Published in final edited form as:

Cancer Res. 2010 February 1; 70(3): 1130–1140. doi:10.1158/0008-5472.CAN-09-0761.

Silencing RON Receptor Signaling Inhibits Growth and Sensitizes Pancreatic Cancer Xenografts to Gemcitabine

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Abstract

The RON receptor tyrosine kinase is overexpressed in pancreatic intraepithelial neoplasia (PanIN) and the majority of pancreatic cancers. Exposure of cultured pancreatic cancer cells to RON ligand enhances migration/invasion and apoptotic resistance and RON was recently identified as a key effector of K-Ras signaling in pancreatic and lung cancer cells. The significance of RON overexpression in pancreatic cancer cells in vivo, however, remains unknown. In this study, we demonstrate that RON signaling mediates a unique transcriptional program that is conserved between cultured cells derived from murine PanIN and 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 STAT-3, and c-jun. We further demonstrate that shRNA silencing of RON in pancreatic cancer xenografts inhibits their growth, primarily by increasing their susceptibility to apoptosis and sensitizes them to gemcitabine treatment. Finally, we show that escape from RON-silencing is associated with re-expression of RON and/or expression of phosphorylated forms of the related receptor c-met or the epidermal growth factor receptor. Taken together, these findings suggest that RON receptor signaling regulates pathways important for pancreatic cancer cell survival and resistance to gemcitabine in vivo and suggests mechanisms by which pancreatic cancer cells may circumvent RON-directed therapies.

Keywords

RON Receptor; Pancreatic Cancer; Gemcitabine; Tyrosine Kinase; Hepatocyte Growth Factor Like Protein

Introduction

The median survival of pancreatic cancer patients remains less than one year and the disease incidence continues to rise, now making it the 4th 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 potential novel therapeutic target in pancreatic cancer, a finding 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 and 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. While RON overexpression has been demonstrated, no mutations or major splice variants of RON have been reported in pancreatic cancer specimens. It even remains unclear whether 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 RON ligand. Our studies revealed that RON regulates the expression of multiple genes that promote cancer cell survival including Bcl-2, STAT-3, VEGF, c-jun and c-fos. In order to further investigate the importance of RON signaling to pancreatic cancer cell survival, we utilized shRNA technology to silence RON expression in two pancreatic cancer cell lines, XPA-1 and FG. 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 both FG and XPA-derived tumors demonstrated enhanced susceptibility to spontaneous apoptosis. In addition, XPA-1-derived RON-deficient tumors, demonstrated enhanced susceptibility to treatment with gemcitabine chemotherapy. Finally, we demonstrate that escape from RON-silencing occurs via re-expression of the receptor and/or upregulation of the receptor tyrosine kinases c-met and EGFR. These studies suggest that RON signaling contributes to pancreatic cancer cell survival and therapeutic resistance in vivo and also suggests 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 and maintained in DMEM (2,7). 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 line were kindly provided by Dr. Michael Bouvet, La Jolla, CA and maintained in RPMI media with 1% sodium pyruvate and 1% non-essential amino acids. The FG cell line was kindly provided by Dr. David Cheresh and maintained in DMEM High Glucose media. All media was supplemented with 1% sodium pyruvate and 1% non-essential amino acids with 10% FBS 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 a 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 puromycin 2.5µg/ml for 5-10 days for the XPA-1-RFP clones, and 1µg/ml for 21 days 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 Nhe1-BamH1 sites was reverse transfected into the cells using Lipofectamine 2000. Following transfection cells were FAC sorted and expanded and maintained in 5µg/ml puromycin.

Genechip Studies

To identify genes whose expression was altered in murine PanIN cells by the RON ligand HGFL, total RNAs were isolated from three independent samples of PanIN cells. At 60-80% confluency, PanIN cells were washed 3 times and then divided into 3 treatment groups: 1) 10% serum media, 2) 400ng/ml of the RON-specific ligand HGFL (recombinant human MSP; Cat # 352-MS; R&D Systems) in 10% serum media for 30 minutes, or 3) 400ng/ml of HGFL in 10% serum media for 12 hours. Cells were then washed 3 times 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 RNA. Biotinylated cRNA was purified with RNeasy columns and as hybridized to Affymetrix MOE430plus2 microarrays using standard procedures (10). The 430 v2.0 array contains 45,000 probe sets representing over 34,000 mouse transcripts.

