MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1

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MUC4 is a type-1 transmembrane mucin differentially expressed in multiple cancers and has previously been shown to potentiate progression and metastasis of pancreatic cancer. In this study, we investigated the molecular mechanisms associated with the MUC4-induced invasion and metastasis in pancreatic cancer. Stable silencing of MUC4 in multiple pancreatic cancer cells resulted in the downregulation of N-cadherin and its interacting partner fibroblast growth factor receptor 1 (FGFR1) through downregulation of partly by pFAK, pMKK7, pJNK and pc-Jun and partly through PI-3K/Akt pathway. The downregulation of FGFR1 in turn led to downregulation of pAkt, pERK1/2, pNF-κB, uPA, MMP-9, vimentin, N-cadherin, Twist, Snail and Slug (11), leading to invasion and metastasis.

Introduction

Despite a welcome decline in mortality rate over the past decade, pancreatic cancer (PC) still remains the 10th most commonly diagnosed cancer and the 4th leading cause of cancer-related death in the USA (1,2). The median survival of PC patients is about 4.1 months with the overall 5-year survival rate being less than 5% (2–4). The clinical manifestations of PC usually occur at a late stage, at which time the disease has already spread to local and distant organs (in 85% of patients) (5). To acquire such invasive abilities, epithelial cancer cells undergo several phenotypic changes, similar to those seen during embryonic development. This process is termed epithelial to mesenchymal transition (EMT). Despite growing knowledge about the EMT process and stabilizing fibroblast growth factor receptor 1 (FGFR1) through the N-cadherin upregulation, the precise molecular mechanisms by which PC cells progress from an epithelial to a mesenchymal-like phenotype) called EMT. This is a phenomenon whereby malignant cells contribute to invasion, metastatic dissemination and acquisition of therapeutic resistance (7,8). The process of EMT involves the disruption of cell–cell and cell–extracellular matrix interactions, loss of cell polarity, reorganization of the actin cytoskeleton, acquisition of a mesenchymal phenotype with reduced intercellular interactions and increased migratory capacity. This is associated with a significant increase in the expression of epithelial markers such as vimentin and cytokeratin-18 (9), downregulation of epithelial markers such as E-cadherin and cytokeratin-18 (10) and upregulation of transcription factors associated with the EMT process such as Twist, Snail and Slug (11), leading to invasion and metastasis.

Previous reports have shown that around 90% of cancer-related deaths are mainly due to metastasis, not due to primary tumors (6). The process of invasion and metastasis in PC is still inadequately understood. Normally, invasion and metastasis occurs in sequential steps, which involves detachment of cancer cells from the primary tumor and invasion into the surrounding healthy tissues followed by intravasation, extravasation and finally colonization at distant sites. However, in recent years, an enormous amount of data has suggested that cancer cells utilize the same mechanisms as healthy embryonic cells (i.e. gravitation by the process of changing from an epithelial to a mesenchymal-like phenotype) called EMT. This is a phenomenon whereby malignant cells contribute to invasion, metastatic dissemination and acquisition of therapeutic resistance (7,8).

