Collagen I Promotes Metastasis in Pancreatic Cancer by Activating c-Jun NH2-Terminal Kinase 1 and Up-regulating N-Cadherin Expression

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Abstract

We have previously shown that N-cadherin expression is associated with tumor invasion, and that some cancer cells respond to specific extracellular matrix molecules by up-regulating N-cadherin. Pancreatic cancer is characterized by excessive deposition of type I collagen. Here, we show that human pancreatic cancer cells respond to collagen I, but not other matrices, by increasing motility and up-regulating mesenchymal markers, including N-cadherin. Both collagen I–mediated motility and metastasis in a mouse model for pancreatic cancer were inhibited by N-cadherin knockdown. Furthermore, inhibiting c-Jun NH2-terminal kinase (JNK) with chemical inhibitors or short hairpin RNA abrogated all collagen I–induced changes. We show that JNK1 is activated in response to collagen I, which increases tumorigenesis by up-regulating N-cadherin expression and by increasing motility. (Cancer Res 2006; 66(24): 11745-53)

Introduction

Epithelial cells undergo a transition from an epithelial phenotype to a mesenchymal phenotype (EMT) to set in motion normal developmental processes (1). Important aspects of EMT include decreased expression of E-cadherin and increased expression of non-epithelial cadherins, such as N-cadherin, cadherin-11, or R-cadherin. Non-epithelial cadherins are expressed by some tumors in situ, and their expression has been correlated with high tumor grade (2–5). In human pancreatic cancer, N-cadherin is expressed in primary and metastatic tumors, and its expression correlates with increased tumor progression, including perineural invasion, advanced histologic grade, and metastasis (6).

EMT is initiated by signals originating from outside the cell, such as epidermal growth factor, hepatocyte growth factor, fibroblast growth factor (FGF), and transforming growth factor β (TGFβ; refs. 7, 8). Recent studies from our lab showed that mouse mammary epithelial cells up-regulate N-cadherin and undergo EMT in response to collagen I through integrin/phosphoinositide-3 kinase (PI3K)/Rac1/c-Jun NH2-terminal kinase (JNK) signaling pathways (9), and that the response to collagen I is much like the response of these cells to other EMT-inducing stimuli. In addition to the obvious morphologic changes, EMTs also induce changes in cellular behavior, including increases in motility and invasion (1, 10). A recent study from our lab showed that cadherin switching is necessary for the increased cell motility that accompanies TGFβ-induced EMT in a classic mammary epithelial cell model of EMT (11), and it was previously shown that N-cadherin expression by tumor cells promotes cell motility, invasion, and metastasis (12, 13).

Pancreatic adenocarcinomas are among the most fatal cancers due to extensive invasion into surrounding tissues and metastasis to distant organs, even at an early stage (14). Pancreatic ductal carcinomas are characterized by a massive desmoplastic reaction or deposition of extracellular matrix, which contains an abundance of collagen I (15–17). Yamanari et al. showed that cultured pancreatic cancer cells were subject to influences by extracellular matrix. For example, S2-013 cells cultured in collagen I gels formed looser aggregates than the same cells cultured in Matrigel (18). Grzesiak and Bouvet showed that the interaction of pancreatic cancer cells with collagen I is mediated by the α2β1 integrin (19). Furthermore, Armstrong et al. reported that Panc-1 and AsPC-1 pancreatic cancer cells attached readily to collagen I and tended to grow as flattened individual cells, in contrast to the small clusters of cells that were observed when the same cells were plated on non-coated dishes (20). In addition, Menke et al. reported that pancreatic cancer cells had reduced E-cadherin expression, increased proliferation, increased migration, and Src-dependent morphologic transformation in response to plating on collagen I (21). Recently, the Menke group showed that plating Panc-1 cells on collagen I activated focal adhesion kinase, which was then associated with the E-cadherin/β-catenin complex (22).

