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Human MUC4 mucin induces ultra-structural changes and tumorigenicity in pancreatic cancer cells

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MUC4 is a type-I transmembrane glycoprotein and is overexpressed in many carcinomas. It is a heterodimeric protein of 930 kDa, composed of a mucin-type subunit, MUC4z, and a membrane-bound growth factor-like subunit, MUC4β. MUC4 mRNA contains unique 5’ and 3’ coding sequences along with a large variable number of tandem repeat (VNTR) domain of 7–19 kb. A direct association of MUC4 overexpression has been established with the degree of invasiveness and poor prognosis of pancreatic cancer.

To understand the precise role of MUC4 in pancreatic cancer, we engineered a MUC4 complementary DNA construct, mini-MUC4, whose deduced protein (320 kDa) is comparable with that of wild-type MUC4 (930 kDa) but represents only 10% of VNTR. Stable ectopic expression of mini-MUC4 in two human pancreatic cancer cell lines, Panc1 and MiaPaCa, showed that MUC4 minigene expression follows a biosynthesis and localisation pattern similar to the wild-type MUC4. Expression of MUC4 resulted in increased growth, motility, and invasiveness of the pancreatic cancer cells in vitro. Ultra-structural examination of MUC4-transfected cells showed the presence of increased number and size of mitochondria. The MUC4-expressing cells also demonstrated an enhanced tumorigenicity in an orthotopic xenograft nude mice model, further supporting a direct role of MUC4 in inducing the cancer properties. In conclusion, our results suggest that MUC4 promotes tumorigenicity and is directly involved in growth and survival of the cancer cells.

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MUC4 is a high molecular weight O-glycoprotein produced by secretory epithelial cells for the lubrication and protection of ducts and lumen (Porchet et al., 1991; Khorrami et al., 2002). MUC4 is expressed in various epithelial tissues including the trachea, colon, stomach, cervix, and lung (Audie et al., 1993, 1995). It is also expressed in goblet, ciliated, and absorptive cells in adults and in poorly differentiated cells of embryos and fetuses (Buisine et al., 1998, 1999). Indeed, MUC4 is expressed early in the primitive gut, before respiratory and digestive epithelial cells have acquired their tissue and cell specificity. For these reasons, morphogenic differentiation and embryonic development functions are attributed to MUC4, and its expression can be regulated by several serum factors including retinooids (Ogata et al., 1992; Choudhury et al., 2000b), growth factors, and cytokines (Andrianifahanana et al., 2005).

The largest size of MUC4 allele is approximately 26.5 kb. Its deduced amino-acid sequence consists of a 27-residue peptide signal sequence followed by three imperfect repetitions of a motif varying in size from 126 to 130 residues and a unique sequence of 554 residues. The central domain is composed of a perfect repetition motif of 16 amino-acid residues, which is repeated 400 times in the largest allele. The C-terminal region contains two domains rich in N-glycosylation sites, three epidermal growth factor-like domains, a transmembrane sequence, and a short cytoplasmic tail. The MUC4 protein is hypothesised to be proteolytically cleaved at a GDPY site generating two subunits, the mucin-type subunit, MUC4z, and the growth factor-like subunit, MUC4β. MUC4 is predicted to be a membrane-associated 2.12 μm long, mature protein where both subunits stay non-covalently bound.

The genomic organisation of MUC4 is fully understood, and its characterisation has been substantially aided by the human genome project (DOE Joint Genome Institute Human Genome Project). MUC4 encompasses 25 exons, the first exon, E1, encoding the 5’-untranslated sequence, the translation start site, and the peptide signal (Escande et al., 2002). Exons range from 65 bp to 22 kb in size, whereas introns range from 94 bp to 16.5 kb. The largest exon, E2, is at the central position and contains a large 2.8 kb unique sequence followed by the repetition of a 48 bp motif. The tandem repeat region in E2 shows a variable number of...
tandem repeat (VNTR) polymorphism, and its size varies from 7.5 to 19 kb. The largest known allele for MUC4 expresses a 26.5 kb transcript that encodes the full-length MUC4 precursor, also called s0-MUC4.

MUC4 is highly expressed in human pancreatic tumours and pancreatic tumour cell lines; however, its expression is undetectable in the normal pancreas or chronic pancreatitis (Balague et al., 1994; Hollingsworth et al., 1994; Choudhury et al., 2000a; Andrianofahandana et al., 2001). MUC4 is expressed by metaplastic ducts and its expression increases with increasing grade in pancreatic intraepithelial neoplasias (Swart et al., 2002; Park et al., 2003). A recent study has shown that MUC4 is a good candidate marker for early diagnosis of pancreatic cancer in fine-needle aspirates, exhibiting a 91% sensitivity and 100% specificity (Jhala et al., 2006). MUC4 expression in a variety of cancer is associated with poor prognosis (Bax et al., 2004; Weed et al., 2004; Alos et al., 2005; Handra-Luca et al., 2005; Jhala et al., 2006). The inhibition of MUC4 expression by an antisense oligonucleotide resulted in decreased in vivo tumourigenicity and metastasis (Singh et al., 2004). Altogether, these data establish a strong association between MUC4 expression and pancreatic cancer pathogenesis. Moreover, in the rat model, sialomucin complex (SMC)/rMuc4, represses cell adhesion, blocks tumour cell killing, promotes the growth of the tumour, and is directly related to the metastatic invasion (Carraway et al., 2000). However, our understanding about the role of MUC4 in the development and progression of pancreatic adenocarcinoma is still unclear.

