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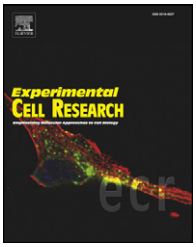

Research Article

MUC6 mucin expression inhibits tumor cell invasion

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ABSTRACT

The MUC6 mucin has a critical protective function in the normal stomach, pancreas and duodenum and is aberrantly expressed during the progression of some gastrointestinal cancers. Our aim was to determine whether MUC6 contributes to the etiology or progression of pancreatic cancer and elucidate the molecular basis of its involvement. Expression of MUC6 glycoprotein was examined in pancreatic cancer tissues by immunofluorescence and loss of MUC6 was observed. Next, to determine whether MUC6 inhibits tumor growth and metastasis by altering cell adhesion and invasion, recombinant MUC6 cDNA and separate MUC6 N-terminal and C-terminal domains were transfected into pancreatic, colorectal and breast cancer cell lines. The recombinant N- and C-terminal proteins were each seen to oligomerize under non-reducing conditions. Overexpression of both domains of the MUC6 glycoprotein significantly inhibited cell adhesion to matrix proteins (collagen I, collagen IV, fibronectin and laminin) in LS 180 but not in PANC-1 cells. Moreover, the N- and C-terminal domains of MUC6 inhibited invasion of both LS 180 and PANC-1 cells by 40% and 70%, respectively, in comparison with controls. These results suggest that MUC6 may inhibit invasion of tumor cells through the basement membrane of the pancreatic duct and slow the development of infiltrating carcinoma.

Introduction

The normal function of the healthy gastrointestinal tract is dependent on the biochemical and biophysical properties of several high-molecular-weight, heavily-glycosylated mucous glycoproteins (mucins). These molecules, which may be cell-surface-associated or secreted, have many important functions. Throughout the digestive system, mucins lubricate the epithelium and provide a selective barrier that protects it from, for example, acidity in the stomach, digestive enzymes, a high pH in the pancreatic duct, and variable insults in the intestine. Moreover, mucins may also function as adhesion/anti-adhesion molecules in invasion and metastasis and in intracellular signaling. Dysregulation of mucin expression and glycosylation may contribute to cancer development and progression [1,2]. The membrane-bound mucins MUC1 and MUC4 are known to be aberrantly expressed and to play a role in the progression of several different types of tumors; in contrast, the secreted mucin MUC2 appears to act as a tumor suppressor in the intestinal epithelium [3]. The potential contribution of other secreted mucins to tumor invasion and metastasis has not been studied in depth. Here, we focus on the MUC6 mucin, which has critical functions in several parts of the digestive system including the stomach, the duodenum and the pancreas.

Four genes encoding secreted mucins (*MUC6*, *MUC2*, *MUC5AC* and *MUC5B*) map to a 400-kb region on chromosome 11p15.5.

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MUC6 and MUC5AC comprise the major secreted gel-forming mucins in the stomach, where they exhibit different cellular distributions [4], with MUC6 expressed in the glands and MUC5AC in goblet cells [5]. The N-terminal domain of MUC6 is similar to that of MUC2 [3,6], and both contain von Willebrand factor (vWF) D-like domains, which have a very high cysteine content. In addition, like other gel-forming mucins, the short C-terminal region of MUC6 contains a cystine knot motif [7,8]. Both the vWF D-like domain and the cystine knot motif are important for mucin dimerization.

MUC6 was first identified in a gastric mucosa cDNA library [9] and is estimated to be 10–20 kb, encompassing 33 exons. The size variation is caused by polymorphisms in the number of tandem repeats within the single tandem repeat exon 31 [10]. The full-length MUC6 cDNA has proven extremely difficult to isolate, mainly due to the problems encountered in cloning a long cDNA encompassing a tandem repeat sequence, which is unstable in *E. coli*. The tandem repeat itself is poorly characterized but can range from 5 to 15 kb encoding repeats of a 169-amino acid sequence.

MUC6 has been implicated in epithelial differentiation through its transient expression in ureteric buds during kidney development [4]. MUC6 is also associated with the progression of gastric carcinomas [11,12], and although this mucin is not expressed by normal breast tissues, it is often upregulated in breast cancer [13]. In pancreatic cancer, published MUC6 mRNA and protein expression data are somewhat inconsistent; however, those data sets in which carefully dissected lesions were evaluated suggested that upregulation of MUC6 expression is an early event in the development of the tumor. Pancreatic ductal adenocarcinomas (PDACs) arise from precursor lesions called pancreatic intraepithelial neoplasms (PanINs), which are graded as PanIN-1A, -1B, -2 and −3 as they progress to infiltrating adenocarcinoma. PanINs accumulate a series of mutations, first in the KRAS oncogene, followed by loss of the tumor suppressors CDKN2A/ INK4A (p16), TP53 and/or SMAD4 prior to the development of carcinomas. Several studies demonstrated upregulation of MUC6 protein or mRNA in PanIN lesions [14–16]; however, no mechanistic studies have yet been undertaken to elucidate the biology of MUC6 expression in pancreatic cancer and its role in tumor progression. To test the hypothesis that a normal function of MUC6 is to inhibit tumor initiation and invasion, we generated epitope-tagged MUC6 and separate N- and C-terminal domains of the molecule and expressed them in relevant cell lines. We found that both domains of the MUC6 protein can inhibit adhesion of cells to extracellular matrix proteins and also hinder invasion of several cell types *in vitro*. These data suggest a mechanism whereby upregulation of MUC6 expression in early lesions associated with pancreatic cancer might be biologically advantageous, for example, by inhibiting invasion of the PanIN lesions through the basement membrane of the pancreatic duct, while later in tumor progression MUC6 expression is lost.

Materials and methods

Microarray expression data

Pancreatic cancer datasets were retrieved from the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nml.nih.gov/geo/) with the GEOmetadb search engine [17] (gbnci.abcc.ncifcrf.gov/geo/), and data were extracted by Array Studio (OmicSoft, Morrisville, NC, USA) software for the analysis of MUC6 RNA expression levels. Two datasets from GEO containing both normal pancreas and pancreatic cancer tissue were used: the first contained 36 pancreatic ductal adenocarcinoma tumors and matching normal pancreatic tissue samples from the same patients (GEO series GSE15471) [18]; the second included 52 samples, of which 16 had both tumor and normal expression data, and 20 only had tumor data (GSE16515) [19]. MUC6 was defined as differentially-expressed between normal tissue and tumors or lesions if (1) the difference between the mean normalized expression values was at least 2.0-fold, and (2) a *P*-value of <0.01 was obtained by using a two-sided *t*-test.

