CD44 Regulates Pancreatic Cancer Invasion through MT1-MMP

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Abstract

Pancreatic cancer is one of the deadliest human malignancies due to its early metastatic spread and resistance to therapy. The mechanisms regulating pancreatic cancer metastasis are so far poorly understood. Here, using both in vitro and in vivo approaches, it is demonstrated that CD44, a transmembrane glycoprotein expressed on a subset of pancreatic cancer cells, is required for the induction of epithelial–mesenchymal transition (EMT) and the activation of an invasive program in pancreatic cancer. Mechanistically, the transcription factor Snail1 (SNAI1), a regulator of the EMT program, is a downstream target of CD44 in primary pancreatic cancer cells and regulates membrane bound metalloproteinase (MMP14/MT1-MMP) expression. In turn, MT1-MMP expression is required for pancreatic cancer invasion. Thus, these data establish the CD44–Snail–MMP axis as a key regulator of the EMT program and of invasion in pancreatic cancer.

Implications: This study sets the stage for CD44 and MT1-MMP as therapeutic targets in pancreatic cancer, for which small molecule or biologic inhibitors are available.

Visual Overview: http://mcr.aacrjournals.org/content/early/2014/09/10/1541-7786.MCR-14-0076/F1.large.jpg

Mol Cancer Res; 13(1); 9–15. ©2014 AACR.

Introduction

Pancreatic cancer is one of the deadliest human malignancies, with a 5-year survival of less than 6%, and a prognosis largely unchanged over the course of the past 40 years. Most patients with pancreatic cancer present with metastases at diagnosis. Thus, understanding the drivers of metastasis in pancreatic cancer is of key therapeutic value.

Pancreatic cancer is almost invariably associated to oncogenic mutations in the Kras gene (1, 2). Expression of mutant Kras in the tumor suppressor p53 in the pancreatic tumors in iKrasmice and cultured as previously described (10). The cells were always maintained in doxycycline (1 µg/mL of doxycycline; Sigma), to activate expression of oncogenic Kras, unless otherwise stated. The human pancreatic cancer cell line Hs766T is available through the ATCC. UM2, UM18, and 1319 are primary human pancreatic cancer cells derived from patients with confirmed diagnosis of pancreatic ductal adenocarcinoma (University of Michigan).

Materials and Methods

Cell culture

The primary cell lines used in this study were isolated from pancreatic tumors in iKrasp53−/− mice and cultured as previously described (10). The cells were always maintained in doxycycline (1 µg/mL of doxycycline; Sigma), to activate expression of oncogenic Kras, unless otherwise stated. The human pancreatic cancer cell line Hs766T is available through the ATCC. UM2, UM18, and 1319 are primary human pancreatic cancer cells derived from patients with confirmed diagnosis of pancreatic ductal adenocarcinoma (University of Michigan).
Western blot analysis
Cells were lysed in RIPA buffer (Sigma-Aldrich; R0278) and protease inhibitor (Sigma-Aldrich; P8340). Western blot analysis was performed as previously described (11). The rat anti-mouse CD44 antibody KM114 from BD bioscience was used at a 1:1,000 dilution.

Quantitative RT-PCR
RNA extraction and quantitative reverse transcription-PCR (qRT-PCR) were performed as previously described (11). Gapdh was used as the housekeeping gene expression control. A list of the primers used is provided in Supplementary Table S1.

Short interfering RNA transfection
Control or target-specific siRNAs were purchased from Sigma (siRNA), Life Technologies (shRNA), and OriGene (ORF) in pCMV6 and control vector pCMV6 were purchased from OriGene. Transfection of CD44s plasmid was used as the housekeeping gene expression control. A list of the primers used is provided in Supplementary Table S1.

Transfection of CD44s plasmid
Plasmids expressing cDNA of CD44s (ORF) or MT1-MMP (ORF) in pCMV6 and control vector pCMV6 were purchased from OriGene.

Collagen invasion assay
Type I collagen was prepared from rat tail (BD Bioscience) in 0.2% acetic acid to a final concentration of 2.7 mg/mL; gelling was induced in upper well of a 6-well transwell dish (3-mm pore size; Corning, Inc.). After gelling was complete (45 minutes at 37°C), 1.5 × 10^5 cells in complete medium were added to the upper well and 2.5 mL of medium was added to the lower chamber. Invasion assays were routinely terminated after 3 days. Invasion depths were measured from digitally captured images of hematoxylin and eosin–stained cross-sections.

Orthotopic pancreatic cancer xenograft
Two groups of cells, control shRNA–infected cells and CD44 shRNA–infected cells (5 × 10^5), were injected in the pancreatic tail of NOD-SCID mice (6 per group). Mice were monitored daily and sacrificed when the control group became moribund.

