Contribution of the MUC1 Tandem Repeat and Cytoplasmic Tail to Invasive and Metastatic Properties of a Pancreatic Cancer Cell Line

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ABSTRACT

MUC1 is a polymorphic, highly glycosylated, type I transmembrane protein expressed by ductal epithelial cells of many organs including pancreas, breast, gastrointestinal tract, and airway. MUC1 is overexpressed and differentially glycosylated by adenocarcinomas that arise in these organs, and is believed to contribute to invasive and metastatic potential by contributing to cell surface adhesion properties [via the tandem repeat (TR) domain] and through morphogenetic signal transduction [via the cytoplasmic tail (CT)]. The large extracellular TR of MUC1 consists of a heavily glycosylated, 20 amino acid sequence that shows allelic variation with respect to number of repeats. This portion of MUC1 may directly mediate adhesive or antiadhesive interactions with other surface molecules on adjacent cells and through these interactions initiate signal transduction pathways that are transmitted through the CT. We investigated the contribution of the TR domain and the CT of MUC1 to the in vivo invasive and metastatic potential, and the gene expression profile of the human pancreatic tumor cell line S2-013. Results showed that S2-013 cells overexpressing full-length MUC1 displayed a less invasive and metastatic phenotype compared with control-transfected cells and cells expressing MUC1 lacking the TR domain or CT. Clonal populations were analyzed by cDNA array gene expression analysis, which showed differences in the gene expression profiles between the different cell lines. Among the genes differentially expressed were several that encode proteins believed to play a role in invasion and metastasis.

INTRODUCTION

Pancreatic carcinoma is the fourth leading cause of death from malignancy in the United States (1). The 5-year survival rate is 3%, and the median survival is <6 months (2). The poor survival is partially attributable to difficulties of early detection and the fact that most patients present with advanced, disseminated disease. MUC1 is a polymorphic, highly glycosylated, type I transmembrane protein expressed by ductal epithelial cells of many organs including pancreas, breast, gastrointestinal tract, and airway (3). MUC1 is overexpressed and differentially glycosylated by different adenocarcinomas. Previous studies demonstrated that tumor cells expressing high levels of MUC1 may have increased invasive and metastatic potential (4–6). It has been proposed that MUC1 mediates antiadhesion activity by interfering with cell-cell and/or cell-ECM interactions, and thereby facilitates detachment of tumor cells from the primary growth (7–9).

Additional studies suggest that MUC1 may be a ligand for selectins (10).

Structurally, MUC1 contains two domains believed to be of functional significance. The large extracellular TR domain of MUC1 is highly O-glycosylated. Glycosylation of the TR domain varies among the different tissues, and tumor-associated MUC1 has been reported to be both less glycosylated (11) or more glycosylated (12) than forms expressed by normal tissues. Differential glycosylation patterns on the TR may affect adhesion properties that result in an increased ability of tumor cells to metastasize. Additional evidence suggests that MUC1 may also confer antiadhesive properties, thereby aiding in the metastatic spread of tumor cells (13). Another important domain of MUC1 is the CT, which is highly conserved across mammalian species and hypothesized to play a role in its post-translational processing, subcellular localization, signal transduction, and intracellular localization (14). Alterations to the CT may affect trafficking through the Golgi and thereby influence glycosylation of the TR domain. The CT also mediates signal transduction events that may contribute to functional differences between tumors and normal cells (15–20). β-Catenin binds to a consensus site found in the MUC1 CT; GSK-3β phosphorylates a serine residue in the CT; and there is a consensus Grb2/Sos binding site in the CT, which includes a potentially phosphorylated tyrosine residue (18–20). Tumor-associated modifications of these sites may affect the ability of tumor cells to associate with components of the ECM or other cells (both malignant and benign).

In addition to the full-length transmembrane isoform, additional variants of MUC1 exist. These isoforms are generated through alternative mRNA splicing, and a number of them lack the TR region (MUC1/X, MUC1/Y, and MUC1/Z) or the CT (MUC1/SEC; Refs. 21–25). Most alternatively spliced variants of MUC1 have been identified in cancer cell lines, and given the potential functional roles of these two domains in the cell, we sought to evaluate the contribution of the TR and CT of MUC1 to the metastatic potential of a human pancreatic carcinoma cell line S2-013 in an in vivo model. S2-013 cells were control-transfected or transfected with constructs resulting in overexpression of full-length MUC1, or overexpression of MUC1 with the CT or the TR portion deleted. We investigated the role of MUC1 in invasion and metastasis using heterotopic implantation of tumor fragments onto the ceca of nude mice. Given the potential role of MUC1 in morphogenetic signal transduction and that the predicted result of alterations in signaling would be to influence patterns of gene expression, we also performed DNA microarray analyses on clonal populations of these cells. Deletion of either the CT or TR of MUC1 resulted in an increased propensity of tumor cells to invade vessels and metastasize to lymph nodes compared with a cell line expressing full-length MUC1. These results suggest a cooperative relationship between the CT and TR domain of MUC1. Deletion of the CT or TR domain produced cell lines with a more aggressive phenotype on implantation onto the cecum. On the other hand, overexpression of full-length MUC1 resulted in the other extreme: a substantially decreased invasive and metastatic character on implantation on the...
Materials and Methods

Reagents. Restriction enzymes and ligase were purchased from Life Technologies, Inc. (Grand Island, NY). Oligonucleotides were synthesized by the Eppley Institute Core Laboratory. Monoclonal antibodies were obtained from the following sources: M2 antibody, FLAG Peptide and M2-agarose were purchased from Sigma (St. Louis, MO); HMFG2 was provided by Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ). Alkaline phosphatase conjugated goat anti-mouse immunoglobulin was purchased from Pierce (Rockford, IL); alkaline phosphatase-conjugated goat-anti-rabbit antibody and enhanced chemiluminescence substrate were purchased from Amersham (Arlington Heights, IL). Lipofectin, cell culture medium, and Geneticin (G418) were purchased from Invitrogen (Carlsbad, CA). All of the other chemicals were purchased from Sigma unless otherwise specified.

Deletion of the MUC1 Tandem Repeat and Cytoplasmic Tail Domain. The region of the MUC1 cDNA encoding the TR domain was deleted by using the restriction enzyme sites BamHI and EcoRl, which flank the region of the cDNA that encodes the TR domain. A double-stranded oligonucleotide linker was designed to join the resulting cDNA fragments while simultaneously introducing sequence encoding the FLAG epitope DYKDDDD and two unique restriction sites, BgIII and ThhI11I. The sequence of the coding strand of the oligonucleotide was 5'-GATGAAAGATGACGACAGATCTTGGGACATGGTGC-3' and the sequence of the complementary strand was 5'-CGACCATGTCAGCTGTCGTCATCCTTGTAATCAG-3'.