One of the hallmarks of cancer development is the inhibition of apoptosis with a central role of the mitochondria in this process. During carcinogenesis mitochondria present an enhanced aerobic glycolysis as a protective mechanism against reactive oxygen species, an acidification of the microenvironment that favours cell invasion and an enhanced resistance to mitochondria-induced apoptosis (Brand and Hermfisse, 1997; Kroemer, 2006). In addition to their role in apoptosis, mitochondria play a pivotal role in cellular metabolism, respiration, and production of ATP essential for the normal function of all human organ systems. Therefore, the mitochondrial content for any given cell is well regulated, presenting variation with the type of the cells and tissues, during cell differentiation, hormone treatment, and exercise (Robinson and Wong, 1988; Renis et al., 1989; Shay et al., 1990). Increase in mitochondrial mass is directly associated with cell division and cell differentiation. In cancer cells, other roles have also been linked to increase in mitochondrial mass, including increased resistance to tamoxifen-induced apoptosis in breast cancer, further suggesting role of mitochondria in cancer progression (Dietze et al., 2001).

To elucidate further the functions of human MUC4, a recombinant MUC4 minigene was generated and stably transfected in pancreatic adenocarcinoma cell lines Panc1 and MiaPaCa. The mini-MUC4 was surface-localised in both the transfected cell lines. Expression of mini-MUC4 increased in vitro growth, motility, and invasiveness of the pancreatic cancer cells. Ultra-structural studies showed increased mitochondrial size in mini-MUC4-transfected cells, which was correlative with increased mitochondrial mass. Furthermore, overexpression of the mini-MUC4 gene in pancreatic cancer cells also resulted in an increase in tumorigenicity of these cells in vivo. Our results demonstrate, for the first time, a direct role of the MUC4 mucin with the cellular changes and tumour progression in human pancreatic cancer cells.

**MATERIALS AND METHODS**

**Generation of mini-MUC4 gene**

The construction of the MUC4 minigene was performed in three steps (Figure 1A). First, the unique sequences of MUC4 were amplified by PCR: two fragments for the 5'-unique sequence, a SalI/EcoRI product from position –71 to 178, and an EcoRI/BamHI product from 179 to 2919 (AJ000281). Two fragments were amplified by PCR for the 3'-unique sequence, an EcoRV/HindIII product from position 1 to 1351 and a HindIII/NotI product from position 1352 to 3471 (AJ010901). Each product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA). Both 5' and 3' fragments were extracted by the introduced restriction endonuclease sites and subcloned into the pBluescript KS(+) vector (Stratagene, La Jolla, CA, USA) to get a SalI/BamHI 5'-full sequence and an EcoRV/NotI 3'-full sequence. In a second step, a new HindIII/BamHI PCR product was performed using the 5'-full sequence as a template, and cloned into the pCR2.1 vector. This fragment is located from position 100 to 2919 (AJ00281). The EcoRI complementary DNA (cDNA) fragment named JER64 coding the repetitive sequence of MUC4 was extracted and subcloned into the pSecTag C vector. Finally, the HindIII/BamHI 3' fragment, as well as the EcoRV/NotI 3'-full sequence, were extracted and subcloned in frame into the pSecTag C containing the sequence repeated in tandem. At each...
Expression of mini-MUC4 cDNA construct

Panc1 and MiaPaCa pancreatic adenocarcinoma cell lines were transfected with plasmid DNA using the Lipofectamine method (Invitrogen). Single colonies were obtained by zeocin selection and expanded for screening. Cell lines were analysed for MUC4 protein expression by western blot with a MUC4 peptide mouse monoclonal antibody (8G7). Growth kinetic and population doubling time of transfected and control cells were determined as published previously (Singh et al, 2004).

Preparation of cell lysates and immunoblotting

Cell lysates were prepared in the lysis buffer (50 mM Tris – HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin and leupeptin, 1 mM Na3VO4, and 1 mM NaF). The protein content of the lysates was determined by using the Bio-Rad Dc protein assay (Bio-Rad, Hercules, CA, USA). Cell lysates were resolved on a 2% sodium dodecyl sulphate (SDS)-agarose gel, transferred to a nitrocellulose membrane, blocked in 5% non-fat milk in phosphate-buffered saline (PBS) for 1 h. The MUC4 antibody was diluted at 1 μg/ml in PBS. The membrane was incubated for 4 h at room temperature, followed by 6 × 10 min washes in TBST (50 mM Tris – HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween-20). The horseradish peroxidase (HRP)-conjugated secondary antibody was diluted at 1:2000 in PBS and incubated for 1 h at room temperature, followed by six washes in TBST. Enhanced chemiluminescence reagents were applied as per the manufacturer’s instructions (Amersham Life Science, Piscataway, NJ, USA), and the blot was exposed to ECL-sensitive film (Kodak, Rochester, NY, USA).

Confocal analysis

The cells were grown at 60–70% confluency on the sterilised coverslip. Cells were first washed with 0.1 M HEPES containing Hank’s buffer and fixed in ice-cold methanol at −20°C for 2 min. Methanol-fixed cells were blocked in 10% goat serum containing 0.05% Tween-20 for 30 min at room temperature before incubation with primary antibody diluted (1:100) in 10% goat serum containing PBS for 60 min at room temperature. Cells were washed four times for 5 min with PBS containing 0.05% Tween-20 (PBST) and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch laboratories Inc., West Grove, PA, USA) for 60 min at room temperature. Cells were again washed with PBST, five times for 5 min and mounted on a glass slide in the anti-fade VECTASHIELD mounting media (Vector Laboratories Inc., Burlingame, CA, USA). Immunostaining was observed under the Zeiss 510 LASER SCAN confocal microscope.