Detailed Experimental procedures are provided in Supplementary Methods.

Results and Discussion
CD44 expression in mouse pancreatic cancer correlates with EMT
CD44 expression was previously detected in a subset of mouse pancreatic cancer cells in the KPCY mouse model (4). To determine its expression in iKras^p53^#1a mice, we stained primary tumors and metastases samples by immunohistochemistry. Both primary pancreatic cancer and liver metastasis contained CD44-positive cells; in contrast, CD44 expression was not detected in normal pancreatic and liver tissue (Supplementary Fig. S1A).

To determine the specific isoform of CD44 expressed in pancreatic cancer cells, we used primary mouse pancreatic cancer cells derived from iKras^p53^#1a mice (10), which can be subdivided in two subgroups based on morphology. The epithelial group forms stroma-rich tumors when transplanted in host mice. In contrast, mesenchymal lines have a fibroblast-like morphology and form tumors largely devoid of stroma (10). Interestingly, these subsets are reminiscent of the "epithelial" or "classical" and "quasi-mesenchymal" subtypes of human pancreatic cancer recently described (12). For the current study, used a panel of four primary cell lines, two classified as epithelial (iKras^p53^#1a, iKras^p53^#3) and two as mesenchymal (iKras^p53^#2, iKras^p53^#1b) based on morphology (Fig. 1A). Of note, the iKras^p53^#1a and iKras^p53^#1b cell lines were derived from the same primary tumor; the mesenchymal iKras^p53^#1b subclone was isolated from a mostly epithelial initial culture. The use of cell lines derived from the iKras^p53^# model gave us the opportunity to modulate the expression of oncogenic Kras by administering, or withdrawing, doxycycline from the growth medium (10).

To determine whether the different morphologic characteristics of the cells correlated with a specific gene signature, we measured the expression of epithelial (E-cadherin) and EMT markers (Snail1, Zeb1, and Twist1) by qRT-PCR (Fig. 1B). Consistent with the noted morphologic features, E-cadherin mRNA level was significantly lower in the mesenchymal cell lines (iKras^p53^#1b and iKras^p53^#2) and higher in the epithelial cell lines. Conversely, Snail1, Zeb1, and Twist1 expressions were higher in the mesenchymal cell lines (iKras^p53^#1b, iKras^p53^#2), compared with the epithelial cell lines (iKras^p53^#1a, iKras^p53^#3). Thus, the gene signature of the cells confirmed the morphology-based classification. These findings are consistent with the notion that human pancreatic tumors can be classified in subtypes with epithelial or mesenchymal characteristics (12).

We then determined the expression of different CD44 isoforms in the pancreatic cancer cell lines. We used RT-PCR and Western blot to measure the expression of CD44s and CD44v, respectively, in the iKras^p53^#2, iKras^p53^#1a, iKras^p53^#3, and iKras^p53^#1b cells (Fig. 1C). Interestingly, the mesenchymal cells (iKras^p53^#2, iKras^p53^#1b) expressed high levels of the standard CD44 mRNA and protein, in addition to the variant isoforms of CD44. In contrast, the epithelial cells (iKras^p53^#1a) did not express CD44s at the mRNA or protein level.

To complete the characterization of these cell lines, we investigated their invasive potential using a Boyden Chamber Matrigel Invasion Assay. In this assay, the epithelial iKras^p53^#1a cells showed no invasive capabilities (Supplementary Fig. S1B). In contrast, the mesenchymal iKras^p53^#1b cells invaded through Matrigel, in a Kras-dependent manner (Supplementary Fig. S1B and S1C). The results were confirmed and further expanded with additional cell lines using a Collagen Invasion Assay, which is more relevant to the in vivo situation given the collagen-rich matrix of pancreatic tumors. The epithelial iKras^p53^#1a and iKras^p53^#3 cells did not invade through the collagen; however, the mesenchymal iKras^p53^#1b and iKras^p53^#2 cells were able to invade (Fig. 1D). Thus, we identified a correlation between the expression of the CD44s isoform, mesenchymal phenotype, and invasive capabilities of the pancreatic cancer cells in vivo. This result parallels previously described findings in mouse models of pancreatic cancer (4).