MUC6 immunohistochemistry

Pancreatic tissues were generously provided by the Rapid Autopsy Program at the Eppley Institute, University of Nebraska Medical Center. Ten normal pancreatic samples and 9 pancreatic tumors, together with 8 adjacent tissues, were examined for MUC6 expression. Frozen tissue sections were fixed in 3% paraformaldehyde for 30 min, washed with PBS, blocked (5% bovine serum albumin and 5% non-fat milk in PBS with 0.5% Tween-20) for 60 min, and then probed with M6P polyclonal antibody (against a 23-amino acid tandem repeat unit of the MUC6 mucin, a generous gift from Dr Samuel Ho) [20] for about 18 h at 4 °C. Pre-immune serum at the same dilution was used as a negative control. After incubation in primary antibody, the sections were rinsed three times in PBS and then incubated in diluted FITC-conjugated secondary antibody (2.5 μg/ml; Sigma, St. Louis, MO, USA) for 1 h in at room temperature. The sections were then washed with PBS, DAPI counterstained and mounted in anti-fade reagent (FluorSave reagent; Calbiochem, EMD Biosciences, La Jolla, CA, USA). Adjacent sections were hematoxylin and eosin Y (H & E)-stained to show the tissue morphology. A specimen was scored as MUC6-positive if M6P immunoreactive cells were found in at least 5% (objective, X10) and up to 25% of low power fields. A specimen was considered to have widespread reactivity if immunoreactive cells were present in >25% of low power fields. The significance of associations was determined using Fisher's exact test. The statistical analyses were performed using SPSS (Chicago, IL, USA).

Cell culture

COS-7 (ATCC CRL-1651; SV40 ori- transformed, African green monkey kidney fibroblasts), PANC-1 [21] (ATCC CRL-1469; a poorly differentiated pancreatic adenocarcinoma cell line), LS 180 [22] (ATCC CL-187; a moderately differentiated colon adenocarcinoma cell line) and MCF7 [23] (ATCC HTB-22; breast adenocarcinoma) cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal calf serum at 37 °C in a 5% CO_2 incubator.

RNA isolation and RT-PCR

RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) from LS 180, MCF7 and PANC-1 cells at 5 days postconfluence. Total RNA was reverse transcribed to synthesize cDNA using SuperScript III (Invitrogen) and oligo$(dT)_{20}$ primer. MUC6 cDNA

PCR was performed using *Taq* DNA Polymerase (New England Biolab, Ipswich, MA, USA) and a primer pair (C1F and C1R, Supplementary Table 1), which amplifies MUC6 exon 31 and generated a 507-bp product.

Generation of MUC6 cDNA constructs

MUC6 N-terminal (MUC6_N), C-terminal (MUC6_C) and tandem repeat (MUC6_TR) cDNAs were cloned by RT-PCR from LS 180 cells using primers designed from the cDNA sequences of human MUC6 (GenBank: AY312160 and U97698; see Supplementary Table 2). For the MUC6_N insert (4.2 kb; amino acid 1–1405), *Eco* RI and *Eco* RV restriction sites were introduced, a FLAG epitope sequence was added at the 3′-end, and a stop codon was generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). For the MUC6_C insert (1.3 kb; amino acid 61–421 [1 relates to the first amino acid of MUC6 C-terminal sequence [9]]), *Xho* I and *Xba* I restriction sites were introduced, and a MUC6 signal sequence (69-bp) and a three-FLAG epitope sequence were added at the 5′-end. For the MUC6_TR, *Eco* RV and *Xho* I restriction sites were introduced, and a 1.8-kb MUC6_TR was generated by RT-PCR. MUC6_N, MUC6_TR and MUC6_C fragments were ligated into a full-length MUC6_F "minigene" and the sequences were verified. The cDNA fragments were cloned into a pcDNA3.1/neo (+) vector.

Cell transfection

The calcium phosphate method [24] was used for the transfection of transient and stable MUC6-expressing cells, as this was found to be more efficient than any of the lipid-based transfection reagents tested. In the transient transfection experiments, COS-7 cell lysates were harvested 48 h after transfection, and for the establishment of stable clones, LS 180, MCF7 and PANC-1 cells were cultured in medium with geneticin (G418 sulfate, Invitrogen) selection at 400, 500 and 700 μg/ml, respectively. Individual resistant clones were then selected and expanded for further analysis.

Protein preparation, immunoprecipitation and western blotting

Cell were lysed in NET buffer (10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% (vol/vol) protease inhibitor cocktail (Sigma) and 1% (wt/vol) Triton X-100. Crude lysates were separated on denaturing polyacrylamide gels and western blotted with M2-antibody against the FLAG epitope to confirm MUC6 protein expression. For immunoprecipitation, MUC6 proteins were immunopurified with M2-conjugated agarose beads (Sigma): 100 μl of M2 beads were incubated with 1 ml of cell lysate or 30 ml of cell culture supernatant at 4 °C for 24 h with vigorous shaking. The agarose beads were then washed three times in TBS buffer (140 mM NaCl, 2 mM KCl and 25 mM Tris, pH 7.4). Protein was eluted from the beads by incubation with 150 μl of FLAG peptide (500 μg/ml; Sigma) for 24 h at 4 °C with vigorous shaking.

Protein concentrations were determined using a detergent-compatible DC protein assay (Bio-Rad, Hercules, CA, USA). Protein samples (crude lysate, 25 μg; purified, 5 μg) were separated on SDS-PAGE gels (3% stacker; 8% resolving for MUC6_C and 5% for MUC6_N and endogenous MUC6 proteins) under reducing or non-reducing conditions, transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA, USA) or nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Freiburg, Germany), and blocked in blocking buffer (5% skim milk powder in phosphate-buffered saline [PBS]) for 1 hr. M2 antibody (anti-FLAG epitope, Sigma) was used at a dilution of 1:3,000 in PBS with 1% skim milk powder and 0.1% Tween-20 (vol/vol). Blots were incubated with the primary antibodies overnight at 4 °C then subjected to three 10-min washes with PBS-0.1% Tween 20, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Dako, Glostrup, Denmark) in PBS with 1% skim milk powder for 1 hr and further washing with PBS-0.1% Tween 20. Blots were visualized using ECL western blotting substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions and exposed to ECL-sensitive film.

Enzymatic deglycosylation

The MUC6_N and MUC6_C proteins immunoprecipitated from the cell culture media or cell lysates were treated with endoglycosidase H (Endo-H) or neuraminidase (both from New England Biolabs, Ipswich, MA, USA) for 3 h according to the manufacturer's instructions. The untreated and enzyme-treated samples were then analyzed by SDS-PAGE and Western blotting as described above. The samples were mixed with sample buffer to a final concentration of 30 mM dithiothreitol (DTT) as the reducing agent.

Cell invasion assay

The in vitro cellular invasion assay is a modification of the Membrane Invasion Culture System (MICS) assay [25]. Briefly, 2×10^5 cells were added to the upper chamber of a 12-well MICS plate and incubated for 36 h, after which the cells that had invaded through the reconstituted matrix barrier to the lower chamber were harvested. Cells were washed twice with medium and resuspended in a 96-well plate. The number of cells was then calculated using a Cell-Titer 96 AQueous MTS assay (Promega, Madison, WI, USA), which measures the enzymatic conversion of a tetrazolium dye during a 2-h incubation period at 37 °C. The absorption OD at 490 nm was determined using a microplate reader. Assaying of each clone was performed in triplicate in each experiment, and experiments were repeated at least three times. Mean ± SEM was calculated in each experimental group and analyzed by the unpaired *t*-test. A *P*-value of <0.05 was considered significant.