Materials and methods

Antibodies

The anti-MUC4 mouse monoclonal antibody (8G7) used in this study was developed by our laboratory (29). The antibodies, cleaved caspase-9 (Asp330), phosphorylated/total pAkt (Serine 473)/Akt, pMKK7 (Ser271/Thr275)/MKK7, pJNK (Thr183/Tyr185)/JNK, pc–Jun(Ser63)/c–Jun and phospho–ERK1/2 (t/Erk1/2, phospho–F AK (pFAK–Tyr 925, Tyr 576/577)/ tFAK, pHER2 (Tyrosine 1248)/HER2, NFκB, and pIL8/IkB were obtained from cell signaling (Danvers, MA, USA). The antibodies against MMP-9 and N and E-cadherin were gifts from Dr. Keith R. Johnson (University of Nebraska Medical Center). The β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO). The secondary antibodies, anti-mouse and anti-rabbit IgGs conjugated to horseradish peroxidase, were obtained from GE Healthcare Biosciences, (Uppsala, Sweden). The fluorescein isothiocyanate–conjugated anti-mouse secondary antibody was obtained from Invitrogen (California, USA).
ShRNA-mediated MUC4 silencing in pancreatic cancer cells Capan1 and BxPC3
Capan1 and BxPC3 PC cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and streptomycin) at 37°C with 5% CO2 in an humidified atmosphere. The shRNA-mediated silencing of MUC4 was carried out as described previously (25). In brief, two 64mer oligonucleotides (forward and reverse) containing the 19-nt target sequence in sense and anti-sense orientation were designed on both sides of the linker TTCAGAGGA. The 
RgI I and Hind III restriction sites were introduced at 5′ and 3′ ends of 64mers, respectively, to allow direct ligation of the annealed MUC4 double-strand insert into the pSUPER-retro-puro vector. The annealed double-stranded oligonucleotides were phosphorylated using polynucleotide kinase (Roche Diagnostics, Mannheim, Germany) and ligated into the digested pSUPER-retro-puro vector. Successful cloning was ascertained by restriction digestion and sequencing. Ecotropic Phoenix packaging cells were transfected with the pSUPER-retro-puro vector containing either the MUC4 shRNA insert (pSUPER-retro-puro-shMUC4) or a scrambled sequence (pSUPER-retro-puro-Scr) using Lipofectamine 2000 (Invitrogen, San Diego, CA), following the manufacturer’s protocol. Supernatant containing infection-competent retroviruses was collected 48 h after transfection and used for stable transduction of Capan1 and BxPC3 cells using polybrene (4 µg/ml) to augment the infection efficiency. Stably clones were then selected in a medium containing puromycin (5 µg/ml; InvivoGen, San Diego, CA).

Real-time PCR
Total RNA was isolated and the cDNA was synthesized by reverse transcription as described previously (30). The real-time primers for MUC4, N-cadherin, FGF1, Slug, Zeb1 and β-actin (Supplementary Table 1, available at Carcinogenesis Online) were designed by using primer 3 software. Real-time PCR was performed on a Roche 480 Real-Time PCR System, Indianapolis, USA. Real-time PCR reactions were performed in triplicate and non-template controls (NTCs) were run for each assay under the same conditions. PCR was then performed in 10 µl reactions containing 5 µl 2x SYBR green Master Mix, 3.2 µl of autoclaved nuclease free water, 1 µl diluted RT product (1:10) and 0.4 µl of each forward and reverse primer (5 pmol). The cycling conditions were comprised of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 58°C for 1 min. Gene expression levels were normalized to the level of β-actin expression and used as a control.