Bioinformatic Analyses

CEL files were generated from GCOS 5.0 and subjected to RMA normalization as implemented in GeneSpring 7.1. Probesets 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 and Students t-test false discovery rate not more than 5%. This yielded a list of 858 probesets that were referenced to the corresponding control values and subjected to hierarchical clustering using Pearson Correlation. Clusters were evaluated for gene co-functional relationships using the Gene Set Enrichment Analysis algorithm as implemented by the Toppgene server (11).

Quantitative RT-PCR

For quantitative RT-PCR, total RNA was purified as described above. 500 to 1000 ng of RNA was converted to cDNA using random hexamers and Superscript III [Invitrogen, Carlsbad, CA]. Amplification was carried out with an ABI 7300 real time PCR system [Applied Biosystems, Foster City, CA] using SYBR green. The reference gene was beta-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' GAATACATAGACCAGGCCCAAGAATCG 3') were designed to span an intronic sequence and were validated by PCR and gel analysis.

Mice and in vivo tumor studies

5–8 week old athymic nude mice (NCI Frederick) were housed in accordance with the NIH guide for 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, the vector control, and the RON-silenced cell line [XPA-1 only]). 200 μ l of RPMI media for the XPA-1-RFP and 200 μ l of DMEM media for the FG-Mcherry with 5×10^6 cells were injected subcutaneously into the flank using a 25 gauge needle. Tumor volume was measured with calipers twice weekly using the formula $(\text{length} \times \text{width}^2)/2$. Mice were anesthetized and photon emission was measured from the tumors twice weekly using a live-animal fluorescence imager (IVIS® Lumina Imaging System) for XPA-1-RFP mice and an OV100 (Olympus housed at AntiCancer Inc. San Diego, CA) for the FG-Mcherry mice. For tumor growth studies, 16 tumor xenografts were developed for each group using bilateral flank injections in 8 mice. Mice were euthanized and tumors harvested 30 days after implantation. For BrdU studies, mice underwent intraperitoneal (IP) injection of 150 μ g/g BrdU (XPA-RFP Sigma-Aldrich, FG-MCherry, BD Biosciences) 2 hours prior to sacrifice. For CD31 and 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 mm³, animals were randomly assigned to 1 of 2 treatment groups: 1) control animals that received no additional treatment or 2) gemcitabine-treated animals that received 65 μ g/g of drug via IP injection twice weekly. For the mice receiving FG-Mcherry cells, a total of 12 xenografts were established in 6 mice. 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 harvested when tumor volume reached 2000 mm³. Three XPA-RFP mice with RON-silenced tumor xenografts were euthanized and sacrificed when treatment with gemcitabine had reduced the tumor size to less than 40 mm³. RON-Silenced FG-Mcherry mice whose tumor xenografts had not grown beyond 400 mm³ after 8 weeks, were euthanized at that time.

Histology and Immunohistochemistry

XPA-1-RFP xenograft tumors from non-therapeutic studies were harvested and immediately fixed in 10% formalin; paraffin embedded and cut into 5 μ m sections. The FG-Mcherry xenograft tumors were harvested and immediately embedded in OCT and cut into 10 μ m sections. Sections were deparaffinized in xylene and rehydrated through a graded series of ethanol/water solutions. Samples were stained for hematoxylin and eosin. A BrdU detection kit (Invitrogen) and CD31 antibody (BD Pharmingen) was used for immunohistochemistry per the manufacturer's instructions. A TUNEL assay (Chemicon International) was performed per the manufacturer's instructions and 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 blinded such that scoring was performed in an unbiased manner. Percent necrosis was determined by calculating the percentage of necrotic area relative to total tumor area. BrdU incorporation was scored by counting the number of cells staining positive for BrdU on 200 $\times$ high-powered field in 4 viable random fields for each tumor specimen. For CD31 counts, 3 areas of peripheral tumor, devoid of necrosis and showing the highest vascularity were identified by evaluating histological sections at 100 $\times$ magnification. Vessels were then counted at 200 $\times$ magnification. For TUNEL scores on the XPA-1-RFP tumors, Axiovision Release 4.5 software was used while for the FG-Mcherry tumors Metamorph software was used to capture and evaluate images at 200 $\times$ 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 (μ m²) of viable tumor.