Here, we used an in vitro model system together with a highly relevant orthotopic animal model to investigate the mechanisms whereby tumor microenvironment regulates N-cadherin expression in pancreatic cancer cells, the influence of N-cadherin on pancreatic cancer cell behavior, and the pathways that pancreatic cancer cells use to coordinate cell-cell interactions with cell-extracellular matrix interactions as they undergo EMT. The Menke study showing that collagen I promotes activation of β-catenin/LEF1 target genes (22) together with our present study showing that collagen I activates JNK, which up-regulates N-cadherin expression, initiates EMT, and promotes invasion and metastasis, point out the highly significant influence of tumor microenvironment on the outcome of pancreatic cancer.

Materials and Methods

Reagents, antibodies, cultured cells and immunofluorescence. All reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher Chemicals (Fairlawn, NJ) unless otherwise indicated. Mouse monoclonal antibody
containing 10% FBS was added to the bottom chamber. Cells were containing 1% FBS was added to the upper chambers, and medium inserts were coated with collagen I or fibronectin. Culture medium chamber of BD BioCoat Control Culture Inserts (six-well plates, pore size = 0.02 Na acetate or fibronectin (50 g/mL) in PBS, washed 2 × with PBS, blocked with PBS 1% bovine serum albumin for 30 minutes at room temperature, and washed 2 × with PBS. Cells were stained for immunofluorescence and examined as described (9). Images were collected and processed using SlideBook software (Intelligent Imaging Innovations, Inc., Santa Monica, CA).

**Detergent extraction, cell surface biotinylation, SDS-PAGE, immunoblot, and in vitro kinase assay.** Monolayers were washed with PBS and extracted on ice with radioimmunoprecipitation assay buffer (9), and protein was determined using a kit (Bio-Rad, Hercules, CA). For surface biotinylation, cells were incubated with sulfo-N-hydroxysuccinimidobiotin (0.1 mg/mL; Pierce, Rockford, IL) for 15 minutes on ice, and 500 μg protein were immunoprecipitated and immunoblotted (24). Gels were quantified using Photoshop. JNK activity was assayed by a kit (BioVision, Mountain View, CA).

**Conventional reverse transcription-PCR and quantitative real-time reverse transcription-PCR.** Total RNA was extracted with TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH) and analyzed by reverse transcription-PCR (RT-PCR) using a kit (BD Biosciences Clontech, Palo Alto, CA) and primers as follows: E-cadherin, 5′-CTCATGCTGGCCAGAAA-3′ and 5′-TGGATCTCTGTTGCTCTGGTA-3′; GAPDH, 5′-TTGATGATGAAAGGACTCATGAC-3′ and 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′. N-cadherin and vimentin primers were reported (25, 26). The conditions for PCR were 94°C for 45 seconds, 60°C for 30 seconds, and 72°C for 90 seconds for 35 cycles. For quantitative real-time RT-PCR, total RNA was analyzed in an Mx3000P Real-time PCR System (Stratagene, La Jolla, CA) as follows: 50°C for 30 minutes and 95°C for 10 minutes followed by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute with primers and probes from Applied Biosystems (Foster City, CA) and a reaction mixture from Stratagene.

**Constricts, transfection, and infection.** Myc-tagged human N-cadherin (27) was subcloned into LZRS-MS-Neo, and infected cells were selected by 1 mg/mL G418 (28). Short hairpin RNA (shRNA) constructs for N-cadherin and JNK1 have been described (11, 29). shRNA targets for JNK2 and MKK7, as suggested by CODEX, were CAAAGATTCCTGGAATCAAA (nucleotides 840–858 of MAPK9; Genbank accession no. NM_139068.1) and GTCATTGCCGTAGAACAA (nucleotides 498–516 of MAP2K7; Genbank accession no. NM_145185.2). Viral expression and cell infection have been described (11, 30).