The deletion of the MUC1 CT has been described (15) and was kindly provided by Sandra Gendler (Mayo Clinic). The epitope tag was added to the constructs by swapping a Muc 1-Flag fragment into the MUC1 (26) cDNA in place of the native sequence.

Expression of Epitope Tagged, Tandem Repeat-deleted and Cytoplasmic Tail-deleted MUC1. Epitope tagged, TR-deleted MUC1 (MUC1F[ΔTR]) and CT-deleted MUC1 (MUC1F[ΔCT]) cDNAs were subcloned into the expression vector pHB-APr1-neo at the BamHI site. S2-013 cells, a SUIT2-derived human pancreatic adenocarcinoma cell line, were transfected with plasmid DNA using the Lipofectin method as described (26). Transfectants were selected in growth medium containing G418 at 800 μg/ml. After 7–10 days, the cells were passaged and plated at limiting dilutions in 96-well plates. Clones were screened for expression of MUC1F, MUC1F[ΔTR], and MUC1F[ΔCT] by analyzing surface immunofluorescence. For screening, cells were plated in 8-well cell culture slides, allowed to grow to 80% confluence, fixed in 4% formaldehyde, and staining using the monoclonal antibody M2 (10 μg/ml) and a goat anti-mouse-FITC secondary antibody (10 μg/ml), and visualized on a fluorescence microscope.

Cell Culture. MUC1F transfectants of the S2-013 cells (S2-013.NEO, S2-013.MUC1F, S2-013.MUC1F[ΔTR], and S2-013.MUC1F[ΔCT]) were maintained in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Valley Biomedical, Winchester, VA), sodium pyruvate (Sigma), penicillin/streptomycin (Biowhittaker, Walkersville, MD), and 12 μg/ml G418 in a humidified incubator at 37°C and 5% CO2.

Preparation of Cell Lysates. Cell lysates were prepared by scraping cells into 1 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) with a rubber cell scraper. Lysates were incubated on ice for 30 min and centrifuged at 4°C for 2 min at 6000 rpm to remove cell debris. Supernatants were transferred to fresh tubes, and protein content was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with BSA as standards. Cell lysates were stored at −20°C.

Immunoblotting. Cell lysate proteins were resolved on 4–20% Novex Tris-Glycine gradient denaturing polyacrylamide gels (Invitrogen) in a 1× SDS-PAGE buffer (1 g/liter SDS, 3 g/liter Tris base, and 14.4 g/liter glycine). Proteins were transferred to polyvinylpyrrolidone difluoride membranes electrothermically and blocked overnight in blotto [5% dry milk in 1× Tris-buffered saline (0.9% NaCl, 10 mM Tris (pH 7.4), and 0.5% MgCl2)]. Primary antibodies were diluted in blotto. Membranes were incubated for 45 min at room temperature with light shaking, followed by three 10-min washes with blotto. Alkaline phosphatase-conjugated goat antirabbit secondary antibodies were diluted in blotto, and incubations were for 45 min at room temperature with light shaking. After incubation with secondary antibody, the membranes were washed three times as above, followed by three washes with 1× Trisbuffered saline. Enhanced chemiluminescence reagent was applied per manufacturer instructions (Amersham Life Science LTD., Buckinghamshire, United Kingdom), and blots were visualized using a Fujifilm LAS-1000 CCD imaging system (Fuji Film Co., Tokyo, Japan). Analysis was performed using IR-LAS-1000 Lite V.1.1.1 software.

Flow Cytometric Analysis of MUC1 Surface Expression on S2-013 Transfectants. Adherent cells were released from culture flasks by treating with 0.05 mM trypsin and 1.5 mM EDTA in PBS for 5 min at 37°C. All of the subsequent steps were carried out on ice. The cells were resuspended in FACS medium (1× PBS, 0.2% BSA, and 0.1% sodium azide) at a concentration of 1×10^6 cells/ml, and incubated with M2 antibody for 20 min at 4°C. The cells were washed with FACS medium and incubated with a phycoerythrin-conjugated rabbit antirabbit secondary antibody (Jackson Immunoresearch, West Grove, PA) for 20 min at 4°C. The cells were washed again and resuspended in FACS medium, followed by analysis on a FACS Calibur (Becton Dickinson, Mountain View, CA). Analysis was performed with Cell Quest software.

Matrigel in Vitro Invasion Assay. Matrigel invasion assays were performed using 24-well BD BioCoat Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA) according to manufacturer directions. Briefly, 2.5×10^6 cells in 250 μl of serum-free DMEM were seeded on BD Falcon Cell Culture Inserts with an 8-μm pore size coated with 12 mg/ml Matrigel Basement Membrane Matrix (in triplicate). The bottom chamber was filled with 500 μl conditioned medium to act as a chemoattractant. After 24-h incubation at 37°C, 5% CO2, cells on the underside of the chambers were removed by treatment with 0.05 mM trypsin and 1.5 mM EDTA in PBS for 5 min at 37°C, and counted under a microscope using a hemacytometer (Hauser Scientific, Horsham, PA). Statistical significance of differences among experimental groups was determined using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. A P < 0.05 was considered significant.

Mice. Congenitally athymic female National Cancer Institute nude mice (NCl-nu/nu) were purchased from the National Cancer Institute (Bethesda, MD). Animals were maintained in pathogen-free conditions, and were fed sterile water and food ad libitum. Mice were treated in accordance with the Institutional Animal Care and Use Committee guidelines.

Tumor Challenge. On the day of tumor challenge, adherent control (S2-013.NEO) and MUC1-expressing S2-013 tumor cell lines were removed from tissue culture flasks with 0.05 mM trypsin and 1.5 mM EDTA in PBS for 5 min at 37°C, counted, and resuspended in DMEM at a concentration of 1×10^5 viable cells/ml. Mice received 1×10^6 viable S2-013.MUC1F, S2-013.MUC1F[ΔTR], S2-013.MUC1F[ΔCT], or S2-013.NEO cells by s.c. injection between the scapulae. Tumor growth was evaluated every 2–3 days, and tumor diameter was measured using a caliper. Mice were euthanized when the tumor diameter reached 8–10 mm. At this time, tumors were harvested and prepared for recipient animals.

