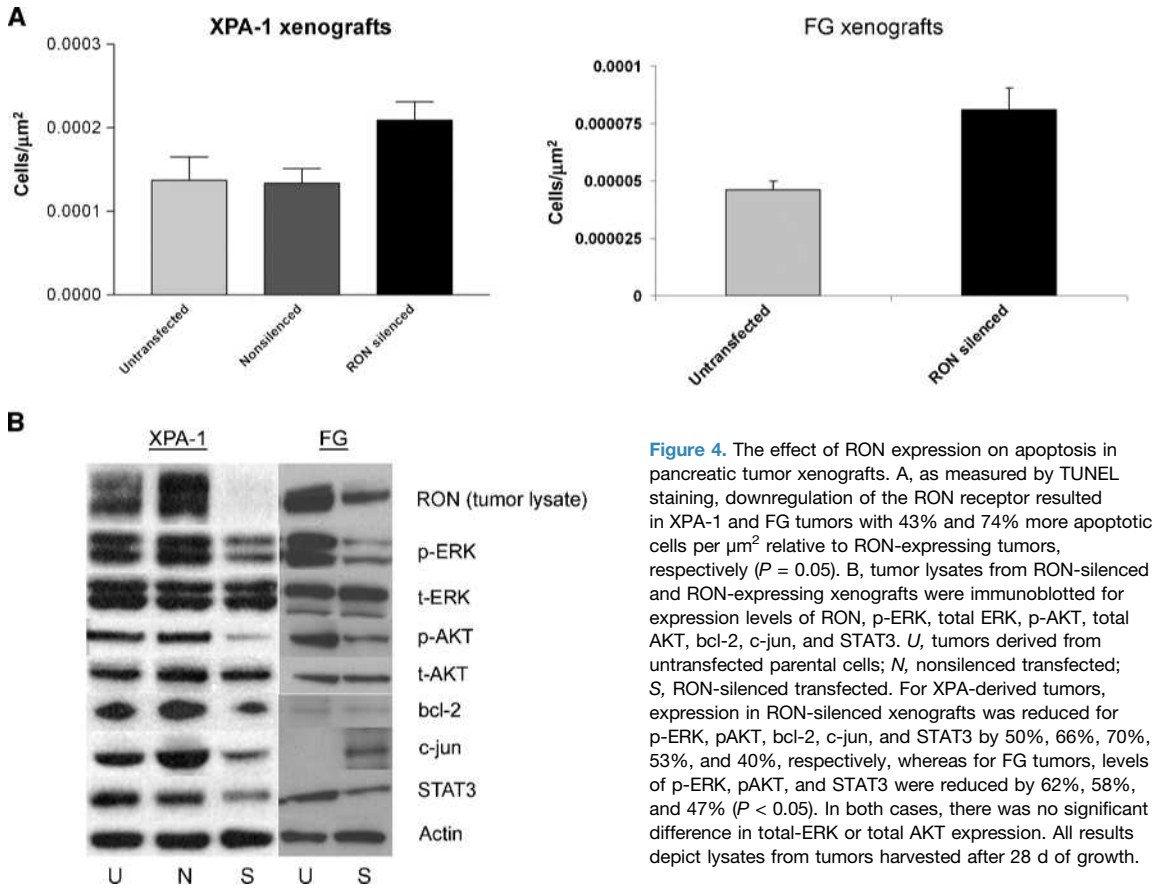

Figure 4. The effect of RON expression on apoptosis in pancreatic tumor xenografts. A, as measured by TUNEL staining, downregulation of the RON receptor resulted in XPA-1 and FG tumors with 43% and 74% more apoptotic cells per μm^2 relative to RON-expressing tumors, respectively ($P = 0.05$). B, tumor lysates from RON-silenced and RON-expressing xenografts were immunoblotted for expression levels of RON, p-ERK, total ERK, p-AKT, total AKT, bcl-2, c-jun, and STAT3. *U,* tumors derived from untransfected parental cells; *N,* nonsilenced transfected; *S,* RON-silenced transfected. For XPA-derived tumors, expression in RON-silenced xenografts was reduced for p-ERK, pAKT, bcl-2, c-jun, and STAT3 by 50%, 66%, 70%, 53%, and 40%, respectively, whereas for FG tumors, levels of p-ERK, pAKT, and STAT3 were reduced by 62%, 58%, and 47% ($P < 0.05$). In both cases, there was no significant difference in total-ERK or total AKT expression. All results depict lysates from tumors harvested after 28 d of growth.

p-ERK, p-AKT, c-Jun, STAT3, and bcl-2, corresponding to a decrease in RON expression in RON-deficient XPA-1–derived tumors (Fig. 4B). In RON-deficient FG-derived tumors, we again observed marked decreases in p-ERK, pAKT, and STAT3 expression. Bcl-2 expression was minimally changed and c-jun expression was actually greater. These data indicate that several powerful mediators of apoptosis are downstream targets of RON in pancreatic cancer and that the transcriptional program regulated by RON is highly conserved between the murine PanIN cell line and invasive human pancreatic cancer xenografts.