To determine whether the vector and MUC6-expressing PANC-1 and LS 180 clones had different replication times, cell proliferation assays were performed by using the Cell-Titer 96 AQueous MTS assay. Cells were counted 24, 36 and 48 h after seeding, in triplicate experiments.

Cell adhesion assay

Cell adhesion assays were performed as described previously [26]. Briefly, 96-well microplates (high-binding EIA plate, Corning-Costar Corp, Corning, NY, USA) were coated with 50 μl of 10 μg/ml matrix protein (collagen I, collagen IV, fibronectin or laminin, all from Sigma) overnight at 4 °C. After air-drying, the microplates were blocked with 500 μg/ml heat-inactivated BSA for 30 min at room temperature, and the plates were then washed with PBS

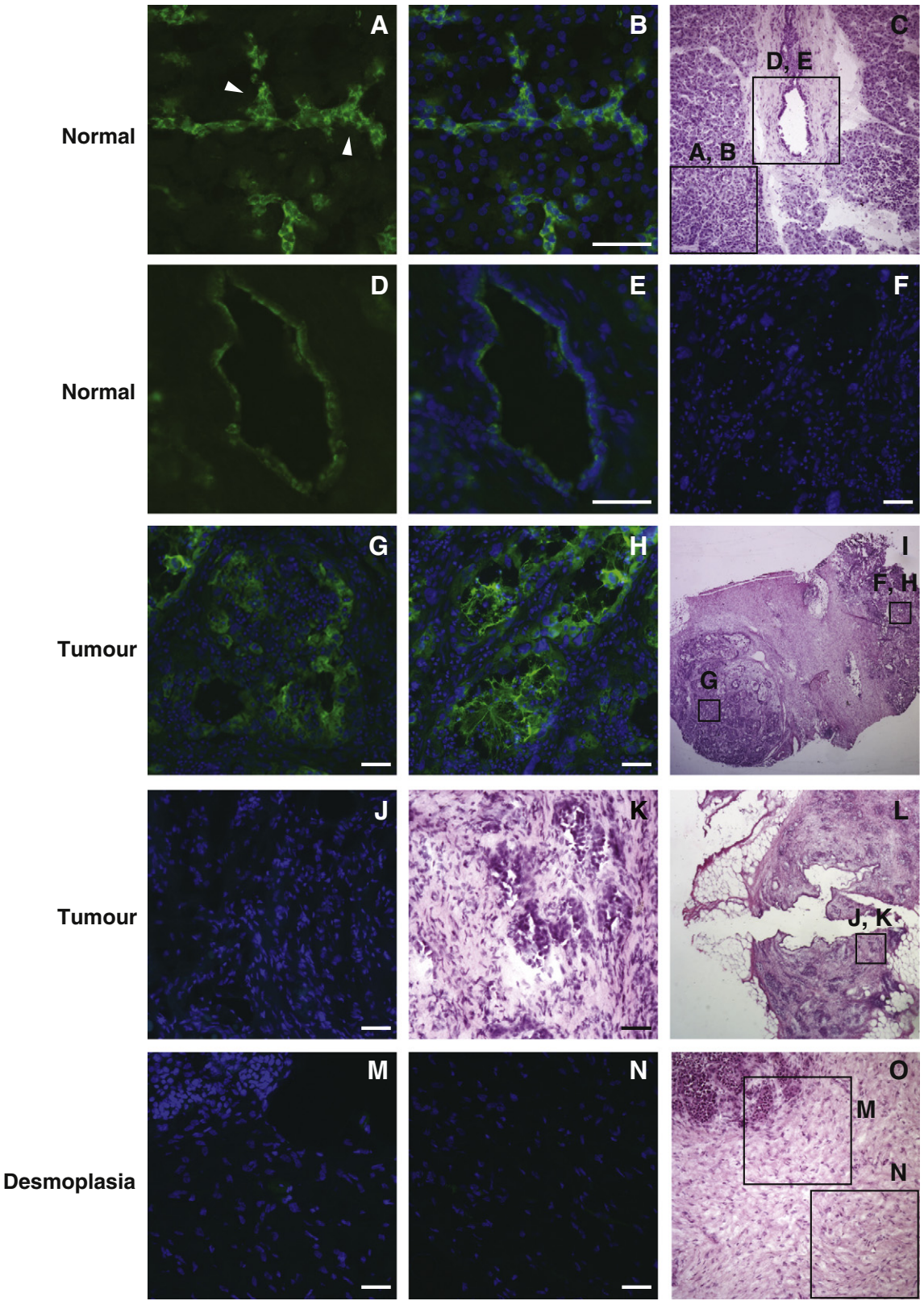

Fig. 1 – MUC6 immunostaining in normal pancreas and pancreatic tumor tissues. MUC6 staining in normal pancreas (A–E). (A, B) acini and intercalated ducts (white arrows); (D, E) interlobular duct; strong MUC6 staining shown in the ductal structures of one tumor sample (G and H), weak staining seen in most tumor samples (J). No MUC6 was detected in desmoplasia (M and N). (F) Pre-immune control from the same section as panel (H). Scale bar = 50 μm. (C, I, K, L and O show H & E staining and the black boxes denote the areas shown in adjacent panels); 4′-6-Diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. (A) and (D) show only MUC6 staining (green); the rest of immunostaining images show a combination of MUC6 (green) an DAPI (blue) staining.

three times before use in adhesion assays. Cells were detached from the plastic using nonenzyme cell dissociation solution (Mediatech Inc., Manassas, VA, USA). 2.5×10^4 cells (PANC-1) or 5×10^4 cells (LS 180) per 100 µl medium were allowed to adhere to the wells of matrix protein-coated plates for 25 min (PANC-1) or 45 min (LS 180) at 37 °C in a humidified CO_2 incubator. Non-adherent cells were gently discarded and washed by triturating with an eight-channel pipette three times with warm medium containing 5% FCS. The same number of cells of each clone was also placed in a replica microplate to calculate the total cell number. The numbers of cells were measured by Cell-Titer 96 AQueous MTS assay, as described above. The percentage of adherent cells was calculated as the OD of the adherent cells divided by the OD of the total cells. Statistical analysis was performed as described in the previous section.

Results

MUC6 mRNA expression data of pancreatic tumors from GEO datasets

We evaluated MUC6 mRNA expression in relevant datasets of the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nml.nih.gov/geo/) database to analyze whether the MUC6 mRNA level differed between normal and tumor tissues. Two datasets, GSE15471 [18] and GSE16515 [19], which contained substantial numbers of samples evaluated from RNA extracted from normal pancreas or tumor tissue, showed no difference in MUC6 mRNA expression (Supplementary Fig. 1). The datasets both utilized the Affymetrix Human Genome U133 Plus 2.0 Array, and though the two MUC6 probes on the array showed differences in overall expression levels, these were not related to normal or tumor tissue.