**Transwell motility assays.** Cells (5 × 10⁴) were plated in the upper chamber of BD BioCoat Control Culture Inserts (six-well plates, pore size = 8 μm; Becton Dickinson, San Jose, CA). The upper and lower sides of the inserts were coated with collagen I or fibronectin. Culture medium containing 1% FBS was added to the upper chambers, and medium containing 10% FBS was added to the bottom chamber. Cells were incubated on the membranes for 4 hours, and motility was quantified as described (9).

**Animal models and tissue preparation.** Congenitally athymic female National Cancer Institute (Bethesda, MD) nude mice (nu/nu) were maintained in pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee guidelines. Animals were anesthetized with ketamine (35 mg/kg) and xylazine (120 mg/kg) by i.p. injection. An incision was created through the left upper abdominal line, and the pancreas was exposed; 40 μL of a single-cell suspension containing 50,000 cells was injected into the pancreas, and the abdominal wall was closed. Animals were kept warm and observed until they recovered from anesthesia. Two months later, the mice were sacrificed, and the pancreas, liver, lung, and disseminated nodules were harvested, fixed in 10% buffered formalin, and embedded in paraffin. Serial 5-μm sections were mounted on slides and stained with H&E. For immunohistochemistry, sections were stained with YS rabbit pAb using a VECTASTAIN Avidin-Biotin Complex kit (Vector Labs, Burlingame, CA) and counterstained with Mayers' hematoxylin for 15 seconds. To assess apoptosis, frozen sections were stained for the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assays using TACS®XL Basic In situ Apoptosis Detection kit (R&D Systems) and counterstained with methyl green (Vector Labs). Sections were examined using a Zeiss Axioskop 40 microscope equipped with an AxioCam MR digital camera and software.

**Statistical analysis.** Statistical analysis was done using Mann-Whitney U and Kruskal-Wallis tests (Statview, version 5.0, Abacus Concepts, Berkeley, CA) with P < 0.05 considered statistically significant.

## Results

**BxPC-3 cells undergo EMT and up-regulate N-cadherin expression in response to collagen I.** The morphogenesis of BxPC-3 cells from ATCC was heterogeneous, with the majority of cells forming epithelial colonial epithelial and with endothelial foci that were observed when cultured on collagen I. Furthermore, conventional RT-PCR showed that both N-cadherin and vimentin were significantly up-regulated when the cells were cultured on collagen I. When cells undergo EMT, they typically up-regulate N-cadherin and concomitantly down-regulate E-cadherin. Because there was only a small difference in E-cadherin expression in cells plated on collagen I, we did immunoblot on extracts of cells plated on non-coated, collagen I–coated, or fibronectin-coated dishes. N-cadherin, fibronectin, and vimentin were up-regulated when BxPC-3 cells were cultured on collagen I–coated dishes (Fig. 1B). Furthermore, conventional RT-PCR showed that both N-cadherin and vimentin mRNA levels were increased, and quantitative real-time PCR showed that N-cadherin mRNA was significantly up-regulated when the cells were cultured on collagen I.

When cells undergo EMT, they typically up-regulate N-cadherin and concomitantly down-regulate E-cadherin. Because there was only a small difference in E-cadherin expression in cells plated on collagen I, we examined its surface localization to determine if there was a difference in E-cadherin available for cell-cell adhesion. We surface labeled cells with biotin and showed that E-cadherin on the cell surface was reduced in cells cultured on collagen I compared with other substrata (Fig. 1C). Quantification showed that ~70% of E-cadherin was on the surface of cells plated on...
non-coated or fibronectin-coated dishes, whereas only 48% was on the surface of cells plated on collagen I. Immunofluorescence staining for E-cadherin also showed a significant difference in its localization (Fig. 1D). In cells plated on non-coated or fibronectin-coated coverslips, E-cadherin was primarily localized at cell-cell borders as expected, whereas E-cadherin staining was reduced at cell borders in cells plated on collagen I–coated coverslips. In contrast, surface labeling and quantification showed that most of N-cadherin was on the cell surface, independent of plating conditions (Fig. 1C).