Apoptosis assay

Apoptosis was measured by using the Annexin V-FITC apoptosis detection kit (Roche Diagnostics, Indianapolis, IN, USA). The cells were grown in serum-free medium and the apoptosis was detected by staining the cells with annexin V and propidium iodide solution, followed by flow cytometry.

Cell motility assay

For motility assays, 1 × 10⁶ cells were plated in the top chamber of non-coated polyethylene teraphthalate membranes (six-well insert, pore size 8 μm; Becton Dickinson, Franklin Lakes, NJ, USA). The cells were incubated for 24 h, and the cells that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells that transversed the membranes were stained with a Diff-Quick cell staining kit (Dade Behring Inc., Newark, DE, USA). Cells in 10 random fields of view at ×100 magnification were counted and expressed as the average number of cells per field of view. Three independent experiments were performed in each case. The data were represented as the average of the three independent experiments with the s.d. of the average indicated.

Cell invasion assay

For Matrigel assay 1 × 10⁵ cells were seeded on Matrigel-coated membrane inserts. The bottom chamber contained 0.75 ml of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After incubation for 24 h at 37°C, the cells remaining inside the insert were removed with a cotton swab, and cells that penetrated the Matrigel and invaded to the lower surface of the membrane were fixed in methanol and stained using a Diff-quick reagent kit. After air-drying the membrane, the cells were visually counted at a magnification of ×100 under a microscope. Assays were performed two times in triplicate wells, and the cells present in 10 fields covering the centre of each membrane were counted. Data were expressed as the number of cells per area and as invasion index.

Electron microscopy

Cells cultured at 70% confluence, were washed twice in ice-cold PBS, trypsinised, and fixed with 2.5% glutaraldehyde in 1 m Sørenson’s phosphate buffer. The cell pellet was then subjected to overnight infiltration in Durepam Acm. Epoxy Resin (Electron Microscopy Sciences, Fort Washington, PA, USA), flat embedding, and polymerisation at 55°C for 48 h. Embedded cells were mounted on resin stubs and sectioned at 90 nm with an Ultracut E ultramicrotome. Sections were viewed and photographed with a Zeiss 600 EM10A transmission electron microscope (TEM) at 60 kV.

Determination of mitochondrial mass

The fluorescent dye 10-n-nonyl-acridine orange (NAO) (Molecular Probes, Carlsbad, CA, USA) was used to monitor the mitochondrial mass. Cells were trypsinised and resuspended in 0.5 ml of DMEM medium containing 5 μM of NAO, incubated for 30 min at 37°C in the dark. After incubation, cells were kept on ice and immediately analysed by flow cytometry. A FACSScan flow cytometer, equipped with a 488 nm argon laser, was used for the flow-cytometric analysis. The forward and side scatters were used to establish the size gates and to exclude cellular debris. The excitation wavelength was at 488 nm and observation at 530 nm for the green fluorescence and 585 nm for the red fluorescence.

Tumorigenicity assay

To test the tumorigenic capacity, mini-MUC4-transfected cells, along with the control cells (parental and mock), were harvested from subconfluent cultures, and resuspended in a normal saline solution at a concentration of 4 × 10⁷ cells/ml. Single-cell suspensions of >90% viability were used for the injections. Immunodeficient mice were purchased from the Animal Production Area of the National Cancer Institute/Frederick Cancer Research and Development Center (Frederick, MD, USA). The mice were housed in specific pathogen-free conditions and were fed sterile water and food ad libitum. The mice were treated in accordance with the Institutional Animal Care and Use Committee.
guidelines. The mice (8- to 12-week old) were anaesthetised with 350 µl of intraperitoneal injection of a mixture (4:1) of ketamine (100 mg/ml) and xylazine (20 mg/ml) diluted 10 times in sterile water. A small left abdominal flank incision was made, and the spleen was exteriorised. Tumour cells (2 x 10⁶) were injected subcapsularly in a region of the pancreas just beneath the spleen using a 1-cc U-100 insulin disposable syringe (Becton Dickinson). A successful subcapsular intrapancreatic injection of tumour cells was identified by the appearance of a fluid bleb without intraperitoneal leakage. To prevent leakage, a cotton swab was held for 30–60 s over the site of injection. Wounds were closed by making a single suture on one layer and three to five independent sutures on the outermost skin. Tumour growth was assessed by daily weighing and palpation of each animal. All mice were killed on day 60 after implantation, and the presence of metastatic lesions in different organs was determined. Pancreatic tumours were excised, weighted, and measured for their dimensions.