Immunoblotting, Immunoprecipitation and ELISA

Tumors were snap-frozen prior to processing. They were placed on dry ice and homogenized in RIPA buffer containing complete protease inhibitors and PhosSTOP phosphatase inhibitors (Roche Applied Science). The lysates were left on ice for 30 minutes followed by centrifugation at 15,000g for 15 minutes and supernatants 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 and analyzed on SDS-PAGE. For immunoprecipitations, 500µg of tumor lysates were incubated with 1µg of RON C-20 (Santa Cruz Biotechnology) for 30 minutes on ice followed by the addition of Protein A/G UltraLink Resin (Pierce) for 1 hour at 4°C with rotation. The beads were washed two quick times followed by two 15 minute washes in RIPA buffer at 4°C with rotation. After removal of the final wash, the beads were resuspended in 1× NuPAGE LDS sample Buffer (Invitrogen) containing 1× NuPAGE sample reducing agent (Invitrogen) and incubated at 60°C for 30 minutes 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), 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 mAb 4G10, anti-EGFR, anti-phospho-EGFR (9H2-Tyr¹¹⁷³) were purchased from Millipore. Goat anti-mouse-HRP (Chemicon/Millipore Inc.) and goat anti-rabbit-HRP (Santa Cruz Biotechnology) were used as secondary antibodies at 1:5000. The reaction was developed with ECL Plus reagent (GE Healthcare).

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

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA). One-way ANOVA or 2-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 PI3K/AKT, 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 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 (Figure 1). As has been seen for the met receptor, a dichotomous pattern of gene expression appeared (15). After 30 minutes, 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 (3–10 fold) of numerous transcription factors including *c-jun*, *c-fos* and *atf-3* of the 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*. Genechip findings were validated using Western blot and quantitative PCR. This data suggests that through 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 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 utilized the human pancreatic cancer cell lines, XPA-1, and FG and stably transfected them with shRNA's specific to the RON receptor transcript or a nonsense control. FG cells are mutant for both KRAS and P53 while, 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 over-expresses RON receptor. Interestingly, we were unsuccessful in our attempt to develop RON-deficient BxPc3 cell lines suggesting that RON may be a critical regulator of cell survival or proliferation in this line. In contrast, RON deficient XPA-1 and FG cells were viable and immunoblot demonstrated that RON expression was reduced by greater than 95% in the shRON-XPA-1 cells relative to controls and approximately 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 utilized 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 (Figure 2A), we next examined their growth characteristics. RON-deficient FG cells immediately demonstrated growth inhibition as compared to controls (Figure 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, BrdU incorporation was not statistically different in RON-deficient cells versus parental cells in either FG or XPA-1, (Figure 2C,D). When tumors were excised, we noted significantly more necrosis in RON-deficient tumors, particularly in the XPA-1 derived subset. We quantified necrosis after hematoxylin and eosin staining and observed an 85% increase in the area of necrosis within RON-deficient tumors as compared to controls ($p=0.001$) (Figure 3A). Evaluation of photon emission from RON-deficient FG and XPA-1 xenografts demonstrated a 3 fold relative decrease ($p=0.002$) in emission from RON-silenced tumors compared to RON-expressing controls, suggesting significantly less viable tumor was present within the RON-deficient tumors. (Figure 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 demonstrate that while 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 anti-angiogenic therapies may result in increased tumor necrosis and associated decreased tumor microvessel counts (16,17). Our GeneChip studies demonstrated 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 non-necrotic 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 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 demonstrated 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 the shRON-XPA-1, shRON-FG and control tumor tissues. When examining only the cellular areas of the tumors, we found 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) (Figure 4A). This finding suggests that an increase in apoptosis was primarily responsible for the decreased 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 (Figure 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 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 pancreas (12). Bcl-2, a potent pro-survival 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 p-ERK, p-AKT, c-Jun, STAT3, and bcl-2 corresponding to a decrease in RON expression in RON-deficient XPA-1 derived tumors (Figure 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 demonstrate 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 demonstrated that inhibiting RON receptor signaling sensitizes pancreatic cancer cells to gemcitabine in vitro (2). Given the new findings that RON signaling appears 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 mm³ 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 125mm³, mice were treated with gemcitabine (65µg/g- approximately half of the maximally tolerated dose) twice weekly. Tumors were evaluated by both caliper measurement and non-invasive 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 to controls, the volume of RON-silenced tumors was reduced by more than 12-fold ($p < 0.05$) (Figure 5A). However, as treatment continued, we noted that all control and the majority of the RON-silenced tumors began to grow again, indicating 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 1000 mm³ as compared to 41 days for RON-expressing, gemcitabine treated tumors ($p < 0.05$), and 15 days for RON-silenced and RON-expressing tumors that went untreated (Figure 5B). Together 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 re-express RON receptor. Immunoblots comparing tumors before and after escape from gemcitabine treatment demonstrated that re-expression of RON occurred in approximately 50% of tumors that acquired gemcitabine resistance. It was apparent that in some tumors, RON re-expression did not explain the acquisition of gemcitabine resistance. Given that cross-talk between RON and the receptor tyrosine kinase's (RTK's) c-met and EGFR has been demonstrated 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 acquisition of gemcitabine resistance (21,22). Immunoblots for phospho-EGFR and phospho-met were initially performed on tumors prior to 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, two re-expressed RON, all three expressed phospho-met and one expressed phospho-EGFR (Figure 6). Because, the phosphoantibodies to EGFR and met recognize mouse antigen, we cannot completely exclude host immune cells as the source of the phospho-forms of these proteins. We believe this to be less likely however, given that tumor xenografts of similar age which had not escaped growth suppression failed to express phosphokinases despite visibly similar host immune cell content.