RON silencing enhances the effects of gemcitabine treatment in pancreatic xenografts. Prior work by our group showed that inhibiting RON receptor signaling sensitizes pancreatic cancer cells to gemcitabine *in vitro* (2). Given the new findings that RON signaling seems to regulate pro-survival pathways in pancreatic tumor xenografts, we next sought to evaluate the effects of RON downregulation on the response of xenograft tumors to gemcitabine treatment. Given that RON-deficient FG tumor xenografts failed to grow beyond 500 mm^3 even after 8 weeks, we performed the next set of experiments with XPA-1 cells only. Tumor xenografts were again initiated by injecting RON-expressing or RON-silenced XPA-RFP cells into the flanks of nude mice. When the tumors reached 125 mm^3, mice were treated with gemcitabine (65 μg/g, approximately half of the maximally tolerated dose) twice weekly. Tumors were evaluated by both caliper measurement and noninvasive fluorescence imaging. Both RON-silenced and control tumors responded to gemcitabine treatment; however, the response in RON-silenced tumors was nearly complete. After 7 weeks of treatment, compared with controls, the volume of RON-silenced tumors was reduced by more than 12-fold ($P < 0.05$; Fig. 5A). However, as treatment continued, we noted that all controls and the majority of the RON-silenced tumors began to grow again, indicating an acquired resistance to gemcitabine therapy. This resistance, however, was significantly delayed in the RON-silenced group relative to the RON-expressing control group. It took 78 days for RON-silenced tumors to reach a mean volume of 1,000 mm^3 compared with 41 days for RON-expressing gemcitabine-treated tumors ($P < 0.05$), and 15 days for RON-silenced and RON-expressing tumors that went untreated (Fig. 5B). These data indicate that downregulation of the RON receptor tyrosine kinase acts to sensitize pancreatic cancer xenografts to the effects of gemcitabine.

Kinase switching occurs following escape from RON silencing. Finally, we sought to determine the mechanism (s) underlying the acquired resistance of RON-silenced

tumors to gemcitabine therapy. Given that cells can escape from shRNA-mediated gene silencing, we hypothesized that gemcitabine-resistant tumors would reexpress the RON receptor. Immunoblots comparing tumors before and after escape from gemcitabine treatment showed that reexpression of RON occurred in ~50% of tumors that acquired gemcitabine resistance. It was apparent that in some tumors, RON reexpression did not explain the acquisition of gemcitabine resistance. Given that cross-talk between RON and the RTKs c-met and EGFR has been shown previously, and the fact that these receptors have been implicated in pancreatic carcinogenesis, we reasoned that upregulation of alternative kinase signaling may be occurring and could potentially explain the acquisition of gemcitabine resistance (21, 22). Immunoblots for phospho-EGFR and phospho-met were initially performed on tumors before escape. No expression of phospho-met or phospho-EGFR was detected. In contrast, when we examined three tumors established from RON-silenced XPA cells that had escaped gemcitabine treatment, we found that two tumors reexpressed RON, all three expressed phospho-met, and one expressed phospho-EGFR (Fig. 6). Because the phosphoantibodies to EGFR and met recognize mouse antigen, we cannot completely exclude host immune cells as the source of the phosphorylated forms of these proteins. We believe this to be less likely, however, given that tumor xenografts of similar age, that had not escaped growth suppression, failed to express phosphokinases, despite a visibly similar host immune cell content.