S2-013 Tumor Specimens and Surgical Microprocedures. Harvesting of tumor specimens and surgical procedures were carried out in a laminar flow hood under sterile conditions. Harvested tumor specimens were halved. One half was flash frozen in liquid N2 and stored for additional analysis. The second half was inspected for necrotic tissue. Necrotic and suspected necrotic tissue was removed, and the remaining specimen was divided into four parts, each 2 mm in diameter. Specimens were kept in HBSS (Life Technologies, Inc.) until recipient animals were prepared. Recipient animals were anesthetized with a mixture of xylazine (35 mg/kg) and ketamine (120 mg/kg) by i.p. injection. The abdomens of recipient animals were sterilized by painting with betadine (The Purdue Frederick Company, Norwalk, CT). A small incision (~5 mm) was made on the lower left quadrant, and the cecum was exteriorized. One 2-mm tumor specimen was implanted onto the distal portion of the cecum with one 5–0 chromic gut surgical suture (Ethicon, Somerville, NJ). The cecum was carefully returned to the abdominal cavity, and the abdominal wall was closed with 5–0 nylon surgical suture (Ethicon). Post-surgery, animals were kept warm and observed until recovered from anesthesia. The animals were observed closely for the next 2 days for recovery. Tumor xenotransplants were allowed to grow for 5 weeks or until the animal...
became moribund (whichever occurred first). At this time animals were sacrificed, and examined for tumor invasiveness and metastasis.

Tissue Specimens and Histological Examination. Tumor, cecum, lungs, kidneys, liver, and mesenteric lymph nodes were harvested from each animal and fixed in a buffered formalin solution ([100 ml formalin, 3.4 g NaH2PO4, and 10.3 g NaHPO4]/1000 ml) (pH 7.3–7.4) and embedded in paraffin. Serial 5-μm sections were cut and mounted on slides, and stained with H&E using standard procedures. For immunohistochemical evaluation of MUC1F or MUC1F deletion construct expression on primary tumor and for metastasis detection, 5-μm thick, paraffin-embedded tissue sections were assessed using a modification of described ABC immunohistochemical methods (27). Briefly, tissue sections were deparaffinized in EZ-DeWax (Biogenex, San Ramon, CA). Antigen unmasking was carried out using 10 mM citrate buffer and boiled for 10 min. The sections were then incubated in blocking serum for 20 min, 1° mAb M2 (Sigma) was added, and the samples were kept in a humid chamber at 4°C overnight. The slides were then rinsed with PBS and incubated for 1 h with biotin-labeled 2° mAb. Endogenous peroxidase activity was blocked by incubating the samples in 3% H2O2 for 5 min. The slides were then incubated for 30 min at room temperature with ABC reagent (Vector Labs, Burlingame, CA). The slides were then rinsed with PBS and incubated for 3–5 min with 3,3-diaminobenzidine substrate (Vector Labs) observing closely for color to develop. Sections were then incubated for 10 min in 50 mM sodium bicarbonate (pH 9.6) followed by a 5–20 s incubation in 3,3-diaminobenzidine enhancing solution (Vector Labs) and counterstained with Meyer’s hematoxylin for 30 s. Coverslips were applied, and the slides were examined under a microscope (Nikon E400; Nikon, Tokyo, Japan). Images were captured using a digital camera (Nikon CoolPix 950; Nikon).

Statistical Analysis. For each cell line, the proportion of animals with tumor in the three experiments was compared using a χ2 test. If there was not evidence of a difference in outcome among the three experiments, data for the three experiments were combined and results of the four cell lines were compared using a χ2 test. Otherwise, logistic regression was used to examine differences among cell lines, adjusting for differences among experiments. Fisher’s exact test was used to additionally examine pairwise differences among cell lines. A P < 0.05 was considered to be statistically significant.

cDNA Array Gene Expression Analysis of S2-013 Constructs. DNA microarrays contained either 2758 or 3157 cDNAs derived from the I.M.AG.E. Consortium cDNA clone set (ResGen; Invitrogen) and prepared as described previously (28). Purified PCR-amplified cDNAs were spotted using a MagnaSpotter robot (BioAutomation, Corp., Dallas, TX) with a six-pin (Telechem, Sunnyvale, CA) configuration in a humidified, HEPA-filtered hood. Approximately 1 nl of PCR product was spotted on poly-l-lysine coated microscope slides at a concentration of 2–10 ng/μl. After spotting, the slides were rehydrated (28), UV-cross-linked in a Stratalinker (Stratagene, Inc., La Jolla, CA), dehydrated (28), UV-cross-linked in a Stratalinker (Stratagene, Inc., La Jolla, CA), and chilled on ice. The labeling mixture for a single sample consisted of 8 μl of 5× Hybridization Buffer (1× SSC, 0.1 mg/ml yeast tRNA, and 12.5 μg of total RNA was incubated with 2 μg of oligodeoxynucleotide dye mixture (1 μM each dATP, dGTP, and dTPP; 0.2 mM dCTP), 2 μl of 1 μM Cy3- or Cy5-labeled dCTP, 30 units of RNaseH (1 μl) and 400 units of Superscript II. After incubation at 42°C for 30 min, an additional 400 units of Superscript II (200 units/μl) was added to the mixture and incubated for an additional 60 min. The reaction was stopped by adding 5 μl of 0.5 M EDTA. Residual RNA was hydrolyzed by adding 10 μl of 1 M NaOH to the mixture followed by incubation at 65°C for 30 min and cooled to room temperature. The reaction was neutralized with 25 μl of 1 M Tris-HCl and desalted using a Microcon-YM30 centrifugal filter device (Amicon-Millipore Corporation, Bedford, MA). Cy3- and Cy5-labeled cDNA were combined and diluted to 60 μl with 3× SSC/0.15% SDS hybridization solution. To reduce nonspecific hybridization, the hybridization solution also contained final concentrations of 10 μg of poly(deoxyadenylate) (Pharmacia), 2.5 μg of yeast tRNA (Sigma), and 12.5 μg of human Cot1 DNA (Invitrogen). The fluorescent probes were boiled for 3 min and cooled to room temperature. After clarification by centrifugation, the probe/hybridization solution was applied to DNA microarrays and incubated at 65°C for 16–20 h. After hybridization, the arrays were washed once for 5 min with 1× SSC/0.1% SDS, once for 5 min with 0.1× SSC/0.01% SDS, and rinsed with 0.1× SSC. The arrays were dried by centrifugation for 5 min at 1000 rpm and scanned with a ScanArray 4000 confocal laser system (Perkin-Elmer, Wellesley, MA).

Microarray experiments were performed using both a reference (2758 cDNA arrays) and block (3157 cDNA arrays) design (29). S2-013.NEO RNA from three independent isolations was pooled and used as reference RNA to hybridize against the three MUC1 constructs. Direct comparisons were also performed: S2-013.MUC1F versus S2-013.MUC1F(ΔTR), S2-013.MUC1F versus S2-013.MUC1F.CT3, and S2-013.MUC1F(ΔTR) versus S2-013.MUC1F.CT3. All of the comparisons were performed in triplicate (including a dye swap experiment). Fluorescent intensities were background subtracted, and normalization and filtering of the data were performed using the QuantArray software package (Perkin-Elmer). For all of the comparisons, differential expression was defined as those genes exhibiting a 2-fold up or down average ratio in signal intensity (after normalization).