MUC6 protein expression in pancreatic tumors by immunocytochemistry

We then performed immunohistochemistry with the M6P antibody [20], which binds to the TR domain of MUC6, in pancreatic adenocarcinoma, tissue adjacent to the primary tumor, and normal pancreas tissue to evaluate the expression of MUC6 protein in these samples (Fig. 1, Table 1). Our results showed that M6P immunoreactivity was localized primarily to small (intercalated and intralobular) ducts of the normal pancreas (Figs. 1A–E/Supplementary Fig. 2). MUC6 was not detected in interlobular ducts, though it was sometimes found in the main duct. Only one of the nine pancreatic tumor tissue samples showed very strong MUC6 staining and this in a region with a densely-packed ductal architecture (Figs. 1G–L), while in the rest of the samples, M6P staining was significantly decreased ($P<0.01$, Fisher's exact test; Table 1). Some MUC6 was evident in the few remaining ductal structures of primary tumors; however, the associated desmoplasitic tissue did not express MUC6 (Figs. 1J–K). We did not observe PanIN lesions in the tumor samples, nor did we see a significant number of these lesions in the normal controls.

To further investigate the findings of others [14–16] of upregulation of MUC6 in PanIN lesions, in contrast to the loss of MUC6 at later stages of pancreatic cancer observed here, we evaluated the effects of MUC6 overexpression on pancreatic and intestinal epithelial cells *in vitro*. Epitope-tagged domains of the MUC6 protein and also an epitope-tagged MUC6 minigene were constructed for expression studies in tumor cell lines.

MUC6 expression in pancreatic cell lines

We evaluated MUC6 mRNA expression in multiple pancreatic adenocarcinoma cell lines (BxPC3, Capan-1, Capan-2, CFPAC-1, HPAF, Hs766, PANC-1, panc89, SW-979 and T3M4), but all lines were negative (data not shown). However, the colon adenocarcinoma cell line, LS 180 was found to express high levels of MUC6 mRNA and low levels were detected in the breast adenocarcinoma cell line MCF7 (Fig. 2A). We therefore chose one pancreatic adenocarcinoma cell line, PANC-1, in addition to LS 180 and MCF7 cells, for use in this study.

Epitope-tagged MUC6 protein expression and oligomerization

The predicted domain-specific proteins expressed from the epitope-tagged MUC6 constructs are illustrated in Fig. 2B. To establish that the MUC6 N-terminal (MUC6_N), C-terminal (MUC6_C) and minigene (MUC6_F) cDNA constructs produced stable proteins, we first performed transient transfections in COS-7 cells. Western blots probed with the M2 antibody, specific for the FLAG epitope tag, revealed one protein species corresponding to MUC6_C at ~50 KDa, and two forms of the MUC6_N protein, one at ~200 KDa and another at greater than 400 KDa (Fig. 2C). These proteins were larger than the predicted unglycosylated MUC6_C (43 KDa) and MUC6_N (152 KDa). Cells transfected with MUC6_F showed two forms, as seen for MUC6_N, though the sizes of the proteins were larger (one species ~250 KDa and the other ~550 KDa, data not shown).

Stably-transfected clones of PANC-1, LS 180 and MCF7 cells carrying the MUC6_C or MUC6_N expression vectors were screened by western blotting of SDS/PAGE gels, separated under standard reducing conditions (Figs. 3A and B). A minimum of three independent clones of each cell line expressing MUC6_N or MUC6_C was used to reduce the chance of artifacts due to clonal variation. MUC6_C and MUC6_N expression were regularly monitored by western blot to ensure that levels did not fall during the experiments. All cell clones showed 160 KDa MUC6_N and ~50 KDa MUC6_C proteins. Multiple small forms of MUC6_C were

Table 1 – MUC6 immunoreactivity in the normal pancreas and pancreatic adenocarcinoma.

Tissues	M6P immunoreactivity Histoscore†				*n*	*P**
	1	2	3	4		
Normal	0	1	8	1	10	
Tumor	5	3	0	1	9	0.0037
Desmoplasia	7	0	1	0	8	0.0006

†MUC6 staining intensity was scored as 1 (−, negative), 2 (+, positive), 3 (++) and 4 (+++). The histoscore was then calculated as (staining intensity) × (percentage of area stained) and graded as 4 intervals (1, 2, 3 and 4). *Fisher's exact test was used for statistical analysis. Comparison was performed by comparing normal *vs.* tumor or normal *vs.* desmoplasia.

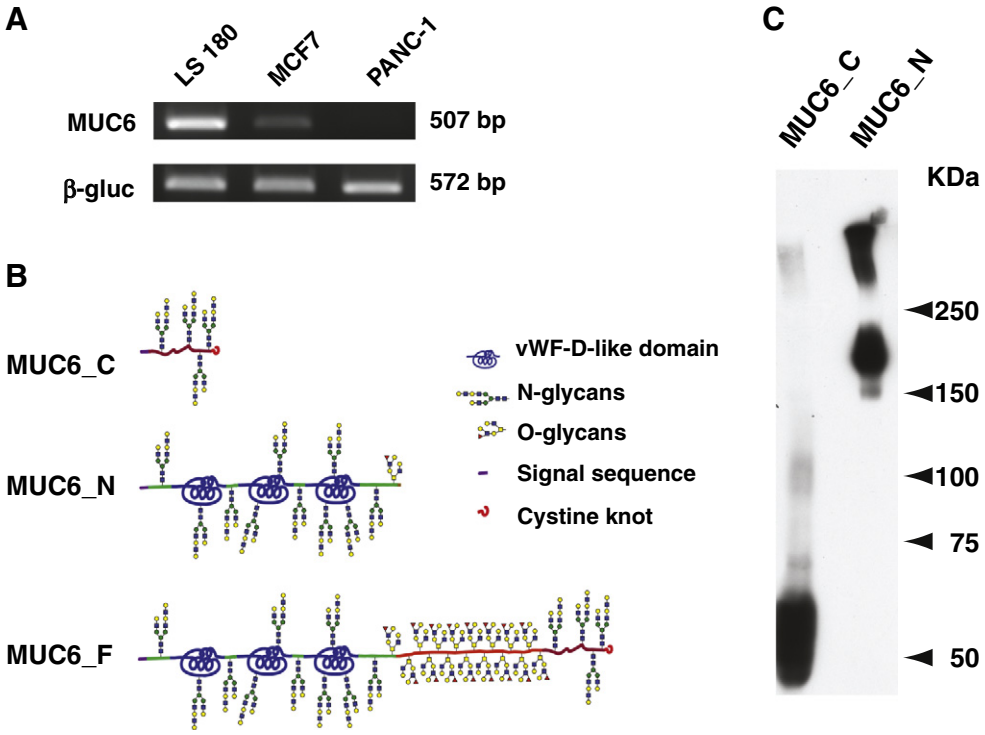

Fig. 2 – Endogenous MUC6 expression in cell lines. (A) Expression of MUC6 mRNA in LS 180, MCF7 and PANC-1 cells. Control gene: *β-glucocerebrosidase* (β-gluc). (B) The proteins encoded by the *MUC6* minigene and the domain-specific constructs. Diagrams illustrate the important structural and functional elements within each domain-specific construct (not to scale). Each construct contains a MUC6 signal sequence and the domain-specific sequence. (C) Transient expression of MUC6 C-terminus (MUC6_C) and N-terminus (MUC6_N) proteins in COS-7 cells. The predicted molecular masses of unglycosylated proteins MUC6_C and MUC6_N are 43 and 152 KDa, respectively. The proteins were analyzed by polyacrylamide gel electrophoresis under reducing conditions. Note: The carbohydrate structures are for illustration only and do not represent actual glycan structures.