Discussion

The RON receptor tyrosine kinase has been implicated as an oncogene in multiple epithelial cancers (23–26). Although only a single instance of a *RON* point mutation has been reported, active splice variants have been identified in colon cancers and in several cell lines of varying histology (27–30). In the majority of tumors, as in pancreatic cancer, the predominant mode of RON dysregulation seems to be overexpression of the receptor protein and/or its ligand. Such overexpression has been shown to confer a poor prognosis in breast and bladder cancer (31, 32). Despite these findings, few studies have directly examined the effects of RON on

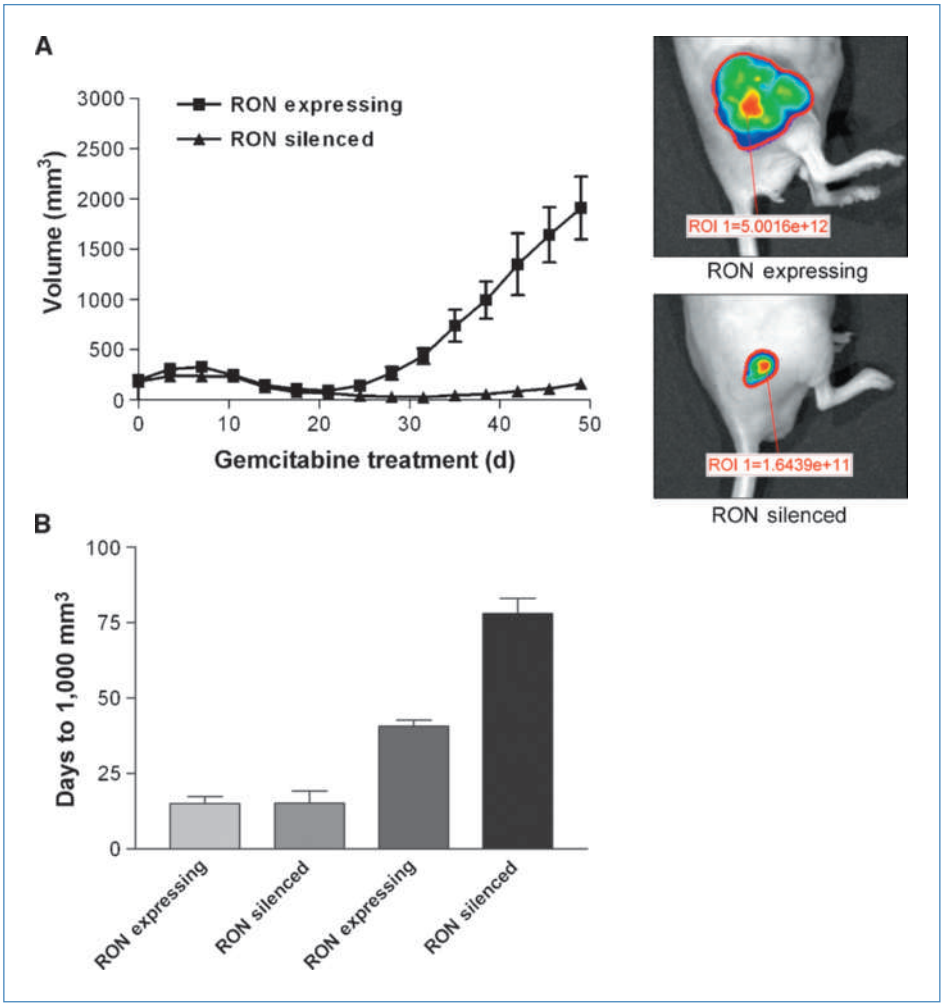

Figure 5. Downregulation of the RON receptor sensitizes pancreatic tumor xenografts to gemcitabine treatment. A, left, tumor regression after treatment with gemcitabine was more profound in RON-silenced tumors than in RON-expressing controls. Right, representative photographs using the IVIS Lumina live-animal imager. B, RON-silenced gemcitabine-treated tumors took nearly twice as long to reach 1,000 mm^3 as RON-expressing tumors and greater than three times longer than untreated tumors regardless of RON expression ($P < 0.05$).

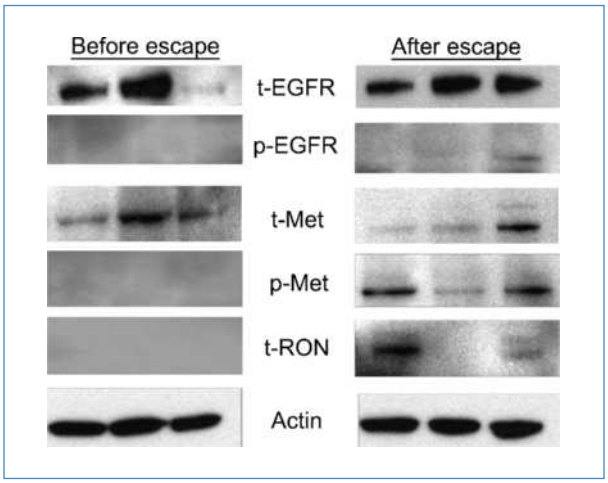

Figure 6. RON-silenced tumors show kinase switching after escape from gemcitabine-induced tumor regression. Before escape, there is no demonstrable presence of RON, p-EGFR, or p-met. Expression of pEGFR, p-met, and total RON is observed in tumors after escape, whereas total met and total EGFR were expressed both before and after escape.

known oncogenic signaling pathways *in vivo*, and none, to our knowledge, have investigated the role for RON signaling in modulating the response to traditional cytotoxic cancer therapy.