RESULTS

Generation of Epitope Tagged, Tandem Repeat-deleted and Cytoplasmic Tail-deleted MUC1. MUC1 cDNA (26) was digested with BsmI and EcoRI restriction enzymes resulting in one fragment encoding the TR domain including the degenerate repeats, and a second fragment containing the cloning vector sequence and the remaining MUC1 sequence. The fragment containing the vector and MUC1 sequence outside the TR was isolated from a gel slice and ligated using a synthetic oligonucleotide linker that encoded the FLAG epitope, two unique restriction enzyme sites, and BsmI and EcoRI compatible ends. Proper ligation and insertion of the oligonucleotides was verified by sequence analysis, and the resulting TR-deleted MUC1 construct was subcloned into the pH-B-Apr-1-neo expression vector. The construction of the MUC1F.CT3 mutant has been described previously (30). A schematic alignment of the altered MUC1F isoforms is shown in Fig. 1. The MUC1F(ΔTR) mutant retains the same NHEJ terminus, CT, membrane spanning domain, and the region containing the proteolytic cleavage site (31). To verify the integrity of the FLAG epitope-tag and cell surface expression of these proteins, stably transfected S2-013 cells were evaluated by flow cytometry and Western blot. As expected, MUC1F, MUC1F(ΔTR), and MUC1F.CT3 were expressed at the cell surface (Fig. 2). Western blot analysis of whole cell lysates using FLAG specific M2 antibody revealed a M, >>250,000 band for MUC1F, a M, ~55,000 band for MUC1F(ΔTR), and a M, ~220,000 band for MUC1F.CT3 (Fig. 3A). Lysates were also probed with an antibody recognizing the TR domain, HMFG2 (Fig. 3B). Both S2-013.MUC1F and S2-013.MUC1F.CT3 exhibited strong reactivity, whereas the
S2-013.MUC1F clone did not, indicating the successful deletion of the TR domain. S2-013.NEO expresses low levels of endogenous MUC1 (Fig. 3B).

**Effect of MUC1F or MUC1F Deletion Construct Expression by S2-013 Cells on In Vitro Invasiveness.** Matrigel invasion assays were used to investigate the effect of overexpression of MUC1F, MUC1F(ΔTR), or MUC1F.CT3 on the *in vitro* invasive potential of the stable transfectants. The results of these studies are shown in Fig. 4. S2-013.MUC1F cells exhibited a higher invasive potential compared with S2-013.MUC1F(ΔTR), S2-013.MUC1F.CT3, and S2-013.NEO cells (*P* < 0.001). Significantly more S2-013.MUC1F.CT3 cells invaded through the Matrigel compared with S2-013.MUC1F(ΔTR) and S2-013.NEO cells (*P* < 0.001). No significant difference of invasive potential was observed for S2-013.MUC1F(ΔTR) and S2-013.NEO cells.

**Histological and Morphological Characteristics of S2-013 Tumors in Nude Mice.** S2-013 tumors were grown s.c. in athymic nude mice. Heterotopically grown tumors were established in all of the cases. S2-013 tumors retained characteristics of a moderately differentiated pancreatic adenocarcinoma. Tissue sections were also stained for the expression of FLAG epitope-tagged MUC1. MUC1F was expressed on the cell surface on the majority of cells (Fig. 5), indicating that S2-013 cells continue to express transfected full-length and domain-deleted MUC1 in *vivo*.

**Lymphatic Vessel Invasion of S2-013 Transfectants.** The distribution of lymphatic vessel invasion in the cecum by cell line is shown in Table 1. There was a statistically significant difference in the percentage of animals that developed lymphatic vessel invasion in the four cell lines. Specifically, fewer S2-013.MUC1F animals developed lymphatic vessel invasion compared with S2-013.MUC1F(ΔTR), S2-013.MUC1F.CT3, and S2-013.NEO animals. There was no statistically significant difference in the distribution of lymphatic vessel invasion among the three cell lines.

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**Fig. 2.** Cell surface expression of MUC1 protein on S2-013 transfectants. Flow cytometry analysis was performed using the anti-FLAG mAb, M2. Open lines indicate the control reaction without M2 and filled peaks represent MUC1 cell surface expression.

**Fig. 3.** Western blot analysis of S2-013 whole cell lysates. SDS-PAGE was run under nonreducing conditions. A, blot with mAb M2 recognizing the FLAG epitope (1:500 dilution). B, blot with monoclonal antibody HMFG2 recognizing the TR domain of MUC1 (1:500 dilution).

**Fig. 4.** Invasion activity of S2-013 transfectants into Matrigel (*n* = 3, mean); bars, ±SD. The S2-013.MUC1F cells showed a significantly greater ability to invade into Matrigel as compared with the other three cell lines. The S2-013.MUC1F.CT3 cells showed a significantly greater ability to invade as compared with the S2-013.MUC1F(ΔTR) and S2-013.NEO cells.

**Fig. 5.** S2-013 primary tumors stained with M2 antibody recognizing the FLAG epitope on MUC1 constructs (counterstained with hematoxylin). All pictures shown at ×100 magnification. A, S2-012.NEO; B, S2-013.MUC1F; C, S2-013.MUC1F(ΔTR); D, S2-013.MUC1F.CT3.
invasion for the S2-013.NEO tumors compared with tumors arising from S2-013.MUC1F.CT3 (P = 0.99) cell lines. The S2-013.MUC1F(ΔTR) tumor cell line showed a trend toward more aggressive lymphatic vessel invasion, but these differences did not achieve statistical significance when compared with S2-013.NEO (P = 0.26) or S2-013.MUC1F.CT3 tumors (P = 0.28). A representative tissue section of vessel invasion is shown in Fig. 6, A and B, for S2-013.MUC1F(ΔTR) and S2-013.MUC1F.CT3, respectively. In summary, the percentage of lymphatic vessel invasion was lower in those animals receiving S2-013.MUC1F tumor implants.

Lymph Node and Lung Metastasis of S2-013 Transfectants.