Microarray analysis
RNA (60 μg) from MUC4-transfected cell lines and control mock cell line were fluorescently labelled with Cy3- and Cy5-conjugated dCTP (Amersham Pharmacia, Piscataway, NJ, USA), using anchored oligo(dT) primers and Superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA). After probe generation, residual RNA was hydrolysed by treatment with NaOH and unincorporated nucleotides were removed by MicroCon YM-30 column (Amicon, Billerica, MA, USA). The latter step also serves to concentrate the labelled cDNAs. Cy3- and Cy5-labelled cDNAs were combined and diluted to 60 μl with 3.5 x SSC/0.15% SDS hybridisation solution. To reduce nonspecific hybridisation, the hybridisation solution also contained 10 μg of poly (da) (Pharmacia, St Louis, MD, USA), 2.5 μg of yeast transfer RNA (Sigma, Ridgefield, CT, USA), and 12.5 μg of human Cot1 DNA (Boehringer-Mannheim, Ridgefield, CT, USA). The fluorescent probes were denatured and hybridised to glass slides featuring approximately 6000 cDNA elements (University of Texas Southwestern Medical Center Microarray Facility, Dallas, TX, USA) overnight at 65°C. Slides were washed successively in 1 x SSC/0.1% SDS, 1 x SSC, and 0.2 SSC for 2 min each to remove excess probe and spun dried. Hybrised arrays were scanned for fluorescent signals using ScanArray (4000 confocal fluorescent laser scanner accompanying ScanArray 3.0 software (Packard Instrument Company, Meriden, CT, USA). For the MUC4-transfected vs mock cell line plot, the log intensity in the Cy3-channel logM(Cy3-) and the log intensity in the Cy5-channel logM(Cy5-) were transformed for normalisation and visualisation purposes. A 45° counter-clockwise rotation of the (logM(Cy3), logM(Cy5))-coordinate system and a scaling of the coordinates were applied to plot the log intensity ratio M = logM(Cy3/Cy5) vs the mean log intensity A = logM(R/CGCy3Cy5). These two values were the features of interest for gene expression microarray data. A print-tip group lowess normalisation (Yang et al, 2002) was applied to the plot. The lowess function is a scatter-plot smoother that performs a robust local linear fit. The printing setup of the arrayer implicated printing genes in 6 x 2 groups. Typically, systematic intensity-dependent differences between such print-tip groups are observed. Separately, for every print-tip group, a lowess normalisation was applied to the plot. The normalisation was performed by subtracting the lowess fit from the log intensity ratios M. This ensured that intensity-dependent effects were removed from the data. All data transformations were performed using the contributed R package Sma (Statistical Microarray Analysis), available from ‘Terry Speed’s Microarray Data Analysis Group Page’; http://www.stat.berkeley.edu/users/terry/zarray/Html/index.html.

Calculated signal intensities and standard deviations for each feature were background-corrected, normalised to total signal intensities, and converted into ratios. The data were further analysed by imposing one or all of the following selection criteria: (1) a minimum level of expression was observed; (2) significant differential expression (twofold or greater) was observed; and (3) the expression pattern was reproducible.

Statistical analysis
Statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). The Mann–Whitney U-test was used to compare tumour weight and tumour volume between groups. Fisher’s exact test was used to compare the frequency of metastasis between groups. A P-value < 0.05 was considered to be statistically significant.

RESULTS
Construction of the mini-MUC4
The full-length coding sequence of MUC4 is difficult to subclone in any known mammalian expression vector because of its dimensions and the repetitive nature of tandem repeat domain. To overcome this problem, we used the first cDNA isolated for MUC4, JER64 (Porchet et al, 1991), to design a mini-MUC4 construct. The JER64 fragment contains a 1.8 kb long MUC4 tandem repeat region, which is composed of the 48 bp motif in repetition. The repetitive insert was subcloned in pSecTag C vector between the EcoRI sites. The complete unique coding sequence and the 5’ and 3’ terminal sequences were then added in frame (Figure 1A) (detailed in the Materials and Methods section). The 8781 bp mini-MUC4 cDNA encoded an estimated 320 kDa protein, composed of 240 kDa mini-MUC4z subunit and the 80 kDa MUC4b subunit (Figure 1B). The mini-MUC4 construct harbors all the unique features of the wild-type MUC4, but contains only 10% of tandem repeat region of the main allele (Nollet et al, 1998). Altogether, the size of the mini-MUC4 represented 58% of the smallest and 30% of the largest MUC4 wild-type alleles, and its size was comparable to its rat homologue, SMC/Muc4 (Carraway et al, 2000).

Expression and subcellular distribution of mini-MUC4
To evaluate the effects of mini-MUC4, multiple clones were selected and characterised for the mini-MUC4 expression. Mini-MUC4 was analysed by resolving the protein lysates isolated from transfected cell lines (Panc1 and MiaPaCa) on a 2% SDS-agarose horizontal gel followed by capillary transfer and immunoblotting with anti-MUC4 antibody. The immunoblotting revealed a band migrating at a distance consistent with the 320 kDa expected molecular weight of mini-MUC4 (Figure 2A). The empty vector-transfected cells were negative for MUC4 expression. CD18/HPAF, a MUC4 overexpressing cell line, was used as a positive control. The subcellular localisation of mini-MUC4 and MUC4 wild type was examined by indirect immunofluorescence and confocal laser microscopy utilising the MUC4 monoclonal antibody. CD18/HPAF cells showed both membrane and cytoplasmic staining for MUC4 (Figure 2B). A similar distribution of MUC4 was observed for mini-MUC4-transfected Panc1 and MiaPaCa cells. Mini-MUC4 and wild-type MUC4 display a similar subcellular localisation, indicating similar processing of MUC4 and mini-MUC4 in these cells.