The prognosis of pancreatic cancer remains dismal, and it is therefore critical that new therapeutic targets for this disease be identified and validated. Several groups have now shown that the majority of pancreatic cancers overexpress the RON protein, yet its importance to pancreatic cancer growth and therapeutic resistance has not been directly shown (2–4). Importantly, RON has recently been associated with KRAS oncogene addiction in pancreatic cancer (5). In the present study, we have shown that RON signaling regulates the expression of numerous genes that encode proteins that promote cancer cell growth and survival. We initially identified this aspect of the RON-regulated transcriptome in cells derived from murine PanIN. This is significant as our prior studies failed to reveal any effect of RON on PanIN or pancreatic cancer cell proliferation, thereby raising the question as to how RON might contribute to pancreatic carcinogenesis. Our studies reveal that the transcriptome induced by RON signaling is highly conserved in pancreatic cancer cells, as we observed concordant effects on gene/protein expression when the RON transcriptome was studied by the exogenous delivery of the ligand to cultured murine PanIN cells, and when RON was silenced in human cancer cells and grown as tumor xenografts in nude mice.

We have identified novel RON-regulated genes, including *Bcl-2* and members of the AP-1 transcription factor complex, *c-jun, c-fos*, and *ATF-3*. The study of RON-silenced tumors confirmed prior *in vitro* data suggesting that RON is not a critical regulator of proliferation, but again pointed to RON as an important regulator of apoptotic resistance. It is noteworthy, however, that the effect on cell proliferation was variable between RON-deficient FG and XPA-1 cells, suggesting additional complexities to RON-regulated phenotypes. It is also notable that regardless of KRAS status, the loss of RON expression resulted in a marked decrease in expression of the activated forms of AKT and ERK1/2, suggesting that RON is an important activator of these pathways in pancreatic cancer cells *in vivo*.

Although our *in vitro* studies revealed that RON regulates VEGF production, our *in vivo* experiments suggest that, at least in this model system, RON signaling is not a critical regulator of angiogenesis. This finding is concordant with the findings of O'Toole and colleagues (6) who reported decreased growth in BxPC3–derived tumor xenografts treated with a RON-specific mAb, but no effect on tumor VEGF levels. Similarly, Jin and colleagues (33) recently described a novel met-directed antibody that reduced the growth of pancreatic cancer xenografts without any effect on tumor microvessel density.

Another important finding of the current study is that in many instances, XPA-1–derived tumor xenografts were able to escape the response to gemcitabine therapy. This mimics the clinical behavior of pancreatic cancers in that even when responses to gemcitabine and/or the EGFR inhibitor erlotinib are obtained, they are generally transient. It is therefore critical that we gain a better understanding of the mechanisms by which pancreatic cancer cells escape initially effective therapy. Stommel and colleagues (34) reported that in glioblastoma, it is often necessary to target multiple RTKs to achieve complete and sustained responses to RTK-directed therapy. We similarly hypothesized that escape from RON silencing was likely occurring through reactivation of RTK signaling, and this possibility is supported by our findings. In each of the RON-silenced tumors that had acquired resistance to gemcitabine, this was accompanied by reexpression of RON and/or activation of EGFR or c-met. We did not examine the entire kinome and obviously cannot rule out the activation of other kinases as well. Although these data do not directly prove RTK switching as the mechanism of resistance, it is highly likely given the lack of any such finding in tumors that remained growth suppressed. In addition, our findings are consistent with those of Shah and colleagues (35) who found that resistance to increasing doses of gemcitabine was accompanied by overexpression of phospho-met. We also cannot rule out the development of additional genetic mutations that underlie drug resistance in this model; however, this seems less likely given the kinetics and uniformity with which tumor escape occurred.

To directly test the hypothesis that kinase switching is underlying the escape from gemcitabine, we plan to pursue additional experiments to interrogate the targeted retreatment of tumors following escape from RON silencing. With the recent development of small-molecule inhibitors directed at RON and met, it will be of great importance to understand the molecular circuitry of tumors so that rational combination therapies can be designed.