CD44s induces EMT in pancreatic cancer cells
To determine whether there was a causative link between CD44s expression and the acquisition of a mesenchymal phenotype, we experimentally manipulated the expression of CD44s. Transfection of iKras^p53^#1a cells with a CD44s expression vector produced a shift to a mesenchymal morphology compared with control
transfected cells (Fig. 2A). We next evaluated whether CD44s expression led to changes in the expression of epithelial and mesenchymal genes. We found that expression of CD44s in iKras\(^{p53}#1a\) cells resulted in near complete inhibition of E-cadherin mRNA levels (Fig. 2B), whereas expression of the mesenchymal marker Vimentin was increased approximately 2-fold. Then, we measured the expression of three transcription factors linked to the EMT process: Twist1, Zeb1, and Snail1. We found no
Figure 2.
CD44 induces EMT and is required for invasion in pancreatic cancer cells. A, qRT-PCR analysis for CD44 in iKras^p53^#1a cells transfected with control or CD44s expression vector (left) and morphology change of iKras^p53^#1a cells expressing control or CD44s vector (right). B, qRT-PCR analysis for EMT genes. C, qRT-PCR analysis for cells transfected with control siRNA or anti-CD44 siRNA. D, Collagen invasion assay. E, Hematoxylin and eosin staining of pancreas (top) and liver (bottom) following orthotopic transplantation of control shRNA or anti-CD44 shRNA infected iKras^p53^#1b cells. Quantification of the metastatic area.
change in expression in the first two genes but a significant increase in Snail1 expression upon overexpression of CD44s. Thus, CD44 expression activated the expression of at least one EMT regulator and caused a change in phenotype consistent with EMT.

Then, taking the opposite approach, namely inhibition of CD44, we sought to determine whether there was a functional relationship between CD44 expression, expression of EMT markers, and invasive ability. Anti-CD44 siRNA transfection effectively reduced CD44 (all isoforms) expression (Fig. 2C, left). Moreover, cells transfected with the anti-CD44 siRNA had only a moderate decrease in Twist1 expression and no change in Zeb1 expression, but a significant decrease in the expression of Snail1, MMP2, and MMP-9, and conversely an increase in E-cadherin expression (Fig. 2D and Supplementary Fig. S2A and S2B). Finally, to further investigate whether CD44s levels regulate Snail, and thus MT1-MMP expression, we targeted the splicing factor ESRP1 (epithelial splicing regulatory protein 1). ESRP1 promotes CD44 alternative splicing, thus reducing the levels of CD44s (13). Indeed, our data show that siRNA-mediated inactivation in iKrasp53"#1a cells results in an increase of CD44s, and a corresponding increase in Snail and MT1MMP mRNA. (Supplementary Fig. S3D). Taken together, these data support the notion that CD44s regulate Snail in pancreatic cancer.

Our results indicate that CD44 is a regulator of Snail expression. We therefore sought to investigate the mechanism of this regulation. Several effector pathways are activated downstream of CD44 in different contexts, including p-AKT, MAPK, and Yap1 (8), but Western blot and qRT-PCR analyses did not reveal significant changes in any of those (Supplementary Fig. S3A and data not shown). In contrast, we observed changes in Wnt/β-catenin signaling following modulation of CD44. The expression of the Wnt target gene LeF1 was reduced upon CD44

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**Figure 3.**

A CD44–Snail1–MT1-MMP axis regulates pancreatic cancer invasion. A, MT1-MMP mRNA level upon CD44 knockdown alone or combined with re-expression of Snail1, P < 0.01. B, Collagen invasion assays in control cells, or cells treated with a MT1-MMP siRNA or inhibitor. C, Collagen invasion assays upon CD44 knock-down alone or combined with re-expression of MT1-MMP. D, CD44S expression in the resected human pancreatic cancer and matched adjacent uninvolved pancreas. E, CD44S and MT1-MMP expression in human pancreatic cancer cell lines. F, Matrigel invasion assay (left) of human pancreatic cancer cell lines treated with MT1-MMP inhibitor and quantification (right).
knock-down; consistently, β-catenin levels were reduced upon CD44 knock-down and increased upon CD44 overexpression. Phospho-GSK-3β (Ser9) levels increased upon CD44 overexpression (Supplementary Fig. S3B), consistent with decreased GSK-3β activity. GSK-3β is a known negative regulator of β-catenin. Moreover, GSK-3β is a negative regulator of Snail1 (14, 15). Thus, our data are consistent with previous work identifying Wnt signaling as a key modulator of the EMT gene program (15). Moreover, it identifies a potential positive feedback loop between CD44 and Wnt signaling in pancreatic cancer.

CD44s regulates invasion in pancreatic cancer cells

Finally, we performed collagen assays to determine whether inhibition of CD44 led to changes in the invasive properties of the cells. As expected, no change was observed in the noninvasive iKras/p53−β1a cell line. However, in both iKras/p53−β1b and iKras/p53−β2 cells, anti-CD44 siRNA transfection led to almost complete abrogation of invasion compared with control siRNA–treated cells (Fig. 2C, right). We then confirmed this result using the CD44-blocking antibody KM114. Thus, CD44 expression is required to confer invasive properties in pancreatic cancer cells in vitro.