MUC4 enhances the in vitro growth of pancreatic cancer cells
An overexpression of MUC4 mucin has been reported in a variety of cancers. In addition, an inhibition of MUC4 mucin in pancreatic cancer cells, using an antisense strategy, diminished in vitro and in vivo growth of cancer cells. To study whether mini-MUC4 has the similar effects on growth as wild-type MUC4, a growth kinetics

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The experiment was performed. The mini-MUC4 overexpressing cells were seeded in six-well plates in triplicate and counted for 5 days. The experiment was carried out for both Panc1 and MiaPaCa transfected with mini-MUC4, in medium containing high (10%) and low (1%) serum. No significant difference was observed for the mini-MUC4-transfected Panc1 and MiaPaca cells grown in 10% serum when compared to the control (Figure 3A). All cell lines presented similar population doubling time. However, when the cells were maintained in medium containing low serum, the mini-MUC4-transfected Panc1 cells grew much faster, with a population doubling time of 18.47 h as compared to 33 and 32.6 h for the parental and mock cells, respectively (Figure 3B). The difference was statistically significant with a $P<0.022$. The results obtained for the MiaPaCa lineage was comparable; however, we were unable to calculate the population doubling time for the parental or mock cells, the majority of the cells did not survive for more than 5 days in low serum (Figure 3B). After 5 days of growth in low serum, the difference in cell number between the control and the mini-MUC4-positive MiaPaCa cells were statistically significant with a $P<0.013$.

**MUC4 induced mitochondrial mass increase in pancreatic cancer cells**

The phenotypic modifications due to mini-MUC4 overexpression were investigated by electron microscopy studies. The studies were performed for both Panc1 and MiaPaCa cells transfected with mini-MUC4 and control cells. The cells transfected for mini-MUC4 expression presented a Golgi system well developed with accumulation of numerous vacuoles, sign of active biosynthesis.
(Figure 4, right panel). In addition, the mini-MUC4-transfected cells showed an increased number of large, elongated, and centrally narrow mitochondria (Figure 4, right panel). The parental cells and the cells transfected with vector alone had mitochondria of normal size and shape (oval and round). The presence of elongated and centrally narrow mitochondria indicates increased mitochondrial divisions in mini-MUC4-transfected cells. The number of mitochondria examined in five fields was counted for Panc1 and Panc1 mini-MUC4 cells. Panc1 cells presented 28 ± 14 mitochondria per field, whereas Panc1 mini-MUC4 presented 70 ± 5 mitochondria (P < 0.005). The mitochondrial abnormalities observed in mini-MUC4-transfected cells by electron microscopic studies were further evaluated using specific fluorescent mitochondrial probes. The fluorescent dye, NAO, which specifically binds to cardiolipin at the inner mitochondrial membrane independent of the membrane potential, was utilised to measure the changes in mitochondrial mass. To monitor the effect of mini-MUC4 overexpression on mitochondrial mass, two independent clones showing different expression levels of mini-MUC4 in Panc1 cells were investigated. The NAO uptake was compared in mini-MUC4-transfected and control cell lines. Figure 5 presents the NAO intensity measured for each cell line, the relative NAO intensity calculated for 20,000 randomly selected cells within each cell population indicated below. As shown in a table in Figure 5, the relative NAO intensity of the MUC4-transfected cells (with a mean of 88.05 and 103.43 for the cell lines expressing low and high levels of transfected mini-MUC4, respectively) was higher than the control cell lines (with a mean of 67.3 and 63.25, respectively, for the parental cells and the cells transfected with vector control). Hence, there was a progressive increase in mean NAO fluorescence of mini-MUC4-transfected Panc1 cells with increasing levels of MUC4 expression, which was also consistent with the increased number of mitochondria observed by electron microscopy. These results indicate a potential correlation between NAO uptake and MUC4 expression. The cell-cycle analysis, investigated by propidium iodide staining, showed no difference in the relative number of cells arrested at the late G1, G0/G1, and G2/M phases. The MUC4-dependent increase in mitochondrial mass appeared to be independent of the cell cycle. The difference in mitochondrial membrane potential between the mini-MUC4-transfected and control cells was analysed by utilising a lipophilic cation JC-I (5,5′,6,6′-tetrachloro 1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide dye). This mitochondrial dye exists as a monomer in solution and the monomers aggregate as a function of the mitochondrial membrane potential. The JC-I monomer fluoresces at 530 ± 17 nm and the JC-I aggregate fluoresces at 590 ± 17 nm. The ratio of aggregate fluorescence to monomer fluorescence is an indication of mitochondrial membrane potential. The ΔΨm was measured in mini-MUC4-expressing Panc1 cells and control cells by! A ratio of 527/590 nm (data not shown). No significant difference was observed in the mitochondrial membrane potential using the JC-I dye, showing that the increase in mitochondrial mass was not associated with an increase in apoptosis. The apoptosis status of the control and mini-MUC4-corrected cells was confirmed by annexin V staining by flow cytometry and poly (ADP-ribose) polymerase (PARP) degradation by western blot (Figure 6), but no significant difference in apoptosis was observed. However, the percentage necrotic cells was significantly increased (P < 0.05) in Panc1 cells overexpressing mini-MUC4 as compared with control cells.
The cDNA microarray hybridisation and analysis

The variations in gene expression between the mini-MUC4-expressing Panc1 cells and mock-transfected cells were analysed by performing cDNA microarray hybridisation. A scattered pattern of expression was obtained when comparing the genes expressed in mini-MUC4-expressing Panc1 cells (presenting the highest levels of expression for mini-MUC4) vs the mock cell line. Among all the genes, 54 genes showed at least twofold change in expression after normalisation. The 33 genes which were over-expressed at least twofold in mini-MUC4-expressing Panc1 cells are listed in Table 1 and the 21 genes which showed at least twofold decrease in expression are listed in Table 2. Among the 33 genes over-expressed in mini-MUC4-expressing cells, four coded for proteins, which are required for mitochondrial functions. These genes are the succinyl CoA synthetase, the succinyl CoA:3-oxoacid