seen in PANC-1 and LS 180 cells, and additional larger forms of MUC6_N (270 KDa) and MUC6_C (150 KDa) were detected in MCF7 clones. The additional larger forms of MUC6_N were also seen in some LS 180 MUC6_N clones (e.g., Fig. 3B, clone 14), but larger forms of MUC6_C were not detected in PANC-1 and LS 180 cells.). To ensure that any phenotypic differences observed in the stable clones expressing MUC6_N or MUC6_C were not due to downregulation of endogenous MUC6 caused by the transgenes, we evaluated endogenous MUC6 protein expression by western blot using the M6P antibody. Since this antibody is specific for the MUC6 TR it does not detect the MUC6_N or MUC6_C proteins. Though there was some clonal variation in endogenous MUC6 expression in the LS 180 clones (Fig. 3C), this did not correlate with MUC6 expression. The level of endogenous MUC6 in MCF7 cells was too low to detect by western blot (Fig. 3C).

The secreted forms of MUC6_N and MUC6_C immunoprecipitated from the cell culture supernatant with anti-Flag (M2) agarose beads were always of slower mobility than the material purified from the cell lysates (Figs. 4A and B). To further investigate the multiple forms of MUC6_N and MUC6_C proteins, we evaluated their migration under non-reducing conditions. MUC6_C generated two predominant oligomers, migrating at 130 and 270 KDa (Fig. 5A). In contrast, MUC6_N migrated as multiple species with apparent molecular masses of 500–560 KDa (Fig. 5B; mass was estimated from the semi-logarithmic graph of the relative mobility of molecular weight markers [range 50 to 300 kDa]). Exo- and endo-glycosidase digestion showed that the small forms seen under reducing conditions (50 KDa and 160 KDa for MUC6_C and MUC6_N, respectively) were sensitive to Endo-H (Figs. 5C and D, left panel), while the large forms (150 KDa and 270 KDa for MUC6_C and MUC6_N, respectively) were resistant to Endo-H. Neuraminidase treatments caused a slight reduction in mobility of the large forms of both MUC6_C and MUC6_N. (Figs. 5C and D). This suggests that the small forms were the precursors of intracellular proteins and the large forms were the secreted protein.

The results suggest that under non-reducing conditions the two forms of secreted MUC6_C represent the monomer (130 KDa) and dimer 270 (KDa). The size of the MUC6_N terminus could not be determined accurately, but was estimated to be 500–560 KDa. Since MUC6_N was estimated to be 270 KDa under reducing conditions, the secreted MUC6_N terminus probably forms an oligomer.

Clones that stably-expressed MUC6_F (the minigene with the N and C terminus together with the reduced tandem repeat) could not be established in any of the cell lines. Although clones initiated growth during the selection process, the cells subsequently failed to proliferate and eventually underwent senescence and death.

Expression of MUC6 N-terminal and C-terminal proteins decreases cell invasion

To further investigate the putative role of the MUC6 glycoprotein in tumor progression, we evaluated the effect of MUC6_C- or

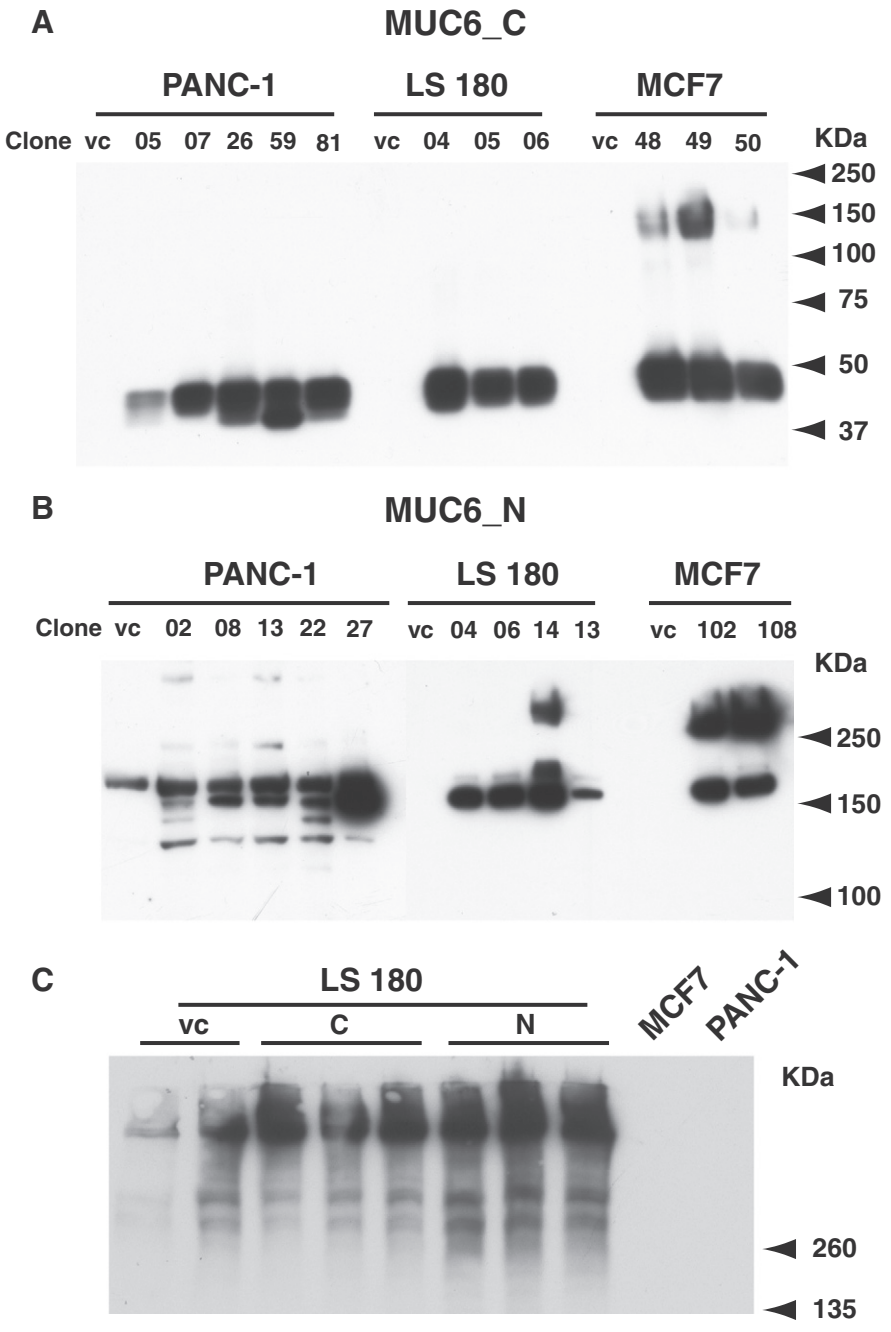

Fig. 3 – Stable clones express MUC6 protein domains. Western blots of cell lysates probed with the M2 anti-Flag antibody. Cells stably-expressing (A) MUC6_C or (B) MUC6_N in PANC-1, LS 180 and MCF7. A ~50-KDa MUC6_C protein and a ~160-KDa MUC6_N protein were seen in all cell lines, while larger forms of the proteins were detected in the MCF7 clones. (C) Endogenous MUC6 protein expression in LS 180, MCF7 and PANC-1 cells, showing clonal variation in expression of MUC6 endogenous protein. The proteins were analyzed by polyacrylamide gel electrophoresis under reducing conditions. vc: vector control.