CD44 regulates pancreatic cancer metastasis in vivo

Next, we evaluated the requirement of CD44 in pancreatic cancer–induced liver metastasis. Mouse pancreatic cancer cell lines iKras/p53−β1b were infected with either control vector or CD44 shRNA and then orthotopically injected to the pancreatic tail of NOD/SCID mice (6 mice per group). Pancreatic, liver, and lung tissue were harvested and analyzed at the time of death or at 2 weeks. All the animals had large primary tumors at the site of injection. Moreover, the mice injected with pancreatic cancer cells infected with control vectors had extensive liver metastasis (in average 10% of the total liver area in each section analyzed). These animals died or reached humane euthanasia endpoints due to elevated disease burden. Mice injected with pancreatic cancer cells infected with CD44 shRNA appeared healthy at the same time point, but were sacrificed to compare tumor growth. Although we did not observe changes in the primary tumor, we observed a significant reduction in the metastatic burden, with only rare, small metastases (Fig. 2E). Thus, this set of experiments provides in vivo evidence that CD44 is required for pancreatic cancer invasion.

CD44 regulates pancreatic cancer cell invasion through MT1-MMP

Our data so far indicate that Snail1 expression is activated downstream of CD44. Snail1 has been shown to regulate the expression of MT1-MMP, a membrane matrix metalloproteinase with strong links to tumor invasion (16). Interestingly, we observed that MT1-MMP was expressed 300- to 750-fold higher in the mesenchymal cell lines compared with the epithelial lines (Supplementary Fig. S2D). Overexpression of CD44 in the epithelial iKras/p53−β1a cells significantly increased the expression of MT1-MMP (Supplementary Fig. S2E). Thus, MT1-MMP expression is activated downstream of CD44 in pancreatic cancer cells. Re-expression of the transcription factor Snail1, in the mesenchymal cells with CD44 knockdown, rescued the expression of MT1-MMP (Fig. 3A). In contrast, re-expression of Zeb1 or Zeb1 knock-down had no effect on MT1-MMP expression (Supplementary Fig. S3C). These data indicate that CD44 regulates MT1-MMP expression through Snail1.

Inhibition of MT1-MMP with either siRNA or a small-molecule inhibitor resulted in significant inhibition of collagen invasion (Fig. 3B), indicating that MT1-MMP is necessary for pancreatic cancer invasion. To determine if MT1-MMP expression could rescue the invasive potential of cancer cells when CD44 was inactivated, we overexpressed MT1-MMP in mesenchymal cell lines treated with either CD44 or control shRNA. As shown previously, inactivation of CD44 in the mesenchymal cell lines inhibited collagen invasion. However, re-expression of MT1-MMP in mesenchymal cell lines with CD44 knockdown rescued the invasive phenotype (Fig. 3C). MT1-MMP drives invasion in breast cancer (16–18). Moreover, MT1-MMP has been associated with pancreatic cancer fibrosis and desmoplastic reaction (18, 19). Our data identifies a CD44–Snail1–MT1-MMP axis that regulates EMT and invasion in pancreatic cancer.

MT1-MMP is expressed in human pancreatic cancer cells and regulates invasion

To determine the relevance of our findings to human pancreatic cancer, we first analyzed the expression of CD44s in RNA extracted from resected pancreatic cancer and matched adjacent uninvolved pancreas. We found that in two of three sample sets CD44s was significantly overexpressed in the tumor compared with the nontumor tissue (Fig. 3D). Similar variability was observed when CD44s expression was analyzed in a panel of four human pancreatic cancer cell lines (Fig. 3E). MT1-MMP expression was detected only in the two human cell lines that had high CD44s expression (Fig. 3E). Finally, we performed Matrigel invasion assays with the two human cell lines that had MT1-MMP expression, with or without MT1-MMP inhibitor. In both cell lines, MT1-MMP inhibition significantly decreased the invasion ability of the cells (Fig. 3F). Thus, MT1-MMP is important in migration at least in a subset of human pancreatic cancer cells. However, human samples have higher variability than their mouse counterpart, possibly due to their higher genome complexity.

Significance

CD44 and MT1-MMP are surface molecules, and as such valid therapeutic targets. In fact, blocking antibodies against CD44 (20–24) and MT1-MMP inhibitors (22) have been described, and new ones are likely to be developed. Our work provides rationale for further exploration of these molecules for pancreatic cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: W. Jiang, D.M. Simeone, M.P. di Magliano, K.T. Nguyen

Development of methodology: W. Jiang, K.T. Kane, K.T. Nguyen

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Jiang, Y. Zhang, K.T. Kane, D.M. Simeone, M.P. di Magliano, K.T. Nguyen

Published OnlineFirst January 7, 2015; DOI: 10.1158/1541-7786.MCR-14-0076

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Acknowledgments
The authors thank Kim Bologna for her assistance with editing the article and Marsha Thomas for laboratory support.

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