Discussion

The RON receptor tyrosine kinase has been implicated as an oncogene in multiple epithelial cancers (23–26). While 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 appears 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 known oncogenic signaling pathways in vivo and none, to our knowledge, have investigated a role for RON signaling in modulating 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 demonstrated that the majority of pancreatic cancers overexpress the RON protein, yet its importance to pancreatic cancer growth and therapeutic resistance have not been directly demonstrated (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 expression of numerous genes that encode proteins which 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 exogenous delivery of 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 RON is an important activator of these pathways in pancreatic cancer cells in vivo.

While 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 et al. who reported decreased growth in BxPc-3 derived tumor xenografts treated with a RON-specific monoclonal antibody, but no effect on tumor VEGF levels (4). Similarly, Jin et al. recently described a novel met-directed antibody that reduced the growth of pancreatic cancer xenografts without any effect on tumor microvessel density (33).

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 as 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 et al. reported that in glioblastoma, it is often necessary to target multiple RTK's to achieve complete and sustained responses to RTK-directed therapy (34). We similarly hypothesized that escape from RON-silencing was likely occurring via 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 re-expression 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. While this data, does 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 et al. who found that resistance to increasing doses of gemcitabine was accompanied by overexpression of phospho-met (35). 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 escape from gemcitabine, we plan to pursue additional experiments to interrogate targeted retreatment of tumors post-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 such 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 utilized cell lines which clearly do not require RON receptor signaling for proliferation, yet interruption of this pathway appears to be highly relevant to cancer cell survival, particularly in the setting of chemotherapy treatment. Currently

there are no biomarkers for tumors which 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 RON's role in promoting cell survival rather than through 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 demonstrate 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.

Acknowledgments

Financial Support: This work was supported by NIH CA89403 (AML), NIH CA 45726 (AML), NIH CA100002 (SEW), and NIH T32 DK64581 (JLC and RMT).

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Figure 1.

Activation of the RON receptor results in 858 differentially expressed genes after 30 minutes and 12 hours. Affymetrix gene chip analyses were performed using PanIN cells treated with 400ng/ml for HGFL for 0 minutes (untreated), 30 minutes or 12 hours. 311 genes were differentially expressed at 30 minutes while 582 were altered at 12 hours. Red areas represent genes that were upregulated and blue areas represent 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 = non-silencing shRNA vector transfected cell line, S = RON-silenced cell line. B) Growth of FG-derived tumor xenografts. Tumors were measured twice weekly via calipers and growth was plotted. C,D) Mice were injected with BrdU 2 hours prior to sacrifice. Sectioned tumors were stained and scored for BrdU 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.

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 days of growth ($p = 0.002$).

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, stat3. U = tumors derived from untransfected parental cells, N = non-silenced 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; while for FG tumors, levels of p-ERK, pAKT and stat-3 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 days of growth.

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 1000mm³ as RON-expressing tumors and greater than 3× longer than untreated tumors regardless of RON expression ($p < 0.05$).

Figure 6.

RON-silenced tumors demonstrate kinase switching after escape from gemcitabine-induced tumor regression. Prior to 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 pre-escape and post-escape.