JNK activation is essential for collagen I–induced N-cadherin up-regulation and cell scattering. To sort out the signals downstream of plating BxPC-3 cells on collagen I, we examined potential pathways using inhibitors of Src family kinases (SU6656), PI3K (LY294002), JNK (SP600125), p38 mitogen-activated protein kinase (MAPK; SB203580), and the extracellular signal-regulated kinase pathway (PD98059). The JNK inhibitor (SP600125) was the only compound to effectively inhibit both collagen I–induced cell scattering (Fig. 2A) and N-cadherin up-regulation (Fig. 2B, lanes 9 and 10). Quantitative real-time PCR showed that collagen I–induced up-regulation of N-cadherin mRNA was completely prevented by SP600125 (Fig. 2C). To confirm a role for JNK in collagen I–induced changes in BxPC-3 cells, we showed that JNK phosphorylation was increased, indicating that JNK was more highly activated, in cells plated on collagen I–coated dishes compared with non-coated dishes or fibronectin-coated dishes (Fig. 3A). We used anisomycin (a small molecule from Streptomyces griseolus) that activates JNK; ref. 31) as a control to help distinguish which of the bands recognized by anti-phospho-JNK were relevant (pointed out by arrows in Fig. 3A). SP600125 inhibits the activity of both JNK1 and JNK2 and anti-phospho-JNK cannot distinguish between these isoforms. Because JNK1 and JNK2 have distinct functions in cells (32), we felt it was important to distinguish which isoform was critical for promoting cell scattering and up-regulation of N-cadherin in response to collagen I. Thus, we expressed shRNA against JNK1, JNK2, or enhanced green fluorescent protein (EGFP; as a control) and assayed the cells for scattering and N-cadherin up-regulation in response to collagen I. Knocking down JNK1 but not JNK2 effectively prevented changes in BxPC-3 cells when they were plated on collagen I (Fig. 3C). Total JNK1 expression was effectively knocked down by shRNA against JNK1 (Fig. 3D), whereas the expression of JNK2 was unaffected (data not shown), and JNK activation in response to plating on collagen I was significantly decreased by shRNA against JNK1 (Fig. 3D). Jun is a common cellular substrate for JNK. To determine whether shRNA against JNK1 prevented phosphorylation of Jun in response to collagen I, we tested extracts of BxPC-3 cells for their ability to phosphorylate glutathione S-transferase-c-Jun (AA1-79) in vitro. shRNA against JNK1 prevented phosphorylation of this in vitro substrate, as did treating the cells with the JNK inhibitor SP600125 (Fig. 3B). Importantly, knocking down JNK1 while preserving expression of JNK2 completely prevented collagen I–induced cell scattering (Fig. 3C) and N-cadherin up-regulation (Fig. 3D). Interestingly, shRNA against JNK2 had no effect on collagen I–induced cell morphology (Fig. 3C) or on N-cadherin up-regulation (Fig. 3D).

In the signaling cascade leading to activation of JNK, there are two members of MAPK kinase family (MKK4 and MKK7) that are immediately upstream of JNK. BxPC-3 cells lack MKK4 (33); thus, we used shRNA against MKK7 to determine if its activity was necessary for cell scattering and N-cadherin up-regulation in response to collagen I. shRNA against MKK7 prevented both the morphologic changes in cells plated on collagen I (Fig. 3C) and the increase in N-cadherin expression (Fig. 3D).