Immunoblot and confocal laser scanning microscopy
The MUC4-silenced Capan1/BxPC3 and scrambled control cells were processed for western blotting and confocal microscopy as described previously (26,30). In brief, a total of 30–60 µg of protein from the cell extracts were resolved by electrophoresis on either a 2.0% sodium dodecyl sulfate–agarose gel (for MUC4) or a 10% sodium dodecyl sulfate–polyacrylamide gel (for other proteins) and transferred to polyvinylidene difluoride membrane (PVDF). After blocking with 5% non-fat milk in Tris-buffered saline (0.1 M, pH of 7.4, 140 mM NaCl and 2.5 mM CaCl2) and protein concentrations determined using a Bio-Rad D/C protein estimation kit, double-stranded DNA from each of forward and reverse primer was added to 1× SBYR green Master Mix and incubated at 72°C for 1 min. Gene expression levels were normalized to the level of β-actin expression and used as a control. Stable silencing of MUC4 expression by shRNA was confirmed by immunoblotting at the protein level and by real-time PCR analysis using primers for effective silencing of MUC4 or to serve as a control, respectively. The pooled populations were selected in 10% DMEM medium containing puromycin (5.0 µg/ml). The stable silencing of MUC4 in Capan1-shMUC4 and BxPC3-shMUC4 cells and their scramble shRNA-transfected cells (Capan1-Scr and BxPC3-Scr) was confirmed by immunoblotting at the protein level and by real-time PCR analysis at the transcript level (Figure 1A and 1B). Western blot analysis showed 80 and 70% downregulation of MUC4 expression at the protein level in Capan1-shMUC4 and BxPC3-shMUC4 cells, respectively, when compared with scramble vector transfected cells (Figure 1A). Subsequently, our real-time PCR analysis revealed
**Fig. 1.** Strategy for shRNA-mediated silencing of oncogenic MUC4 in Capan1 and BxPC3 cells. (A) Analysis of MUC4 expression in BxPC3/Capan1-shMUC4, BxPC3/Capan1-Scr by immunoblotting shows significant inhibition in protein expression (70–80%), respectively. β-actin was used as a loading control. (B) Real-time PCR analysis using primers that specifically amplify the MUC4 gene. Capan1 and BxPC3 pooled population cells show a 50–60% decrease in the expression of MUC4 at the mRNA level compared with the control population. No amplification was observed in HPDE, which is negative for the MUC4 expression. (C and D) Confocal analysis showed a decreased expression of MUC4 on the cell membrane in Capan1 and BxPC3-shMUC4 cells compared with scramble cells. (E and F) A morphological comparison between the Capan1/BxPC3-shMUC4 and Capan1/BxPC3-Scr cells. The MUC4 knockdown Capan1 and BxPC3 cells grow in aggregates compared with the scramble cells. (G and H) The phallolidin-rhodamine (phallolidin-RITC) staining of actin-cytoskeleton. MUC4-expressing Capan1 and BxPC3-Scr cells revealed the presence of more microspikes, lamellopodia and filopodia-like cellular projections compared with the MUC4 knockdown Capan1/BxPC3 cells.
consistent results with the western blot analysis, confirming the reduced expression of MUC4. RNA from immortalized but untransformed human pancreatic ductal epithelial HPDE cells was used as a negative control for MUC4 expression (Figure 1B). In addition, to strengthen the aforementioned results, we also examined the expression of MUC4 using immunofluorescence, with results congruent with immunoblot and real-time PCR analysis (Figure 1C and 1D).

**MUC4 knockdown leads to altered morphology in PC cells**

We next examined the cellular morphology and growth patterns of Capan1-shMUC4/BxPC3-shMUC4 and scramble vector transfected cells. The Capan1-shMUC4 and BxPC3-shMUC4 cells showed a spherical (roundel) morphology and a tendency to grow in clumps compared with the Capan1-Scr and BxPC3-Scr cells which displayed a flat- and spindle-shaped morphology, with loose association (Figure 1E and 1F). Further, we also observed that MUC4 knockdown resulted in a significant reduction in lamellipodia and filopodia in BxPC3-shMUC4 and Capan1-shMUC4 cells (Figure 1G and 1H) when compared with scramble vector transfected cells.

**MUC4 knockdown alters the expression of EMT markers in PC cells**

To investigate the mechanism underlying the morphological changes in MUC4 knockdown Capan1 and BxPC3 cells, we examined the expression of known epithelial markers E-cadherin (CDH1), Occludin, Cytokeratin-18 (CK-18) and mesenchymal markers, such as N-cadherin (CDH2), Twist, Slug, Zeb1, p53, and vimentin, by western blotting QPCR and immunofluorescence. Both western blot and immunofluorescence analysis revealed that the expression of E-cadherin, Occludin and CK-18 significantly increased (Figure 2A and 2B), whereas expression of N-cadherin, Twist, p53, vimentin, Slug and Zeb1 significantly decreased in both Capan1-shMUC4 and BxPC3-shMUC4 cells (Figure 2A–2C) when compared with their respective control cells. Therefore, these results demonstrate that the MUC4 mediates the EMT in PC, leading to increased invasion and metastasis.