Figure 4  Electronmicroscopy. Cells, cultured at 70% confluency, were fixed with 2.5% glutaraldehyde in 1 M Sorenson’s phosphate buffer. The cell pellet was then subjected to overnight infiltration in Duereap Acm. Epoxy Resin (Electron Microscopy Sciences), flat embedding, and polymerisation at 55°C for 48 h. Embedded cells were mounted on resin stubs and sectioned at 90 nm with an Ultracut E ultramicrotome. Sections were viewed and photographed with a Zeiss 600 EM10A TEM at 60 Kv. The pictures presented are representative for the experiment. Twenty cells for each cell line were observed in two independent experiments. The black arrows indicate dividing mitochondria in the MiaPaCa cell line transfected with mini-MUC4 construct and the red arrows indicate the golgi complex.
transferase, the glycerol-3-phosphate dehydrogenase 2 (GPD2), and the isovaleryl CoA dehydrogenase (IVD). Five other genes were found to be overexpressed with a level close to the twofold cutoff: the alcohol dehydrogenase (ADH), the ATP-binding cassette transporter (ABC2), the thioredoxin reductase (TrxB2), the outer mitochondrial membrane 34 kDa translocase (TOM34), and the pyruvate kinase. Altogether, nine genes coded for proteins located and functionally active within the mitochondria. The overexpression of mitochondrial genes in mini-MUC4-transfected Panc1 was consistent with the increased number of mitochondria observed by electron microscopic studies. This indicated increased energy generating ability of MUC4-expressing cells that might enhance the tumorigenicity and metastatic potential of pancreatic adenocarcinoma cells.

MUC4 overexpression enhances cell motility and invasiveness of pancreatic cancer cells

To examine the effect of mini-MUC4 expression on cancer cell properties, a motility and invasion assay was performed. A cell motility assay was performed by utilising uncoated porous membranes of 8.0 μm pore diameter. The number of cells migrated to the lower surface of the porous membrane under chemotactic stimulus of FBS in the lower chamber was approximately twofold greater in both mini-MUC4-expressing pancreatic cancer cells in comparison with control transfected cells (Figure 7A). An in vitro cell invasion assay was performed based on the principle of the Boyden chamber assay. The Matrigel matrix served as a reconstituted basement membrane in vitro. The cells migrating through the Matrigel matrix under chemotactic stimulus of FBS in the lower chamber were counted and the result is presented in Figure 7B. The mini-MUC4 overexpressing cells showed significantly enhanced invasiveness (~1.5-fold) as compared with the control cells (P < 0.05). These data indicated that the enhanced expression of MUC4 in pancreatic cancer cells is associated with increased invasiveness.

Overexpression of mini-MUC4 results in an increase in tumorigenicity of the Panc1 and MiaPaCa cells

To determine the effect of MUC4 expression on tumorigenicity and metastatic potential of pancreatic adenocarcinoma cells, tumorigenicity, and metastatic index were determined by transplanting the tumour cells orthotopically in the pancreas of the nude mice. The MUC4 overexpressing cells showed significantly increased tumorigenicity (~1.5-fold) as compared with the control cells (P < 0.05). These data indicated that the enhanced expression of MUC4 in pancreatic cancer cells is associated with increased invasiveness.
We have previously reported that the inhibition of MUC4 expression by antisense technique in the well-differentiated cancer cell line, CD18/HPAF, reduced tumorigenicity, and metastasis in vivo (Singh et al, 2004). To confirm that mini-MUC4 plays a similar role in tumour development compared with the wild-type MUC4, we examined the changes in the tumorigenicity and metastatic potential of mini-MUC4-transfected Panc1 and MiaPaCa cells in vivo. Two million cells were implanted into the pancreas and tumour formation was checked twice a week in the first 2 weeks and daily thereafter. Primary tumours were resected, and data were recorded for tumour weight and volume (Table 3). Seventy-five percent and 64% of the mice injected with the Panc1 parental or vector-transfected control cells, respectively, developed primary tumours as compared with 100% for the cells expressing mini-MUC4 (Table 3). However, this difference in the percentage of animals harbouring tumours was not statistically significant. Among animals with tumour present, the tumour weight was significantly higher for the Panc1 cells expressing mini-MUC4 as compared with the parental cells (P<0.01) and the vector-transfected cells (P<0.01). Similar results were obtained for the tumour volume for Panc1 cells expressing mini-MUC4 vs Panc1 parental (P<0.01) and for Panc1 transfected with mini-MUC4 vs vector-transfected cells (P<0.01). No statistically significant difference was observed for the metastasis frequency. Only lymph node involvement was detected by macroscopic examination of the mice in 27% of the cases for the Panc1 cells expressing mini-MUC4 and 17–18% for the control cells. The number of macroscopic metastases per mice was comparable for all the three cell lines investigated. The results for the mini-MUC4-expressing MiaPaCa cells are not presented here, but their implantation in mice provided similar results as for the mini-MUC4-expressing Panc1 cells.