Up-regulation of N-cadherin is necessary for collagen I–induced pancreatic cancer cell aggression. To determine if N-cadherin up-regulation is a critical component of the collagen I–induced scattering of BxPC-3 cells, we used an shRNA directed against N-cadherin to manipulate the cells so that they could not up-regulate N-cadherin when plated on collagen I. In addition, we overexpressed N-cadherin in BxPC-3 cells to ask if up-regulation of N-cadherin was sufficient to promote the morphologic changes
even in the absence of collagen I (Fig. 4A). Importantly, the overexpressed N-cadherin was homogenous throughout the culture (Fig. 4B). When cells were cultured on non-coated dishes, the morphology of N-cadherin knockdown and N-cadherin–overexpressing cells was almost identical to that of parental BxPC-3 cells, in spite of significant differences in N-cadherin expression (Fig. 4C). Interestingly, collagen I–induced cell scattering was completely inhibited by N-cadherin knockdown compared with Mock-infected BxPC-3 cells or N-cadherin–overexpressing cells. It is interesting to note that cells overexpressing N-cadherin did not become scattered on non-coated dishes, although the overexpression of N-cadherin was significant.

We have shown in other tumor types that expression of N-cadherin during tumorigenesis promotes cell motility. Thus, we used Transwell filters coated with collagen I or fibronectin to ask whether plating BxPC-3 cells on collagen I increased cell motility. Figure 4D shows that Mock-infected cells plated on collagen I were thrice as motile as cells plated on fibronectin. Furthermore, overexpression of N-cadherin resulted in increased cell motility on collagen I–coated filters, whereas N-cadherin knockdown resulted in decreased cell motility. These data suggest that collagen I–induced cell motility depends, at least in part, on the expression of N-cadherin. However, one question that still remained was whether activating JNK by plating BxPC-3 cells on collagen I was sufficient to promote motility. To address this, we compared collagen I–mediated motility in Mock knockdown cells with that of JNK1 knockdown cells. Figure 4D shows that knocking down JNK protein and thus preventing its activation significantly reduced collagen I–mediated cell motility. Interestingly the JNK inhibitor SP600125 reduced cell motility even in N-cadherin–overexpressing BxPC-3 cells, suggesting that the influence of JNK on cell motility includes factors in addition to N-cadherin up-regulation.

To ensure we were not studying a cell type–specific phenomenon, we tested another human pancreatic cancer cell line (Panc-1) for response to collagen I. Panc-1 cells grew as colonies on non-coated or collagen I–coated dishes but were more scattered in response to collagen I (Supplementary Fig. S2A). In addition, Panc-1 cells up-regulated N-cadherin, fibronectin, and vimentin in response to plating on collagen I (Supplementary Fig. S2B).
N-cadherin expression promotes invasion and metastasis in a mouse model for pancreatic cancer. To determine if the expression of N-cadherin influences the behavior of tumors in vivo, we turned to an orthotopic injection model for pancreatic cancer. We used the BxPC-3 cells with manipulated N-cadherin expression and injected cells into the pancreas of athymic nude mice (9–10 mice per group). Two months later, the mice were sacrificed; the tumor size in the pancreas was estimated macroscopically, and peritoneal dissemination nodules that were over 5 mm were counted. BxPC-3 cells knocked down for N-cadherin formed significantly smaller tumors in the pancreas than did Mock-infected cells (Fig. 5B). Neither Mock-infected cells nor N-cadherin knockdown cells showed high levels of tumor dissemination (Fig. 5B). BxPC-3 cells overexpressing N-cadherin formed pancreatic tumors larger than Mock-infected or N-cadherin knockdown cells, and most interestingly, they showed many dissemination nodules that were significantly more numerous than Mock-infected cells or N-cadherin knockdown cells (Fig. 5A and B). To compare the levels of expression of N-cadherin in the tumors, we extracted tumor tissue and immunoblotted for N-cadherin using two different antibodies: the 13A9 monoclonal anti-N-cadherin, which recognizes all N-cadherin in the tumors, the endogenous N-cadherin expressed by the BxPC-3 cells, and the overexpressed N-cadherin; and the anti- myc antibody, which recognizes only the myc-tagged N-cadherin that is overexpressed (Fig. 5C). Tumors formed by Mock-infected cells expressed a small amount of N-cadherin; those knocked down for N-cadherin had no N-cadherin; and tumors formed by N-cadherin-overexpressing cells had significant N-cadherin. Immunoblotting of the same tumor tissue showed that each tumor expressed equivalent levels of E-cadherin (Fig. 5C).