**MUC4 upregulates N-cadherin expression through the FAK pathway in Capan1 and BxPC3 cells**

N-cadherin is a key mediator of the EMT process in PC cells (32), and FAK activates N-cadherin expression in PC cells through the c-jun N-terminal kinase (JNK)-mediated pathway (33). A previous study from our laboratory has also shown that downregulation of MUC4 leads to decreased activation of FAK in pancreatic and ovarian cancer cells (22,28,31). As such, we were interested in looking at the effect of MUC4 knockdown on N-cadherin expression and the signaling pathways involved. Interestingly, our results showed a significant decrease in the activation of downstream signaling molecules such as pERK1/2 and pAkt (Ser473) (Figure 4A and 4B). Further, downregulation of pNf2B, pIKBα, MPP-9, p53, and vimentin and upregulation of E-cadherin (Figure 2A and 2B) when compared with scramble vector transfected Capan1 and BxPC3 cells was also observed. The level of total ERK1-2, and Akt, however, remained unchanged (Figure 2A, 4A and 4B). These results support the hypothesis that MUC4 knockdown results in decreased activation of downstream signaling molecules, such as N-cadherin, Twist, Slug, Zeb1, p53, and vimentin. Moreover, the knockdown of MUC4 led to a significant reduction in lamellipodia and filopodia in Capan1-shMUC4 and BxPC3-shMUC4 cells when compared with scramble vector transfected cells. Phosphorylated HER2 and pSrc are the key molecules involved in FAK activation by MUC4 (31). We checked the expression of these molecules in MUC4 knockdown Capan1/BxPC3 cells. Our western blot analysis showed a significant decrease in pHER2 and pSrc expression in Capan1 and BxPC3-shMUC4 cells when compared with scramble vector transfected cells (Figure 3A and 3B). Difference in the levels of total FAK and HER2 was not observed (Figure 3A and 3B). We also analyzed the expression of downstream signaling molecules of FAK and observed that MUC4 knockdown leads to a decreased expression of activated pMKK7, pJNK and pc-Jun in both Capan1 and BxPC3-shMUC4 cells (Figure 3A and 3B); however, no change was observed in their total protein level (Figure 3A and 3B). Further, a recent study has shown that cadherin switch regulated at transcriptional level by Twist, Snail through PI3K/PTEN pathway (34). In the present study, we also observed the downregulation of pAkt, Twist, Slug and Zeb1 in MUC4 knockdown Capan1 and BxPC3 cells (Figures 2A, 2C, 4A and 4B). Therefore, N-cadherin expression is regulated partly through PI3K and JNK pathways.

**MUC4 knockdown reduces motility/invasive potential and induces apoptosis in PC cells**

Previous studies have demonstrated an important role of EMT in tumor progression where in tumor cells become more invasive and metastatic (42,43). We were also interested in examining the effect of MUC4 knockdown on the motility of Capan1 and BxPC3 PC cells. MUC4 knockdown (Capan1 and BxPC3-shMUC4) showed a significant decrease in motility (P<0.0001) when compared with control Capan1-Scr and BxPC3-Scr cells as studied by trans-well migration assay (Supplementary Figure 1A and 1B, available at Carcinogenesis Online), as well as scratch assay (Supplementary Figure 1C and 1D, available at Carcinogenesis Online). MUC4 knockdown cells also demonstrated a significant decrease in their invasive potential of (Capan1 and BxPC3-shMUC4) when compared with scramble vector transfected cells (Supplementary Figure 1E and 1F, available at Carcinogenesis Online).