Traditional evaluations of cancer therapies have relied on their ability to disrupt proliferation and induce objective tumor regression. Our study used cell lines that clearly do not

require RON receptor signaling for proliferation, yet the interruption of this pathway seems to be highly relevant to cancer cell survival, particularly in the setting of chemotherapy treatment. Currently, there are no biomarkers for tumors that are dependent on RON/met signaling for proliferation and/or survival. Given that proliferation alone does not seem to be an adequate marker for the potential utility of RON-directed therapy, clearly this is an area that demands investigation.

In summary, our studies reveal that RON receptor signaling mediates the viability of pancreatic cancer xenografts and that decreased RON signaling sensitizes cells to the effects of gemcitabine therapy. These effects seem to be mediated by the role of RON in promoting cell survival rather than through the effects on angiogenesis. Regardless of the KRAS status, RON signaling remains a potent regulator of both MAPK and PI3K/AKT signaling *in vivo*. RON also regulates the transcription of numerous genes involved in apoptotic resistance, such as *Bcl-2* and *STAT-3*. Finally, we show that pancreatic cancers can escape from RON silencing and gemcitabine therapy, suggesting mechanisms by which pancreatic cancer cells may circumvent RON-directed therapies. These findings suggest that further investigations into RON-directed therapies for pancreatic cancer are warranted and that RON-targeted agents may ultimately form part of an effective multidrug approach to pancreatic cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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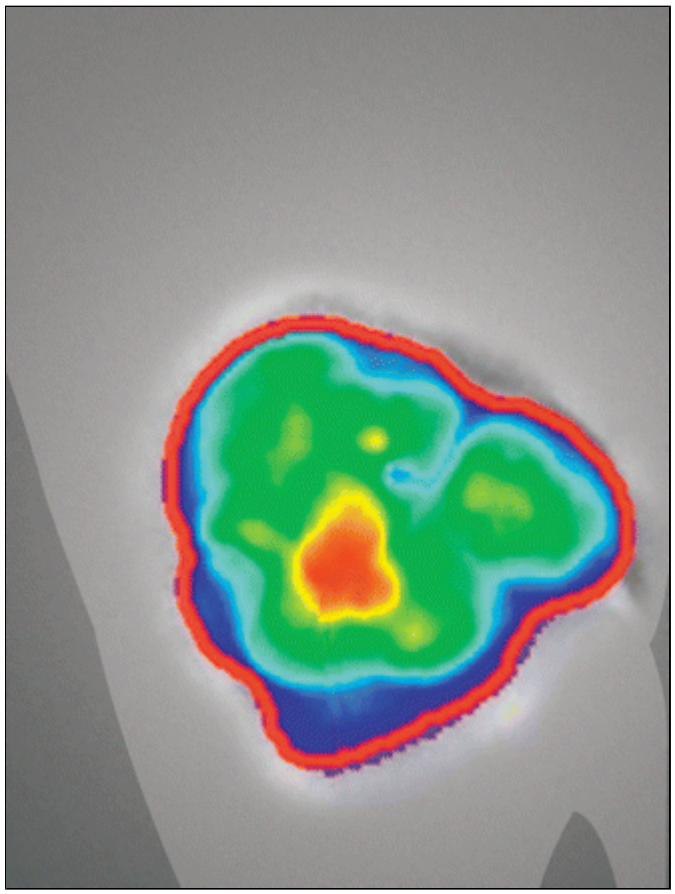
Cancer Research

About the Cover



The RON receptor tyrosine kinase is overexpressed in premalignant pancreatic intraepithelial neoplasia and in the majority of pancreatic cancers; however, the pathobiological significance of RON overexpression in pancreatic cancers has yet to be fully established. In this study, Logan-Collins and colleagues show that in both murine and human pancreatic cancer cells, RON signaling regulates the expression of genes implicated in cancer cell survival. shRNA-mediated silencing of RON in pancreatic cancer xenografts inhibited their growth, primarily by increasing susceptibility to apoptosis and by

sensitizing them to gemcitabine treatment. In addition, the authors show that escape from RON silencing is associated with re-expression of RON and/or expression of phosphorylated forms of the related c-Met or epidermal growth factor receptors. Given these findings, the authors propose that RON signaling mediates cell survival and *in vivo* resistance to gemcitabine in pancreatic cancer, and they reveal mechanisms through which pancreatic cancer cells may circumvent RON-directed therapies. For details, see the article by Logan-Collins and colleagues on page 1130 of this issue.

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