MUC6_N-terminus expression on cell invasion using MICS assays [25]. To exclude the potential contribution of clonal differences in cell proliferation rates these were first compared. No differences in cell growth rates were observed at 24, 36 and 48 h between the vector control, MUC6_C and MUC6_N clones in each cell line (Supplementary Table 3). We then evaluated at least three clones of each cell line expressing a MUC6 domain and vector transfected controls for invasion through a reconstituted matrix barrier (containing collagen IV, laminin and gelatin) on 10-μm-pore-size membranes. For MCF7 cells, which are known to be poorly-invasive [27], less than 1% of invasive cells were seen in both vector controls and MUC6_C- and MUC6_N-expressing clones, suggesting that expression of MUC6 alone does not affect poorly-invasive cell behavior (data not shown). In contrast, expression of either the MUC6_C or _N terminal proteins in PANC-1 and LS 180 cells inhibited migration. Migration of MUC6_C-expressing PANC-1

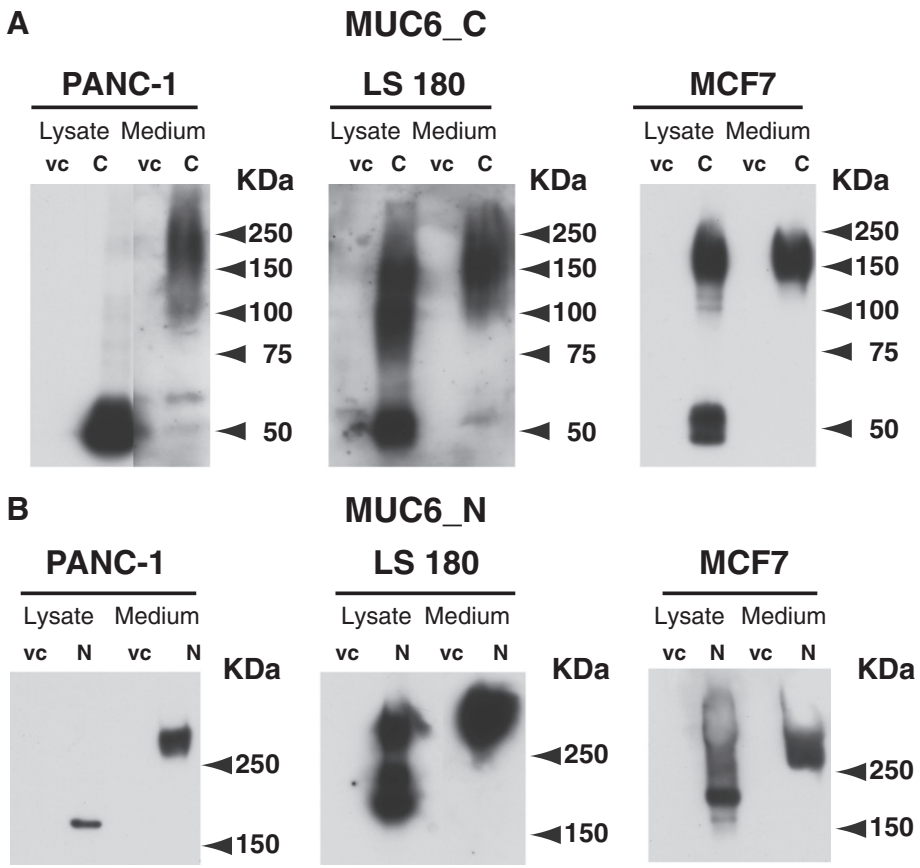

Fig. 4 – MUC6_C and MUC6_N proteins are secreted from transfected cells. Cell lysates or culture supernatant from PANC-1, LS 180 and MCF7 cells stably-expressing (A) MUC6_C and (B) MUC6_N were immunoprecipitated with M2-agarose and the bound protein eluted with FLAG peptide. The proteins were analyzed by polyacrylamide gel electrophoresis under reducing conditions. Western blots were probed with M2 antibody. vc: vector control; C: MUC6_C; N: MUC6_N.

cells was consistently decreased by 57% ($P<0.01$, unpaired t-test) compared with the vector-transfected controls (Figs. 6A and B). Similarly, migration of MUC6_N-expressing PANC-1 cells was consistently decreased by 70% ($P<0.01$) compared with the controls (Figs. 6A and B). Expression of MUC6_C or MUC6_N proteins in LS 180 cells also decreased invasion by 46% ($P<0.01$) and 41% ($P<0.01$), respectively, as compared with the vector control clones (Figs. 6C and D).

MUC6 N-terminal and C-terminal proteins alter the adhesion properties of LS 180 cells

To investigate the mechanism by which MUC6 expression decreases cell invasion, we assayed cell adhesion to different substrates by plating MUC6_C- and MUC6_N-expressing PANC-1 and LS 180 cells onto plastic microplates coated with collagen I, collagen IV, fibronectin or laminin. Although there was a significant decrease ($P<0.05$, unpaired t-test) in the adhesion of PANC-1 cells expressing MUC6_C and MUC6_N to collagen I and collagen IV as compared with the vector control clones, no difference was seen on fibronectin or laminin (Fig. 7A). In contrast, expression of MUC6_N significantly reduced adhesion of LS 180 cells to all matrices ($P<0.01$), as did expression of MUC6_C, though to a lesser degree ($P<0.05$), with the exception of on collagen I ($P<0.01$) (Fig. 7B).

Discussion

MUC6 is a secreted gel-forming mucin that plays an important role in protecting the gastrointestinal tract epithelium. We hypothesized that the normal functions of MUC6 may be usurped in cancer, as previous studies showed that a loss of MUC6 accompanies aggressive tumor behavior. Gastric tumors undergo malignant transformation following downregulation of MUC6 [12]. Aberrant expression of MUC6 has been reported in well-differentiated cholangiocarcinomas [28] (biliary tract tumors) and colonic adenomas [29]. Moreover, colonic sessile serrated adenoma, which is likely a precursor lesion to the development of invasive colorectal adenocarcinoma, showed a 100% correlation between MUC6 expression and progression to malignancy [30].