We have reported previously that MUC4 knockdown cells have reduced growth (25). Because the overall rate of cell growth is determined by the balance between cell proliferation and apoptosis, we were interested in looking at the effect of MUC4 knockdown on the apoptotic indices of Capan1 and BxPC3 cells. To analyze this, MUC4 knockdown and control cells were grown for 48h and trypanized. The extent of apoptosis and necrosis was determined by Annexin-V and propidium iodide staining, followed by flow cytometry. The apoptotic, as well as necrotic, cells of both Capan1-shMUC4 and BxPC3-shMUC4 were significantly higher (P<0.01 and 0.05, respectively) as compared with their respective scramble vector transfected cells (Supplementary Figure 1G, available at Carcinogenesis Online). Taken together, our results showed that MUC4 knockdown led to a decrease in cell survivability, as well as a decrease in the migration/invasion potential, of PC cells. Consistent with the enhanced apoptosis, we also observed an increased expression of cleaved Caspase-9.
Fig. 2. Role of MUC4 in the EMT process. (A) The immunoblot analysis showed significant upregulation of epithelial markers such as E-cadherin, Occludin and CK-18, and downregulation of mesenchymal markers, such as N-cadherin, Twist, uPA and vimentin, in Capan1/BxPC3-shMUC4 cells compared with Capan1/BxPC3-Scr vector cells. (B) Confocal microscopy showed increased staining for E-cadherin and CK-18 and a faint expression of N-cadherin and vimentin was observed in Capan1/BxPC3-shMUC4 compared with Capan1/BxPC3-Scr vector cells. FITC-conjugated goat anti-mouse IgG for secondary antibody and DAPI was used for nuclear staining. β-actin was used as a loading control in immunoblotting. (C) Real-time PCR analysis using primers that specifically amplify the Slug and Zeb1 genes showed reduced expression Slug and Zeb1 in MUC4 knockdown Capan1 and BxPC3 cells.
In the MUC4-silenced cells when compared with scramble vector transfected cells (Figure 4A and 4B).

In order to strengthen the aforementioned results, we knocked down N-cadherin in both Capan1 and BxPC3 cells and then checked the expression of pERK1/2, FGFR1, N-cadherin, uPA and E-cadherin. Our western blot analysis showed that knockdown of N-cadherin resulted in the downregulation of FGFR1, N-cadherin, uPA, inactivation of pERK1/2 and upregulation of E-cadherin (Figure 5A and 5J). Our confocal analysis of BxPC3 cells also revealed that N-cadherin knockdown leads to an increased expression of E-cadherin (Figure 5B). Further, we also observed that N-cadherin knockdown also resulted in a significant reduction in the motility of BxPC3 and Capan1 cells when compared with scramble vector transfected cells (Figure 5C).

Inhibition of MUC4 expression in PC cells results in the suppression of tumorigenicity and metastasis

To examine the effect of oncogenic MUC4 knockdown in vivo, Capan1-shMUC4 and Capan1-Scr cells (5 × 10⁵ cell/animal) were orthotopically implanted into the pancreas of nude mice (six mice per group). The animals were killed at 21 days postimplantation, and the pancreatic tumors were removed and weighed (Figure 5D, 5E and 5F). The liver, lung, diaphragm, intestine, kidneys and mesenteric lymph nodes were carefully examined for the presence of metastatic lesions. A primary pancreatic tumor and metastatic lesions in the spleen, mesenteric lymph nodes and on the peritoneal wall were found in the majority of the mice implanted with Capan1-Scr cells. In this group, some animals also had metastasis in the liver (n = 3) and/or diaphragm (n = 1). In contrast, animals injected with Capan1-shMUC4 cells had significantly smaller tumors (P < 0.03) and had fewer or no metastatic lesions when compared with scramble vector transfected cells injected animals (Table 1, Figure 5G, 5H and 5I).

Discussion

MUC4 is aberrantly overexpressed in various carcinomas, including PC (12–18,20,22,23). Previous studies by our group showed a significant role of MUC4 in facilitating pancreatic and ovarian cancer cell motility by altering morphology, actin-cytoskeleton and downstream signaling events (25,28). Cancer invasion and metastasis occurs as a result of epithelial to mesenchymal transition and its underlying mechanisms that harmonize these processes in PC are not fully understood. In order to decipher the role of the glycoprotein MUC4 in regulating EMT in PC cells, we selectively silenced MUC4 expression in Capan1 and BXPC3 cells using specific shRNA. Subsequently, we studied the effect of MUC4 knockdown on EMT by performing various assays in vitro (motility, invasion, and apoptosis) and in vivo (tumorigenesis and metastasis). In addition, the downstream signaling pathways involved in EMT, which leads to invasion and metastasis of PC, were also observed. The stable expression of a shRNA against MUC4 resulted in a significant decrease in MUC4 expression at the mRNA, protein and cellular levels in Capan1/BxPC3-shMUC4 compared with scramble vector transfected cells (Figure 1A, 1B, 1C and 1D).