Immunohistochemistry on sections of tumors with an antibody against human N-cadherin showed tumors formed by Mock-transfected BxPC-3 cells had a small amount of staining (Fig. 6A, arrows), and tumors formed by BxPC-3 cells knocked down for N-cadherin had no detectable staining, whereas tumors formed by BxPC-3 cells overexpressing N-cadherin had high levels of N-cadherin. Figure 6B shows H&E staining of the intestine and a disseminated nodule of a typical mouse bearing an N-cadherin-overexpressing tumor. Villi on the top surface of the intestine are present at the left, and the tumor occupies the space under the villi. Micro-metastases were found in the lungs of 7 of 10 mice bearing tumors formed by BxPC-3 cells overexpressing N-cadherin, whereas no metastases were seen in mice from the other groups. Figure 6B shows H&E staining and anti-N-cadherin immunohistochemistry of micrometastases (arrows) in the lung from a mouse bearing a tumor formed by BxPC-3 cells overexpressing N-cadherin.

Inhibition of JNK1 prevented invasion and metastasis in the mouse model for pancreatic cancer. To determine if inhibiting JNK1 prevented cancer invasiveness, BxPC-3 cells infected with shRNA against JNK1 were injected into the pancreas of athymic nude mice. BxPC-3 cells knocked down for JNK1 formed significantly smaller tumors than cells knocked down for EGFP as a control (Fig. 6D). Furthermore, cells knocked down for JNK1 formed fewer abdominal dissemination nodules less than control cells, and N-cadherin expression was not detectable in these
tumors (Fig. 6D). The tumor from control cells expressing shRNA against EGFP expressed small amounts of N-cadherin (Fig. 6C), comparable with that shown in Fig. 6A, whereas the tumor formed by BxPC-3 cells knocked down for JNK1 showed no staining for N-cadherin and was comparable with the tumor formed from cells knocked down for N-cadherin (Fig. 6C).

Discussion

Plating human pancreatic cancer cells on collagen I resulted in the loss of epithelial cell-cell junctions without a change in total E-cadherin expression. However, the level of E-cadherin on the cell surface was reduced, and the localization of E-cadherin was clearly altered in BxPC-3 cells plated on collagen I. In contrast, Menke et al. and Koenig et al. reported that plating pancreatic cancer cells on collagen I reduced the expression of E-cadherin (21, 22).

We observed that parental BxPC-3 cells were a heterogeneous mixture of cells with an epithelial morphology and cells with a more fibroblastic phenotype. Thus, we selected an epithelial clone for the studies reported here, which may explain the differences between our observations.

A role for JNK1 in collagen I–induced N-cadherin up-regulation. When we investigated signaling downstream of plating human pancreatic cancer cells on collagen I, the JNK inhibitor completely prevented collagen I–induced cell scattering and N-cadherin up-regulation. This is consistent with a previous study from our lab showing that inhibiting JNK activity abrogated collagen I–induced cell scattering and N-cadherin up-regulation in mouse mammary epithelial cells (9). However, in the mammary epithelial cells, a PI3K inhibitor completely inhibited JNK signaling in response to collagen I, whereas it was only partially effective in BxPC-3 cells. In addition, neither dominant-negative Src nor dominant-negative Rac prevented morphologic changes in BxPC-3 cells (data not shown). Thus, it seems that collagen I–mediated JNK activation occurs via different pathways in different cell types. A number of studies suggest that integrins signal to JNK (34–37). Furthermore, JNK phosphorylation of transcription factors promotes expression of matrix metalloproteinases, which implicates integrin-JNK signaling in tumor cell invasion (38, 39). Thus, JNK is clearly associated with cell migration and invasion via numerous pathways. Our study shows that JNK signaling promotes invasion and metastasis of pancreatic
cancer partly due to its role in up-regulation of N-cadherin expression.