DISCUSSION

Mucins are characterised by the presence of mucin domain, which is the largest domain and rich in serine, threonine, and proline residues. It is composed of highly glycosylated repetitive units that confer an extended conformation to the mucins due to the presence of multiple proline residues. For instance, the largest MUC4 allele identified, so far, is transcribed into RNA of 26.5 kb (Nollet et al, 1998). Within this 26.5 kb, 19 kb is composed of a 48 bp motif repeated up to 400 times, and this RNA encodes an estimated 2.12 µm-long protein. Although the structural organisation of most of the human mucins has been elucidated, their functions in human are still not well understood. This lack of knowledge is partly due to the complexity of their transcripts and the large size of the encoded proteins. One of the classic techniques to investigate the protein functions consists of overexpression of the corresponding gene in a cell line model and to study the phenotypic changes. The large size of MUC4 RNA, however, makes this technique very difficult to perform. In this study, the ectopic expression of mini-MUC4 construct was generated, containing the complete 5′- and 3′-unique sequences of MUC4, but with 1.8 kb of its repetitive domain instead of the original 7–19 kb. The MUC4 minigene is 8781 bp long and encodes an estimated 320 kDa protein. It represents one-third of the estimated size of the largest MUC4 allele and two-third of the smallest MUC4 allele reported. Mini-MUC4 is almost equal in size to its rat homologue, SMC/Muc4 (Carraway et al, 2000).

In our previous studies, inhibition of MUC4 expression reduced in vitro growth of pancreatic cancer cells (Singh et al, 2004). Another study performed on rat Muc4/SMC also proves the involvement of MUC4 mucin in growth advantage to cancer cells (Komatsu et al, 2001). In this study, the ectopic expression of...
mini-MUC4 showed an increased in vitro growth. Our in vivo studies also indicated that mini-MUC4 expression is associated with increased tumorigenicity of human pancreatic cancer cells. To evaluate the role of MUC4 in cell behavioral properties, we performed cell motility and invasion assays. The mini-MUC4 containing cells were more motile on uncoated 8 μm pore membrane. A decrease in motility was observed when MUC4 was downregulated, using an antisense technology in CD18/HPAF cells. Furthermore, the mini-MUC4-transfected cells also presented an increased capacity to form tumors compared to mock-transfected cells. Additionally, we showed that MUC4 induces an increase in mitochondrial mass (Dietze et al., 1998). The transfection of mini-MUC4 in Panc1 cell line (a poorly differentiated cell line) increased the mitochondrial mass but was not accompanied by an increase in the apoptotic index and change in the polarisation state of the mitochondrial membrane. A similar observation has been reported in breast cancer, where resistance to tamoxifen-induced apoptosis was correlated with an increase in mitochondrial mass (Dietze et al., 2001). In this paper, we showed that MUC4 induces an increase in mitochondrial mass without changing the apoptotic index. However, in our MUC4 knockdown cell line model, we showed that endogenous over-expression of MUC4 is preventing apoptosis of the CD18/HPAF pancreatic cancer cells after 48–72 h serum starvation (Chaturvedi et al., 2007). The lack of detection of any significant change in apoptosis and PARP activity in mini-MUC4-transfected cells might be due to the fact that apoptosis was induced by serum starving the cells for less than 24 h. The shorter duration of serum starvation was applied owing to the reason that Panc1 cells and their derivatives cannot survive for more than 24 h in serum-free media. However, we found a significant upregulation in necrosis of Panc1 (Pagliacci et al., 1993; Mancini et al., 1997). For instance, oxidative stress induced by H2O2 leads to an increase in mitochondrial mass in fibroblast. An increase in mitochondria can occur even in the absence of nuclear DNA replication (Sazer and Sherwood, 1990). Apart from that, mitochondria and mitochondrial DNA replication are also observed in the G2/M cell-cycle arrest. The increase in number of mitochondria occurs during cellular differentiation. The progress of differentiation in a normal cell depends on the increase in the ratio between the mitochondrial differentiation-promoting activity and decrease in nuclear-preventing activity (von Wangenheim and Peterson, 1998). During the differentiation process, the mitochondrial growth occurs at a rate that doubles its amount each cycle. The high cytoplasmic to nuclear ratio determines the cells to differentiate, followed by apoptosis. The relationship between mitochondria and apoptosis is well established (Green and Reed, 1998). The mitochondria is known to regulate apoptosis by releasing the apoptogenic factors such as cytochrome c from their intermembrane space into the cytoplasm (Kluck et al., 1997). The release of cytochrome c initiates caspase activation (Liu et al., 1996). An increase in mitochondria mass is, therefore, considered as one of the final steps during differentiation, just prior cell death by apoptosis (von Wangenheim and Peterson, 1998). The transfection of mini-MUC4 in Panc1 cell line (a poorly differentiated cell line) increased the mitochondrial mass but was not accompanied by an increase in the apoptotic index and change in the polarisation state of the mitochondrial membrane. A similar observation has been reported in breast cancer, where resistance to tamoxifen-induced apoptosis was correlated with an increase in mitochondrial mass (Dietze et al., 2001). In this paper, we showed that MUC4 induces an increase in mitochondrial mass without changing the apoptotic index. However, in our MUC4 knockdown cell line model, we showed that endogenous over-expression of MUC4 is preventing apoptosis of the CD18/HPAF pancreatic cancer cells after 48–72 h serum starvation (Chaturvedi et al., 2007). The lack of detection of any significant change in apoptosis and PARP activity in mini-MUC4-transfected cells might be due to the fact that apoptosis was induced by serum starving the cells for less than 24 h. The shorter duration of serum starvation was applied owing to the reason that Panc1 cells and their derivatives cannot survive for more than 24 h in serum-free media. However, we found a significant upregulation in necrosis of Panc1.