Fig. 3. MUC4-mediated upregulation of N-cadherin through FAK signaling. (A and B) Western blot analysis showed a significant upregulation of pHER2, pScr, pFAK, pMKK7, pJNK, pc-Jun and upregulate N-cadherin in Capan1/BxPC3-Scr vector cells as compared with Capan1/BxPC3-shMUC4. The total form of FAK, MKK7, JNK1/2, c-Jun molecules remains unchanged. β-actin was used as a loading control.

Table 1. Incidence of metastases developed by orthotopic implantation of pooled population Capan1-Scr cells and Capan1-shMUC4 in immune-deficient mice

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Spleen (%)</th>
<th>Liver (%)</th>
<th>Peritoneum (%)</th>
<th>Mesentric lymph nodes (%)</th>
<th>Diaphragm (%)</th>
<th>Intestinal wall (%)</th>
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<tbody>
<tr>
<td>Capan1-Scr</td>
<td>5/6 (83.33)</td>
<td>3/6 (50)</td>
<td>4/6 (66.66)</td>
<td>5/6 (83.33)</td>
<td>1/6 (16.66)</td>
<td>2/6 (33.33)</td>
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<tr>
<td>Capan1-shMUC4</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>2/6 (33.33)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>P-value (chi-square test)</td>
<td>0.015</td>
<td>0.18</td>
<td>0.57</td>
<td>0.015</td>
<td>1</td>
<td>0.45</td>
</tr>
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which were further related to decreased activation of Akt, FAK, phospho and total ERK, phospho and total Akt and phospho and total ERK1/2, NFκB, FGFR1, MMP-9) in Capan1/BxPC3-shMUC4 and Capan1/BxPC3-Scr vector cells. The results showed upregulation of Caspase-9 and downregulation of pSrc, pFAK (Tyr576/577, Tyr925), pERK1/2, PAkt, NFκB, FGFR1 in Capan1/BxPC3-shMUC4 compared with scramble vector transfected cells. The total form of FAK, Akt and ERK molecules remains unchanged. β-actin was used as a loading control. (C) Reciprocal co-immunoprecipitation analysis to show the interactions between N-cadherin and FGFR1. Lysates from the MUC4-expressing Capan1/BxPC3 cell lines were utilized for immunoprecipitation with N-cadherin and FGFR1 antibodies. The immunoprecipitates were electrophoretically resolved on 10% polyacrylamide gel and immunoblotted with anti-N-cadherin or anti-FGFR1 antibodies. The isotype antibodies were used as controls. (D) Real-time PCR analysis using primers that specifically amplify the FGFR1 and N-cadherin genes showed reduced expression of FGFR1 and N-cadherin in MUC4 knockdown Capan1 and BxPC3 cells lines were utilized for immunoprecipitation with N-cadherin and FGFR1 antibodies.

Fig. 4. Effect of MUC4 knockdown on key signaling molecules in PC cells (A and B) involved in apoptosis (caspase 9), metastasis and invasion (pScr, phospho and total FAK, phospho and total Akt and phospho and total ERK1/2, NFκB, FGFR1, MMP-9) in Capan1/BxPC3-shMUC4 and Capan1/BxPC3-Scr vector cells. The results showed upregulation of Caspase-9 and downregulation of pSrc, pFAK (Tyr576/577, Tyr925), pERK1/2, PAkt, NFκB, FGFR1 in Capan1/BxPC3-shMUC4 compared with scramble vector transfected cells. The total form of FAK, Akt and ERK molecules remains unchanged. β-actin was used as a loading control. (C) Reciprocal co-immunoprecipitation analysis to show the interactions between N-cadherin and FGFR1. Lysates from the MUC4-expressing Capan1/BxPC3 cell lines were utilized for immunoprecipitation with N-cadherin and FGFR1 antibodies. The immunoprecipitates were electrophoretically resolved on 10% polyacrylamide gel and immunoblotted with anti-N-cadherin or anti-FGFR1 antibodies. The isotype antibodies were used as controls. (D) Real-time PCR analysis using primers that specifically amplify the FGFR1 and N-cadherin genes showed reduced expression of FGFR1 and N-cadherin in MUC4 knockdown Capan1 and BxPC3 cells lines were utilized for immunoprecipitation with N-cadherin and FGFR1 antibodies.