MUC6 protein expression levels in normal pancreas are much lower than in stomach. Several studies examined MUC6 expression in pancreatic cancer, though the results were inconsistent. The earliest cellular markers predictive of pancreatic adenocarcinoma are PanIN lesions. PanIN-1A and -1B demonstrated the highest levels of MUC6 protein [14–16,31], which then fell to lower levels in adenocarcinoma. These data mirror those obtained by RNA *in situ* hybridization [14]. MUC6 expression was also examined in the intraductal papillary mucinous neoplasms (IPMN) pathway, which is apparently distinct from the PanIN pathway and

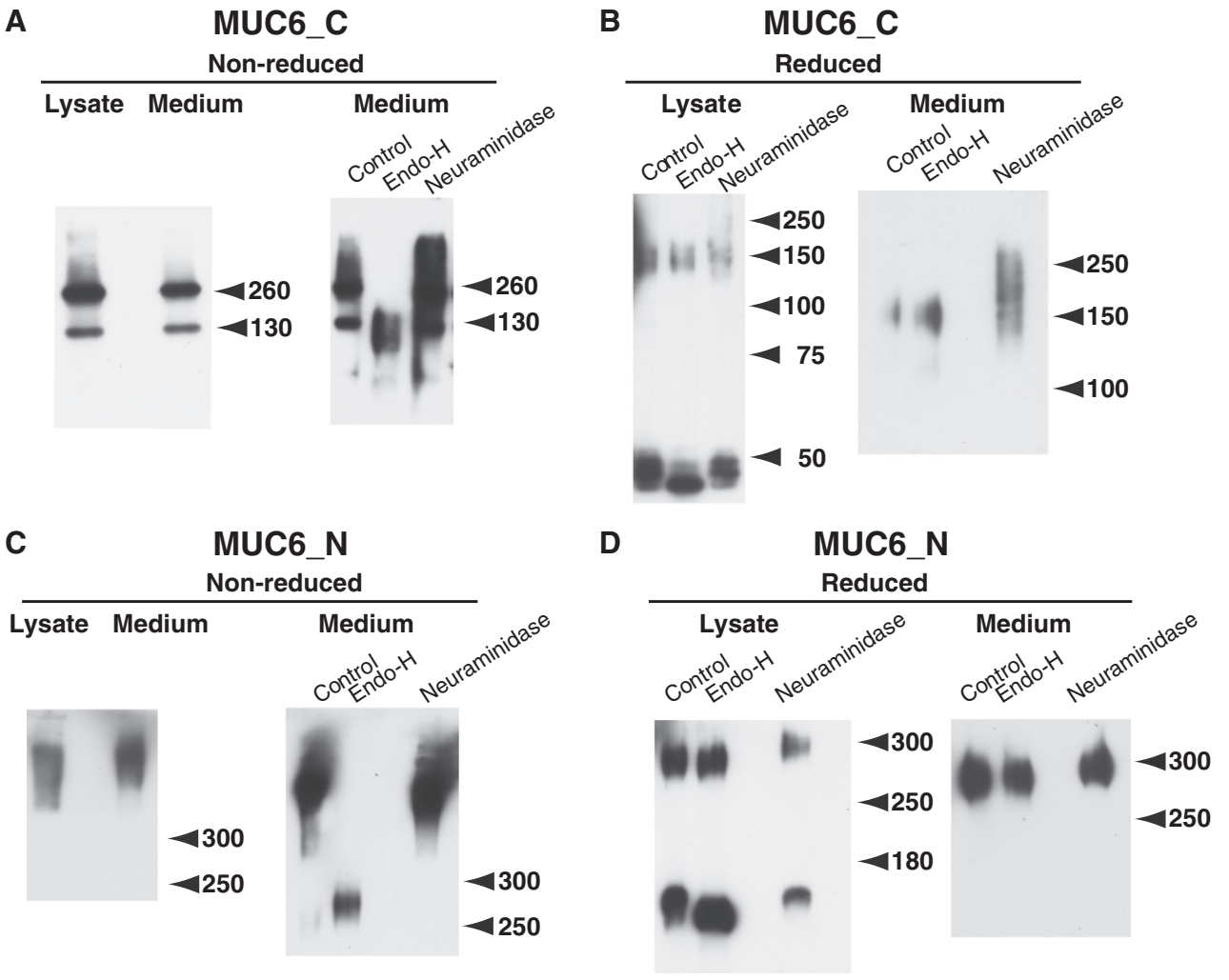

Fig. 5 – Oligomerization of MUC6N and MUC6_C. Immunoprecipitated MUC6_C (A, B) and MUC6_N (C, D) proteins from cells lysate and medium with or without Endo-H or neuraminidase treatment were analyzed under (A, C) non-reducing or (B, D) reducing conditions. MUC6_C and MUC6_N immunoprecipitated proteins were from MCF7 and LS 180, respectively and the reciprocal results were similar.

only gives rise to a minority of invasive ductal adenocarcinoma (IDC) [15,32]. MUC6 expression was higher in IPMNs-C (gastric type) than in IPMNs-D (intestinal type) and IDC. Other groups also reported MUC6 to be highly-expressed in intraductal tubular carcinoma [33,34]. The observations that MUC6 expression is elevated in early PanIN lesions but lost from later stages as they progress to invasive ductal adenocarcinoma [16] and that in intestinal-type (IPMNs) MUC6 is only present at early non-invasive stages and is later lost in all lineages [31], led us to evaluate *MUC6* gene expression in relevant gene expression databases.

In the analysis of two datasets from NCBI GEO, which compared RNA extracted from normal pancreas and pancreatic cancer tissues, no significant differences in MUC6 expression were seen. The results illustrated the potential pitfalls of comparing gene expression patterns in pancreatic tumors and adjacent tissues without performing careful pathological analysis of the sample followed by microdissection. This point is reinforced by our immunofluorescence data generated with the M6P antibody against the MUC6 mucin. In normal pancreas, the majority of MUC6 expression is found in intercalated ducts and possibly in centroacinar cells, with some MUC6 in intralobular ducts, but not in interlobular ducts. This contrasts with other work [31] that reported MUC6 expression in intercalated ducts and in some tributary ducts, but not in intralobular ducts. Moreover, there appears to be individual variation in the pattern of MUC6 expression in normal pancreatic tissue, which may be in part due to limitations of antibody specificity, since these are mainly targeted to the TR of MUC6 where O-glycosylation may mask the epitope. In pancreatic cancer, we observed aberrant distribution of MUC6 expression. Within the primary tumors, some MUC6 was evident in tumor-associated ductal structures; however, expression was lost in the abundant desmoplasia generated in the organ. Since MUC6 mucin is only synthesized in certain epithelial cells, comparisons of *MUC6* gene expression between normal and tumor tissue would be of limited value in the absence of detailed knowledge of the cellularity of the sample.