**Role of N-cadherin up-regulation in pancreatic cancer.** Knocking down N-cadherin in BxPC-3 cells interfered with collagen I–mediated morphologic changes, indicating that N-cadherin expression is essential for the cells to become scattered. Furthermore, the motility of N-cadherin-overexpressing cells was not significantly different when the assay was done on fibronectin-coated Transwell but was significantly different when the assay was done on collagen I–coated dishes, suggesting that N-cadherin up-regulation is associated specifically with collagen I–mediated cell motility. This is in contrast to previous studies by our lab and others showing that human breast cancer cells expressing N-cadherin are more motile and invasive, independently of matrix interactions (12, 13). Pancreatic carcinomas exhibit a severe fibrotic response with high levels of collagen I deposition, and our findings...
indicated that interactions of pancreatic cancer cells with collagen I increases N-cadherin expression and promotes invasion, which enhances the malignant phenotype of pancreatic cancer.

We also showed that N-cadherin expression increased the invasive and metastatic capacity of BxPC-3 cells in an orthotopic mouse model for pancreatic cancer. Immunostaining with N-cadherin showed that BxPC-3 cells in the tumors expressed N-cadherin mainly at edges of the tumor, where cells were migrating into the tissue. This pattern of N-cadherin staining is similar to what we have previously reported for N-cadherin localization in invasive human oral squamous cell carcinoma (2). Both the Mock-transfected cells and the N-cadherin-overexpressing cells within tumors showed border staining for N-cadherin as well as cytosolic staining. Nakajima et al. reported that N-cadherin expression by primary human pancreatic tumors was predominantly observed in a cytoplasmic but not a membranous pattern (6).

In humans, pancreatic cancer metastasizes to liver, lung, and other tissues; thus, we examined numerous tissues for metastases and found extensive lung micro-metastases. Bouvet et al. reported that parental BxPC-3 cells develop tumors very rapidly but do not show distant metastases due to cell type-specific "seed and soil" interactions governing the chronology and sites of metastatic targets (40). In our study, we found micro-metastasis in the lungs of 7 of 10 of the mice bearing tumors that overexpress N-cadherin but no metastases in mice from the Mock-transfected cells (essentially the same cells as those from the Bouvet study) or from the cells knocked down for N-cadherin. Hazan et al. showed that exogenous expression of N-cadherin in weakly metastatic breast cancer cells significantly increased metastasis in vivo (13). This group further showed that expression of these cells caused sustained FGFR-1 signaling due to stabilization of the receptor on the cell surface (41). In our system, N-cadherin expression did not change the proliferation rate of cells in vitro (data not shown), whereas it did cause larger tumors to grow in a s.c. injection model (data not shown) and in the orthotopic pancreatic cancer model. Furthermore, TUNEL staining showed that apoptosis was very low in all tumors and was similar among tumors formed from Mock, N-cadherin knockdown, and N-cadherin-overexpressing BxPC-3 cells (Supplementary Fig. S3). These data suggest that N-cadherin likely influences tumorigenesis not only by increasing invasion but also by increasing tumor growth, which may be due to growth factors specific to the in vivo tumor microenvironment. Furthermore, N-cadherin has been reported to promote interactions between tumor cells and endothelial cells, which facilitates metastasis (13, 42). These findings strongly indicate that up-regulation of N-cadherin promotes cancer cell proliferation, migration, invasion, and metastasis in vivo. Given the highly fibrotic nature of pancreatic cancer, our data suggest that collagen I promotes the malignant phenotype of pancreatic cancer partly by activating JNK1, which promotes invasion and metastasis via up-regulation of N-cadherin expression together with activation of cell motility.

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**References**


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