which were further related to decreased activation of Akt, FAK and ERK pathways, downregulation of MMP9, uPA and an upregulation of E-cadherin, Occludin and CK18 (Figures 2A, 2B, 3A, 3B, 4A and 4B). These findings reinforce previous observations by our group that MUC4 plays a significant role in modulating cell motility, invasion and re-reorganizing the cytoskeleton of cancer cells (22,25–28,31).

EMT results in phenotypic changes that are accompanied by an increased cellular motility (8) and production of proteolytic enzymes resulting in the disruption of cell–cell adhesions, which are mediated by E-cadherin (45,46). In most solid tumors, the function of epithelial E-cadherin is altered due to various genetic and epigenetic mechanisms, such as activation of ERK1/2 signaling pathway. This, in turn, activates signaling molecules that promote tumor-cell migration, invasion and dissemination (8). We observed a downregulation of pERK1/2 (Figure 4A and 4B) and an increased expression of E-cadherin in the MUC4 knockdown cells (Figure 2A and 2B). These results suggest that oncogenic MUC4 inhibits E-cadherin function partly by upregulating Twist, Slug, Zeb1 via Akt pathway (Figures 2A, 2C, 4A and 4B), as well as N-cadherin and its interacting partner FGFR1 via JNK1/2 mediated pathways (Figures 2A,
4A, 4B), thus effecting downstream molecules such as ERK1/2 and MMP9. Further, this study, we also observed a decreased expression of matrix metalloproteinase-9 (MMP9) in MUC4 knockdown Capan1 and BxPC3 cells (Figure 4A and 4B), which is a key mediator of the invasive property of tumor cells (47). Similarly, MUC4-mediated activation of the ERK1/2 pathway also promotes production of MMP-9, which in turn causes cleavage of E-cadherin, leading to the disruption of cell–cell contacts. Our results suggest that MUC4-mediated downregulation of E-cadherin may contribute to the highly metastatic property of Capan1 and BxPC3 cells.

Suppression of MUC4 expression in Capan1 and BxPC3 cells led to a significant reduction in the expression of mesenchymal markers, such as Twist, Slug, Zeb1, N-cadherin, and vimentin, and an upregulation of epithelial markers, such as E-cadherin, Cytokeratin18, and Occludin (Figure 2A, 2B and 2C). During cancer metastasis, EMT is initiated and the process upregulates mesenchymal markers such as Twist, Slug, Zeb1, N-cadherin, and vimentin, and downregulates epithelial markers, such as E-cadherin, Cytokeratin18, and Occludin.
as vimentin, N-cadherin and Vitronectin-75 while reducing the expression of epithelial markers such as E-cadherin and cytokeratin (9,10,48–50). Similarly, stable ectopic expression of MUC4 in SKOV3 cells led to the downregulation of E-cadherin and CK-18 and upregulation of N-cadherin and vimentin expression in cells (31).