Peritoneal organs, lungs, mesenteric lymph nodes, and liver were examined for distant metastases. No metastases were detected in the kidney or spleen, and three total liver metastases were detected [one each in S2-013.NEO, S2-012.MUC1F(ΔTR), and S2-013.MUC1F.CT3]. The majority of metastases were found in the mesenteric lymph nodes (Fig. 6C) and lungs (Fig. 6D). A summary of the prevalence of lymph node and lung metastasis for each cell line is shown in Table 2. It is worth noting that the percentage of animals that developed lymph node metastasis was lower for the S2-013.MUC1F cell line compared with the S2-013.NEO cell line. Using S2-013.MUC1F as the reference group, the odds of having lymph node metastasis is increased for the S2-013.MUC1F(ΔTR) cell line (P = 0.02). The percentage of animals that developed lung metastasis was different in the four cell lines as shown in Table 2 (P = 0.01). The percentage of lung metastasis was lower for the S2-013.MUC1F cell line compared with the S2-013.MUC1F(ΔTR) cell line (P = 0.004). This appears to be primarily because of differences in lung metastasis in experiment 2 [MUC1F, 0% versus MUC1F(ΔTR), 56%; P = 0.01]. There was also some evidence that lung metastasis was more prevalent in S2-013.MUC1F(ΔTR) tumors compared with S2-013.NEO tumors (8% versus 30%; P = 0.08).

Gene Expression Analysis of S2-013 Transfectants. cDNA arrays representing 2.7K and 3.2K genes were used to identify potential genes influenced by MUC1 expression, and responsible for the invasive and metastatic differences observed in the S2-013 transfectants. Transfected cells expressing full-length MUC1 (S2-013.MUC1F), MUC1 without the CT (S2-013.MUC1F.CT3), and MUC1 devoid of a TR domain [S2-013.MUC1F(ΔTR)] were compared with a reference RNA of control-transfected S2-013.NEO cells. Table 3 lists the genes differentially expressed between S2-013.MUC1F and S2-013.MUC1F(ΔTR) cell line (exp. 1, 0% versus exp. 2, 56% versus exp. 3, 30%; P = 0.04). There was no difference in the percentage of animals that developed lymph node and lung metastasis across the three experiments from the MUC1F, MUC1F.CT3, and NEO cell lines.

Logistic regression was used to examine the differences in lymph node metastasis across cell lines after accounting for differences by experiment. The S2-013.NEO cell line was used as the reference group. The odds of lymph node metastasis is increased for the S2-013.MUC1F(ΔTR) cell line compared with the S2-013.NEO cell line after accounting for differences by experiment (odds ratio, 13.8; P = 0.02). There was no difference in lymph node metastasis for the S2-013.MUC1F or the S2-013.MUC1F.CT3 when compared with the S2-013.NEO cell line. Using S2-013.MUC1F as the reference group, the odds of having lymph node metastasis is increased for the S2-013.MUC1F(ΔTR) cell line (P = 0.02). The percentage of animals that developed lung metastasis was different in the four cell lines as shown in Table 2 (P = 0.01). The percentage of lung metastasis was lower for the S2-013.MUC1F cell line compared with the S2-013.MUC1F(ΔTR) cell line (P = 0.004). This appears to be primarily because of differences in lung metastasis in experiment 2 [MUC1F, 0% versus MUC1F(ΔTR), 56%; P = 0.01]. There was also some evidence that lung metastasis was more prevalent in S2-013.MUC1F(ΔTR) tumors compared with S2-013.NEO tumors (8% versus 30%; P = 0.08).

Table 1. Lymphatic vessel invasion by cell line

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<th>Cell line</th>
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<th>S2-013.CT3</th>
<th>S2-013.ΔTR</th>
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<td>14 (54%)</td>
<td>6 (23%)</td>
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*p < 0.05 compared to MUC1F (Fisher’s exact test).
*p < 0.001 compared with MUC1F (Fisher’s exact test).

Fig. 6. H&E tissue stains of lymphatic vessel invasion and metastases. A, lymphatic vessel invasion of S2-013.MUC1F(ΔTR) tumor (×100). B, partial lymphatic vessel invasion of S2-013.MUC1F.CT3 tumor (×100). C, lymph node metastasis of S2-013.MUC1F(ΔTR) tumor (×100). D, lung metastasis of S2-013.MUC1F(ΔTR) tumor (×100).
have structural similarities with a number of alternatively spliced forms; however, there are important distinctions. Both MUC1F(ΔTR) and MUC1F/Y are expressed at the cell surface, but MUC1F(ΔTR) retains a proteolytic cleavage site and is expressed as a heterodimer, and studies have shown that MUC1F/Y is not proteolytically cleaved (35). MUC1/SEC lacks the transmembrane domain and CT, and is secreted, whereas MUC1F.CT3 retains the transmembrane domain and is, therefore, expressed at the cell surface. The aim of our studies was not to use MUC1F(ΔTR) and MUC1.CT3 to simulate MUC1/Y or MUC1/SEC, but instead to evaluate the contribution of the TR and CT of MUC1 to the metastatic potential of the tumor cells when full-length MUC1 is overexpressed at the cell surface.

There is conflicting evidence in the literature regarding the role of MUC1 in metastasis. Numerous studies found that high levels of MUC1 expression correlate with invasive cancers of the colon, pancreas, papillary thyroid, gallbladder, and oral epithelium (36–41). However, Rahn et al. (42) reported that in breast carcinoma higher overall MUC1 expression was associated with a better prognosis and lower grade tumor, and that this reflected similar findings in an extensive review of the literature. Similarly, Biemer-Huttmann et al. (43) concluded that there was no correlation between MUC1 expression and metastasis in a study of breast cancer cell lines. Many groups have documented the apparent adhesive and antiadhesive properties of cells expressing MUC1 (9, 44–47). Initially, we hypothesized that the S2-013 cell line expressing full-length MUC1 would exhibit the greatest invasive and metastatic potential, and that removal of the CT or TR domains would diminish these characteristics.

An in vitro invasion assay showed that S2-013.MUC1F cells had significantly greater ability to invade through Matrigel-coated transwells as compared with the other two transfectants and NEO control (Fig. 4). Cells expressing full-length MUC1 were five times more invasive in vitro than cells expressing MUC1F devoid of the TR. This result is in agreement with previous results from our laboratory (46), which showed that MUC1 expression enhanced in vitro invasiveness and decreased the ability of S2-013 cells to bind to Type I collagen, Type IV collagen, and laminin. Similar results were described by

**DISCUSSION**

In this report we investigated the invasive and metastatic properties of a pancreatic cancer cell line, S2-013, transfected with human MUC1 or altered isoforms. S2-013 is a subline of the human pancreatic cancer cell line, SUIT-2, that produces a moderately differentiated adenocarcinoma cell line, SUIT-2, that produces a moderately differentiated cancer cell line, SUIT-2, that produces a moderately differentiated cancer cell line. S2-013 is a subline of the human pancreatic cancer cell line, S2-013, transfected with human MUC1 or mutant forms. The full-length FLAG-tagged MUC1 construct has been described previously (26). Two additional constructs encoded a CT deletion (MUC1F.CT3; Ref. 15) or a complete deletion of the TR (MUC1F(ΔTR)) lacks all of the TR domain, as well as the degenerate repeat sequence that flanks the TR domain on both ends (Fig. 1).