To further examine the significance of MUC6 up-regulation early in the development of pancreatic cancer, we utilized an *in vitro* invasion assay using cells over-expressing MUC6 domain-

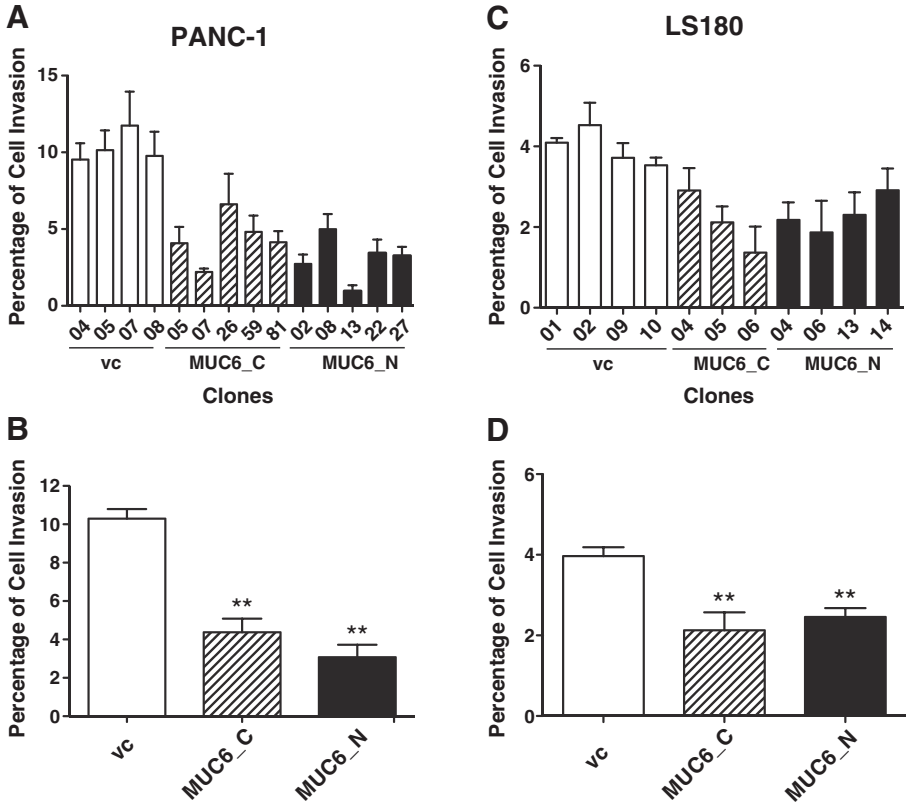

Fig. 6 – Effect of MUC6_C and MUC6_N expression on PANC-1 and LS 180 cell invasion. Cell invasion was assayed with a matrix barrier (collagen IV, laminin and gelatin) on a 10-μm-pore-size membrane. Invasion of individual MUC6_C- and MUC6_N- expressing clones and vector control clones of (A) PANC-1 cells and (C) LS 180 cells; (B) and (D) combined data from all the clones shown in (A) and (C), respectively. $^{**}P<0.01$, unpaired *t*-test.

specific proteins. Our results revealed that expression of both the MUC6 N-terminus and C-terminus proteins suppressed the invasion of pancreatic and intestinal carcinoma cells and that this was not the result of differences in cell proliferation rates between the clones. We also determined that this inhibition of invasion was not due to modulation of endogenous MUC6 expression levels in LS 180 clones where the MUC6 domains were overexpressed. Adhesion assays were carried out in parallel, and revealed some differences between the behavior of LS 180 and PANC-1 cells expressing MUC6_N and MUC6_C domains. The adherence of PANC-1 cells was influenced less by MUC6_N and MUC6_C expression than that of LS 180 cells. We also observed differences in the speed of adherence of these two cell lines: LS 180 cells adhered slowly, while PANC-1 cells adhered more quickly

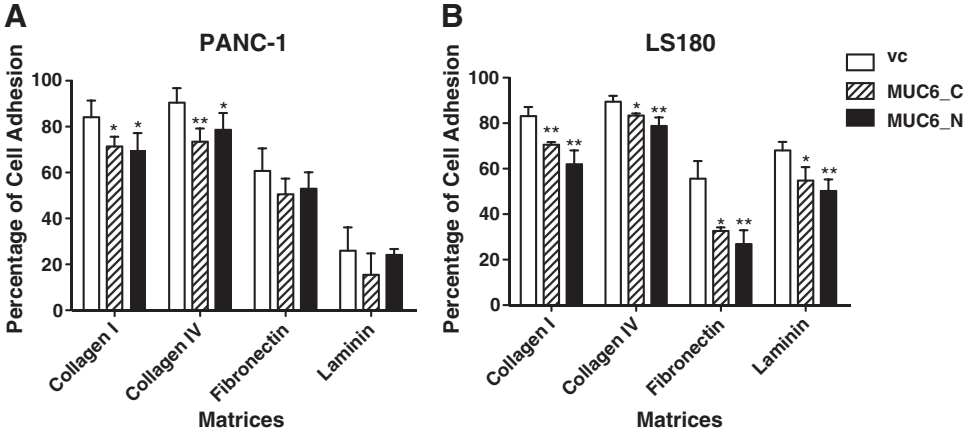

Fig. 7 – Effect of MUC6_C and MUC6_N expression on cell adhesion to collagen I, collagen IV, fibronectin and laminin substrates. (A) PANC-1 or (B) LS 180 cells were seeded on to the matrix-coated microplate, and 25 min (PANC-1) or 45 min (LS 180) after incubation the percentage of cell adherence was calculated. $^{**}P<0.01$; $^{*}P<0.05$, unpaired *t*-test.

(>90% cell adherence to the matrix protein surface within 30 min). It is possible that in the fast-adherent cell type, expression of MUC6 domains had a substrate-dependent, smaller or insignificant effect on adhesion because other cell surface adhesion molecules influenced substrate binding. Moreover, the LS 180 line expresses endogenous MUC6 and thus might have constitutively active pathways to affect adhesion through MUC6-mediated interactions.

The MUC2 mucin glycoprotein dimerizes through its C-terminal cystine knot and further oligomerization occurs through interactions of motifs in the N terminus [6,35]. The MUC6 and MUC2 mucins have many structural features in common, including the N-terminal vWF-D domains and a C-terminal cystine knot, so we predicted that MUC6 could also form oligomers. In stably transfected human epithelial cell lines we noted a difference in the molecular mass of intracellular and secreted forms of the MUC6_N- and C-terminus domains. MUC6_N and MUC_C proteins purified from cell culture supernatants were larger than those isolated from cell lysates and these secreted forms of MUC6_N and MUC6_C carried post-translational modifications such as glycosylation. The secreted forms having passed through the Golgi apparatus are resistant to Endo-H treatment under reducing conditions, while the smaller intracellular forms are likely incompletely modified precursors, as they are sensitive to Endo-H. Two forms of MUC6_C were observed under non-reducing conditions, the monomer and the dimer. Under the same conditions, the MUC6 N-terminus exhibited multiple forms of protein with an apparent molecular mass of 500–560 KDa, suggesting that the N-terminus forms disulfide bonded oligomers and the range of mobilities is due to differences in glycosylation. We did not see a monomeric form of secreted MUC6_N, suggesting that unlike MUC6_C, this N-terminal protein exists largely as an oligomer.

It is probable that these biochemical properties of the MUC6 mucus gel may have a profound impact on epithelial cell behavior *in vivo*, particularly when the glycoprotein is overexpressed in the early stages of pancreatic cancer. The cause of altered MUC6 expression in cancer is still unclear and since acidic conditions can up-regulate MUC6 [36], the low pH associated with inflammation and tumor cell metabolism might be critical. However, our studies suggest that MUC6 influences cell adhesion in the local tumor microenvironment. Further studies should be undertaken to examine whether MUC6 would serve as a useful biomarker for pancreatic cancer progression.

Supplementary materials related to this article can be found online at doi:10.1016/j.yexcr.2011.07.021.

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