Recent reports have shown that upregulation of N-cadherin through the activation of FAK (33) in PC cells. Subsequently, it has been shown that MUC4 upregulates N-cadherin in OC cells through FAK activation (31). In the current study, we further analyzed the mechanism(s) underlying MUC4-mediated upregulation of N-cadherin through the
FAK signaling pathway in human PC cells and their further downstream signaling pathways (Figure 3A and 3B). The results of the current study provide a mechanistic basis for our previous studies on the role of MUC4 in PC (22,25) and OC (31). The results of the present study revealed that knockdown of oncogenic MUC4 resulted in decreased stabilization of pHER2, followed by decreased activation of pSer, FAK and its downstream molecules, such as MKK7, JNK1/2 and c-Jun, which further result in the downregulation of N-cadherin in PC cells (Figure 3A and 3B). Further, we also observed the downregulation of pAkt, Twist, Slug and Zeb1 in MUC4 knockdown Capan1 and BXPC3 cells (Figures 2A, 2C, 4A and 4B). Our results further corroborate the recent result showing role of PI3K/PTEN pathway role in cadherin switch at transcriptional level via regulating Twist and Snail in melanoma cells (34). Therefore, the present study indicated that MUC4 mediates N-cadherin upregulation partly through Akt pathway and partly by the JNK1/2 pathway in PC cells and EMT resulted in a switch between E-cadherin and N-cadherin expression levels (51,52). N-cadherin stabilizes FGFR1 by interacting with first FGFR1 Ig-like domain 1 (D1) and two (D2) extracellular domains (39). This interaction activates MAPK/ERK1/2, which in turn upregulates MMP-9 (39) Akt, AP1, uPA, and downregulates E-cadherin expression. We also observed an interaction between N-cadherin and FGFR1 in BxPC3 and Capan1 cells (Figure 4C) and effects downstream signaling molecules (Figure 2A, 4A and 4B).

Our data further showed that MUC4 has an anti-apoptotic effect, which is mediated by activation of the PI3-K/Akt pathway (53). In our study, inhibition of MUC4 also resulted in the suppression of ERK1/2 and pAkt (Figure 4A and 4B). Similarly, there was a significant decrease in the cellular levels of pNFkB and pIκB and an increase in the levels of caspase-9 in both Capan1-shMUC4 and BXPC3-shMUC4 cells (Figure 4A and 4B). These results suggest that MUC4 has a potential role in facilitating survival, proliferative ability, motility and invasive ability of PC cells. Thus, MUC4 emerges as a significant player in controlling important cellular processes during PC progression and metastasis, reinforcing its importance as a target for anticancer therapy.

Upon orthotopic implantation of MUC4 knockdown cells into the pancreas of nude mice, the Capan1-shMUC4 cells formed smaller tumors when compared with the Capan1-Scr control cells and did not cause morbidity (Figure 5D–5F, Table 1). However, there was
no decrease in the incidence of tumors formed by Capan1-shMUC4 cells, but a significant decrease in metastasis to the vital organs was observed, suggesting that MUC4 may play a role in progression rather than the initiation of PC (Figure 5G–5I). The higher apoptotic and antitumorigenic effects of MUC4 knockdown cells were partially compensated by other unknown signaling pathways. The present study revealed that oncogenic MUC4 not only regulates tumor cell invasion but also plays an important role in modulating the invasive nature of the malignant cells (Table 1).

Finally, we conclude that silencing of oncogenic MUC4 results in decreased expression of HER2, Src and FAK, which in turn leads to downregulation of the signalling pathway, resulting in reduced levels of N-cadherin partly through Akt- and JNK-mediated pathways. N-cadherin then stabilizes FGFR1 through its direct association and potentiates the downstream signaling pathways involved in promoting cell proliferation, inhibition of apoptosis, breaking cell–cell contacts and regulating expression of proteases such as MMP-9 and uPA. The observed inhibition of cell proliferation in the MUC4 knockdown cells may be mediated through the inhibition of the MAPK pathway, whereas the increase in metastatic properties of MUC4-expressing cells might be at least partly due to the activation of FAK and its decrease in the expression of E-cadherin (Figure 6). Overall, our studies demonstrate that PC cells that overexpress MUC4 promote the EMT process, leading to increased invasion and metastasis. The results of this study could be useful for targeting novel proteins downstream of MUC4 in order to disrupt signaling pathways involved in proliferation, anti-apoptosis, motility, invasion and metastasis.

**Supplementary material**

Supplementary Table 1 and Figure 1 can be found at http://carcin.oxfordjournals.org/

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**References**


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