Several MUC1 isoforms can be generated by alternative splicing from the MUC1 gene (21–25, 33, 34). Most alternatively spliced forms of MUC1 have been identified in tumor cell lines or a few cancers. MUC1/Y is a transmembrane isoform lacking the entire TR domain (21). Results of some studies suggest that MUC1/Y plays a role in oncogenesis (24). Two other splice variants lacking the TR have been identified: MUC1/X and MUC1/Z (21, 23, 24). An additional secreted form of MUC1 lacking the transmembrane domain and CT (MUC1/SEC), but containing the full TR region, has been identified (22, 25). Constructs of MUC1 used for studies presented here

---

**Table 2. Lymph node/lung metastasis by cell line**

<table>
<thead>
<tr>
<th></th>
<th>NEO</th>
<th>MUC1F</th>
<th>CT3</th>
<th>ΔTR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>20 (77%)</td>
<td>23 (88%)</td>
<td>23 (74%)</td>
<td>19 (70%)</td>
</tr>
<tr>
<td>Positive</td>
<td>6 (23%)</td>
<td>3 (12%)</td>
<td>8 (26%)</td>
<td>8 (30%)</td>
</tr>
<tr>
<td><strong>Lung metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24 (92%)</td>
<td>26 (100%)</td>
<td>27 (87%)</td>
<td>19 (70%)</td>
</tr>
<tr>
<td>Positive</td>
<td>2 (8%)</td>
<td>0%</td>
<td>4 (13%)</td>
<td>8 (30%)</td>
</tr>
</tbody>
</table>

*P = 0.02 compared with NEO or MUC1F cell line (logistic regression).

*P = 0.08 compared with ΔTR cell line (Fisher’s exact test).

*P = 0.004 compared with ΔTR cell line (Fisher’s exact test).

013.NEO in order of magnitude. As indicated, a total of 14 genes are unique to this comparison and are not detected as differentially expressed in the CT3 or ΔTR mutants (versus NEO). The remaining genes exhibit differential expression in at least one other S2-013 mutant (CT3, ΔTR, or both). There were a total of 6 genes differentially expressed in all three of the mutant cell lines (versus NEO; Table 3). Few genes were exclusively differentially expressed in the ΔTR versus NEO and CT3 versus NEO comparisons (data not shown).

Direct comparisons between the transfected S2-013 cell lines provided additional evidence of genes affected by the deletion of the MUC1 CT or TR domain. Tables 4 and 5 show lists of genes differentially expressed between S2-013 cells expressing full-length MUC1 and MUC1 deletion constructs lacking the CT or TR domain. Differences in gene expression observed between these sets of comparisons are hypothesized to represent downstream effects mediated by these separate domains of MUC1. Differential expression of genes similar to both comparisons is more likely because of the effects of unregulated overexpression of full-length MUC1 (compared with mutant forms). Interesting similarities that may account for the marked increase in invasive and metastatic potential of the S2-013 mutants are discussed in the following section. There were few genes differentially expressed between the ΔTR and CT3 transfectants (data not shown).

---

**Table 3. Differentially expressed genes between S2-013.MUC1F and S2-013.NEO**

| Genes differentially expressed between S2-013.MUC1F and S2-013.NEO |
|---|---|---|---|---|
| S2-013.MUC1F/S2-013.NEO > 2.0 |
| Metallothionein 1H  
| Nucleosome assembly protein 1-like b  
| Metalloproteinase 10G  
| KIAA0544 protein  
| N-acetylglucosaminyltransferase (GalNac-T)  
| Glucose regulated protein, 58 kDa  
| Thioredoxin reductase 1  
| Connective tissue growth factor  
| Apolipoprotein E  
| MvP17 transgene, murine homolog, glucuronosyltransferase  
| Collagen, type XIV, alpha 1; undulin  
| Spermine synthase  
| Transferrin receptor (p90, CD71)  
| Tropomyosin 1 (alpha)  
| Annexin A1  
| Clathrin assembly lymphoid-myeloid leukemia gene  
| Nucleoprin 88 kDa  |
| S2-013.NEO/S2-013.MUC1F > 2.0 |
| Lipocalin 2 (oncogene 24p3)  
| Keratin 19  
| Matrix metalloproteinase 7 (matrilysin, uterine)  
| Myelin basic protein  
| RNA binding motif protein 3  
| Suppression of tumorigenicity 14 (colon carcinoma)  
| Leukemia inhibitory factor (cholinergic differentiation factor)  
| Transmembrane 4 superfamily member 4  
| Proprotein convertase subtilisin/kexin type 5  

*Genes differentially expressed in CT3 versus NEO and ΔTR versus NEO comparison.

Genes differentially expressed in CT3 versus NEO comparison.

Genes differentially expressed in ΔTR versus NEO comparison.

Genes unique to MUC1F versus NEO comparison.
It was reported previously that the number of lung metastases was increased for S2-013.MUC1F cells compared with control (NEO) and TR deleted transfectants (ΔTR; Ref. 46). These results were obtained by injecting tumor cells s.c. into the left flank of BALB/c nude mice and subsequent evaluation of pulmonary metastasis. Lymph node invasion and metastasis was not evaluated. The differences observed between our previous report and this report are most likely because of the organ site used (s.c. versus cecum implantation). It is likely that the local cellular and stromal environment, and interactions between the tumor and this environment affect metastatic and invasive potential of tumor cells. Thus, it is possible that specific interactions between MUC1 and local elements have different effects at different sites (e.g., pancreas, cecum, or s.c.). Previous studies using animal models demonstrated different metastatic properties of oral and colon tumors when implanted at a s.c. or orthotopic site (53). At the molecular level, interactions between the glycosylated TR of MUC1 and molecules on opposing cells could be mediated by protein-protein interactions, by carbohydrate-protein interactions (13), or both. In any case, the net functional effect of overexpressing MUC1 would be, in part, dependent on the nature of the ligands that were available in the local environment.

The similarity in behavior between tumors expressing the CT3 or ΔTR construct suggests a cooperative relationship between the intracellular and extracellular domains of the protein. Previous studies investigating the role of MUC1 in metastasis have primarily focused on the TR domain. The MUC1 TR binds to intercellular adhesion molecule 1, and may, therefore, aid in the binding of tumor cells to epithelium and invasion into stromal tissue (54). Underglycosylated forms of MUC1 may contribute to the initial attachment of breast carcinoma cells to ECM proteins and lung tissue (47). There also evidence that the CT is important in regulating the adhesive/antiadhesive properties of the molecule. The association of MUC1 with β-catenin is thought to alter cadherin-catenin interactions and, thus,

Table 5 Differentially expressed genes between S2-013.MUC1F and S2-013.MUC1F.CT3

<table>
<thead>
<tr>
<th>S2-013.MUC1F/S2-013.MUC1F.CT3</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein E</td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor 2 (somatomedin A)</td>
<td></td>
</tr>
<tr>
<td>Protein tyrosine phosphatase, receptor type, N</td>
<td></td>
</tr>
<tr>
<td>N-acetylglactosaminyltransferase (GalNac-T)</td>
<td></td>
</tr>
<tr>
<td>Annexin A1</td>
<td></td>
</tr>
<tr>
<td>Protein phosphatase 2, regulatory subunit B (B56), α isoform</td>
<td></td>
</tr>
<tr>
<td>Histone deacetylase 3</td>
<td></td>
</tr>
<tr>
<td>Proteasome (prosome, macrorn) subunits, α, type 4</td>
<td></td>
</tr>
<tr>
<td>DEATH (Asp-Glu-Ala-Asp-His) box polyepitope 5 (RNA helicase, 68kD)</td>
<td></td>
</tr>
<tr>
<td>Keratin 19</td>
<td></td>
</tr>
<tr>
<td>Keratin 8</td>
<td></td>
</tr>
<tr>
<td>Matrix metalloproteinase 7 (matrixin, uterine)</td>
<td></td>
</tr>
<tr>
<td>Transmembrane 4 superfamily member 4</td>
<td></td>
</tr>
<tr>
<td>High-mobility group (nonhistone chromosomal) protein isoforms I and Y</td>
<td></td>
</tr>
<tr>
<td>Hexokinase 1</td>
<td></td>
</tr>
<tr>
<td>Lipocalin 2 (oncogene 24p3)</td>
<td></td>
</tr>
<tr>
<td>Actin related protein 2/3 complex, subunit 1B (41 kDa)</td>
<td></td>
</tr>
<tr>
<td>Tat interactive protein (60kD)</td>
<td></td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td></td>
</tr>
<tr>
<td>Integrin, β 4</td>
<td></td>
</tr>
<tr>
<td>Isovaleryl Coenzyme A dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>Integrin, β 4</td>
<td></td>
</tr>
<tr>
<td>Isovaleryl Coenzyme A dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>Suppression of tumorigenicity 14 (colon carcinoma)</td>
<td></td>
</tr>
<tr>
<td>Keratin 13</td>
<td></td>
</tr>
<tr>
<td>CD59 antigen p18–20</td>
<td></td>
</tr>
<tr>
<td>Discoidin domain receptor family, member 1</td>
<td></td>
</tr>
<tr>
<td>Histidine ammonia-lyase</td>
<td></td>
</tr>
<tr>
<td>IbV (bacterial acetolactate synthase)-like</td>
<td></td>
</tr>
<tr>
<td>ESTs, Highly similar to CGI-52 protein [H. sapiens]</td>
<td></td>
</tr>
<tr>
<td>Integrin, β 5</td>
<td></td>
</tr>
<tr>
<td>CD9 antigen (p24)</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
<tr>
<td>Serine protease inhibitor, Kunitz type, 2</td>
<td></td>
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</tbody>
</table>

Hudson et al. (44), which showed that MUC1-transfected cells demonstrated reduced adhesion to Type I collagen.

Surgical implantation of tumors into nude mice is a viable strategy to investigate invasion and metastasis of human tumors in vivo (48, 49). One advantage is that transplanted tumors in nude mice maintain cellular characteristics in the context of stromal elements (50). On implantation of S2-013.MUC1 tumors onto the cecum, the invasive and metastatic properties did not correlate with the in vitro Matrigel invasion results. Both S2-013.MUC1F(ΔTR) and S2-013.MUC1F.CT3 exhibited a greater invasive and metastatic phenotype at the cecum in vivo compared with S2-013.MUC1F. S2-013.MUC1F also showed a marked decrease in vessel invasion compared with the control (S2-013.NEO; Table 1). One interpretation is that overexpression of MUC1 in this system skews the adhesive/antiadhesive balance toward adhesion, preventing the cells from detaching from the primary tumor. Unregulated overexpression of MUC1 devoid of the TR or CT had the same effect in this model: increased propensity to invade lymph vessels and metastasize to the lymph nodes and lungs. Conversely, unregulated overexpression of full-length MUC1 decreased the invasive and metastatic potential of S2-013 cells at the cecum in vivo.

Organ microenvironment is known to affect tumor growth and metastatic spread (51). In this study, the cecum wall was chosen as the site of surgical implantation for a number of reasons. The orthotopic site (pancreas) poses technical and surgical problems that could result in severe damage to the pancreas and loss of function not because of tumor burden. The cecal wall provides a more rigid cellular environment and still resides in the peritoneal cavity (as opposed to s.c. injection). The sites of spontaneous metastases we observed for S2-013 tumors in this study were the same as those initially reported for the S2-013 subline after s.c. implantation (52). It could be argued that heterotopic implantation of the S2-013 tumors contributes to the observed differences in invasion and metastasis between the four cell lines used in these studies. However, if the site of implantation was the primary factor in the results reported here, we would not have observed differences in invasion and metastases with the S2-013 transfectants. Thus, we conclude that the cecum serosal and subserosal surfaces provide a suitable site for heterotopic implantation and investigation of invasion and metastasis caused by overexpression and structural differences of MUC1.
cell-cell adhesion (55, 56). Phosphorylation of the CT correlates with changes in cell-cell adhesion (45), suggesting that MUC1 is involved in intracellular signaling.

It is too simplistic to conclude that the TR, CT of MUC1, or simple overexpression of the full-length molecule are sole factors determining invasive and metastatic properties of tumor cells. The role of MUC1 in invasion and metastasis is not limited to direct physical interactions of the TR portion of the MUC1 protein with surrounding tissue, because cells expressing MUC1 without the TR exhibit an aggressive phenotype. Overexpression of MUC1 in cancer is generally considered detrimental; however, our results with S2-013.NEO, which expressed low levels of endogenous MUC1, demonstrated a greater invasive and metastatic phenotype at the cecum than cells overexpressing MUC1 in an unregulated manner (S2-013.MUC1F). One hypothesis that would explain these findings is that MUC1 contributes to a complex regulated process that configures the overall adhesive properties of the cell. If MUC1 functions as a sensory and signaling molecule in addition to contributing to cell surface adhesion properties, via the TR and CT, then it is not surprising that deletion of either domain or unregulated overexpression of the full-length protein alters the adhesive/antiadhesive properties of tumor cells.

cDNA array analysis provided evidence that overexpression of MUC1 and mutant MUC1 isoforms in S2-013 cells resulted in differential expression of mRNA for other proteins that may influence invasive and metastatic potential. cDNA array analysis was performed using two experimental designs. First, each of the three cell lines expressing forms of MUC1 was compared with S2-013.NEO as a reference. Because S2-013.NEO expresses low levels of endogenous MUC1, these array analyses provided insight into the effect of unregulated overexpression of MUC1. As seen in Table 3, a total of 26 genes were differentially expressed (up or down) between S2-013.MUC1F and S2-013.NEO. Of these, 14 genes were unique to this comparison, whereas the remaining genes were also differentially expressed in the CT3 and ΔTR constructs. Genes of which the expression was affected by all three of the constructs can be considered to be affected by overexpression of MUC1. For example, genes from the metallothionein family (1G and 1H) are up-regulated in cell lines expressing any of the three constructs as compared with the NEO control. Metallothionein expression in pancreatic cancer has been shown to be related to metastasis and poor prognosis (57) and corresponds to the presence of MMPs in pancreatic islets (58, 59). Another example is Trx reductase, the enzyme responsible for reduction of Trx to its active state. Trx, once activated, stimulates cell growth, inhibits apoptosis, and has been shown to be overexpressed in numerous cancers, including pancreatic tumors (60–62). Trx is also thought to contribute to chemotherapy resistance (60). Genes that were specifically affected by overexpression of full-length MUC1 (compared with NEO) included a N-acetylgalactosaminyltransferase, which may play a role in the cellular mechanisms responsible for the glycosylation of full-length MUC1.

We were interested in those genes that might help explain the *in vivo* invasion and metastasis results. Direct comparisons of differences in gene expression between cells expressing the different mutated MUC1 constructs should provide insight into different functions that are mediated by the separate domains of MUC1. As shown in Tables 4 and 5, there were numerous genes differentially expressed between the mutant forms and full-length MUC1. One of the more interesting candidates is MMP7, also known as matrilysin. MMP7 was one of the most highly differentially expressed genes in cells expressing the CT3 and ΔTR constructs compared with full-length MUC1. MMP7 is differentially expressed between S2-013.MUC1F and the NEO control as well (Table 3). These results were confirmed via Northern blot, Western blot, and immunohistochemistry, and these data will be presented in more detail elsewhere. It was confirmed that MMP7 expression was almost completely absent in S2-013.MUC1F. In contrast, the other three cell lines (NEO, ΔTR, and ΔCT3) expressed high levels of MMP7 at the RNA and protein level. MMP7 has been shown to be overexpressed in pancreatic cancer (63, 64). On overexpression of full-length MUC1, MMP7 was down-regulated, indicating that MUC1 can directly influence the expression of proteins responsible for invasion and degradation of the extracellular matrix. Moreover, this effect required both the CT and TR domains of the protein, because overexpression of either of the deletion constructs did not affect MMP7 expression.

Several other genes were up-regulated in cells expressing the CT3 and ΔTR constructs that may account for their increased propensity for vessel invasion and metastasis. These include integrin subunits β4 and β5. Integrins mediate adhesion between the cell membrane and extracellular matrix, and play a role in signal transduction mechanisms (65, 66). Abdel-Ghany *et al.* (67) showed that β4 integrin mediates adhesion to breast cancer cells and metastasis to the lungs. It is also known that β4 integrin can be cleaved by proteolytic enzymes (MMP7), and this cleavage event may aid in metastatic spread (67, 68). Integrins also form complexes with tetraspanins, which are transmembrane proteins that can affect cell motility, proliferation, and metastasis (69). Tetraspanins form complexes with integrin proteins, and these associations are thought to play a role in signaling and intracellular trafficking of integrins (69, 70). Two tetraspanin proteins were also identified as being differentially expressed in S2-013 cells: CD9 (up in MUC1F.CT3 versus MUC1F) and transmembrane 4 superfamily member 4 (up in both MUC1F.ΔTR and MUC1F.CT3 versus MUC1F). CD9 expression was confirmed by flow cytometry (data not shown). Expression of tetraspanin proteins may contribute to the observed phenotypes via regulation of cell-cell and cell-ECM interactions.

One of the more surprising findings was the expression of the gene encoding CD24 antigen (Table 4). Western blot, flow cytometry, and immunohistochemistry confirmed CD24 differential expression. CD24 is a differentiation antigen expressed on developing B cells, and is thought to play a role in signal transduction and migration (71–74). There is a growing body of evidence that CD24 is expressed in numerous types of cancer and may facilitate metastasis via binding to P-selectin (75–79). To our knowledge CD24 has never before been shown to be expressed in pancreatic cancer. Its function is not completely known, but the fact that it is expressed, suggests the identification of a protein that may be involved in pancreatic cancer metastasis.

In summary, we have investigated the role of MUC1 in invasion and metastasis using a surgical implantation model in nude mice. Alterations in MUC1, specifically deletion of either the CT or TR, resulted in an increased propensity of tumor cells to invade vessels and metastasize to lymph nodes compared with a cell line overexpressing full-length MUC1. These results suggest a cooperative relationship between the CT and TR domain of MUC1. Deletion of one or the other altered adhesive/antiadhesive MUC1 functions in the cell and created cell lines with more aggressive phenotypes *in vivo*. Unregulated overexpression of full-length MUC1 resulted in an opposite effect: a substantially decreased invasive and metastatic character *in vivo*. Gene expression analyses suggested that numerous candidate genes are differentially expressed on MUC1 overexpression and that a distinct set of genes is affected when different domains of MUC1 are altered. Genes that might help explain the invasion and metastasis results include MMP7, CD9, and cytoskeletal proteins. Additional studies on the role of these genes in invasion and metastasis are currently being pursued.

---

MUC1 were deleted (either the CT or TR). Taken together, our results suggest that MUC1 assumes a more comprehensive and complex role in invasion and metastasis than was espoused previously. Instead of simply facilitating invasion and metastasis, we hypothesize that MUC1 serves to regulate invasive and metastatic properties of adenocarcinomas on which it is expressed by both contributing to cell surface adhesion properties, sensing the local extracellular environment by ligand receptor interactions and responding to that environment by engaging in morphogenetic signal transduction. The sum of this process ultimately affects expression of a number of additional genes and gene products that play a role in the invasive properties of the tumor cell.

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Contribution of the MUC1 Tandem Repeat and Cytoplasmic Tail to Invasive and Metastatic Properties of a Pancreatic Cancer Cell Line

Karl G. Kohlgraf, Andrew J. Gawron, Michiyo Higashi, et al.


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