### Table 1

| Accession number | Name                                                                 | Fold upregulated |
|------------------|----------------------------------------------------------------------|------------------|
| W38923           | Transmembrane receptor (ror2)                                        | 4.08             |
| N90368           | Succinyl-CoA synthetase                                               | 4.01             |
| R25074           | Transmembrane 4 superfamily protein (SAS)                            | 3.61             |
| W60015           | Translation initiation factor elf-2α                                  | 3.57             |
| N72452           | RAD52 (Sachihoraymes cernoviose) homolog                              | 3.54             |
| H11692           | Neurospor-specific vesicle coat protein and cerebellar degeneration    | 3.50             |
| T51895           | Hepatoma transmembrane kinase                                         | 3.44             |
| R59579           | Prostaglandin D2 synthase                                             | 3.30             |
| AA490680         | Transcobalamin II                                                    | 3.19             |
| H07991           | Vascular endothelial growth factor related protein VRP                | 2.99             |
| H29256           | Neuronal DHP-sensitive, voltage-dependent calcium channel α-1D subunit | 2.97             |
| AA410680         | Tropomodulin                                                         | 2.88             |
| AA453105         | Histone 2A-like protein (H2Afl)                                      | 2.81             |
| AA037014         | Prostaglandin transporter hPGT                                        | 2.75             |
| H11792           | Putative splice factor transformer-2β                                 | 2.70             |
| R22274           | Phospholipomamine cytidyltransferase                                  | 2.69             |
| N80617           | Mitochondria-specific protein homolog                                 | 2.45             |
| H54686           | Mitogen-responsive phosphoprotein (DOC-2)                             | 2.38             |
| AA491302         | Inlet cell autoantigen 1 (69kDa)                                     | 2.30             |
| T64134           | Monocyte chemoattractant protein-4 precursor (MCP-4)                  | 2.26             |
| AA457051         | Lyophiloid-restricted membrane protein (Jak1)                         | 2.26             |
| R40897           | Succinyl CoA-3-oxoacid CoA transferase precursor (OXCT)               | 2.19             |
| AA489743         | Interferon-inducible 56kDa protein                                   | 2.16             |
| AA482128         | Protein-tyrosine kinase tyk2 (non-receptor)                          | 2.10             |
| AA169176         | Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)                 | 2.09             |
| N79051           | Transmembrane tyrosine-specific protein kinase (M2/PO)                | 2.08             |
| AA464149         | Isovaleryl coenzyme A dehydrogenase                                   | 2.08             |
| AA489714         | Homosporina mRNA for ZYG homologue                                   | 2.08             |
| H37761           | Mitogen induced nuclear orphan receptor (MNOR)                        | 2.06             |
| AA455712         | Kruppel-related zinc finger protein (ZNF184)                         | 2.03             |
| W23757           | Keratin 13                                                           | 2.03             |
| A5521453         | Human flightless-I                                                   | 2.01             |
| R19878           | Reelin (RELN)                                                        | 2.00             |
| R19878           | Reelin (RELN)                                                        | 2.00             |

### Table 2

| Accession number | Name                                                                 | Fold upregulated |
|------------------|----------------------------------------------------------------------|------------------|
| H63077           | Annexin I (Ispocortin I)                                             | 5.33             |
| AA478589         | Apolipoprotein E                                                     | 4.64             |
| AA167222         | Collagen type XIV                                                    | 2.91             |
| H60549           | Ribosomal protein S26                                                | 2.89             |
| T94626           | Ribosomina 3a chain precursor                                        | 2.74             |
| H77766           | Myotochloidine                                                       | 2.72             |
| R43544           | Ribosomal protein L32                                               | 2.59             |
| H71847           | Flavin-containing monoxygenase 2                                     | 2.58             |
| R90561           | Decay accelerating factor                                         2.52             |
| AA489569         | Mesothelial keratin K7 (type II)                                     | 2.51             |
| AA284668         | Urokinase-type plasminogen activator                                  | 2.50             |
| AA132090         | CD53 antigen                                                          | 2.43             |
| AA412053         | CD9 antigen                                                          | 2.36             |
| H72272           | Metallothionein 1-B gene                                             | 2.26             |
| T96083           | Low-Mr GTP-binding protein (RAB31)                                   | 2.26             |
| R05336           | β-1,4 N-acetylgalactosamintransferase                                 | 2.21             |
| T72877           | Interleukin-1 receptor antagonist                                     | 2.14             |
| AA598478         | Complement component 7                                               | 2.13             |
| N711659          | Metallokinase 2                                                      | 2.11             |
| RR4153           | Inositol 1,4,5-trisphosphate-3-kinase B                              | 2.03             |
| H16633           | Vascular cell adhesion molecule I                                    | 1.84             |
Expression of mini-MUC4 increases the motility and invasiveness of pancreatic cancer cells. (A) Cells were plated onto non-coated membranes for motility assays. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab and the remaining cells were stained. Cells that migrated through the pores were counted, and representative fields were photographed under bright field microscopy. Quantitation of cells invaded through the membrane in the above experiments is presented as a bar diagram. The mini-MUC4-expressing cells showed significant increase in cell motility as compared to control cells (*P < 0.001). (B) Matrigel invasion assay to measure the invasive potential of mini-MUC4-expressing cells. The invasion of mini-MUC4-expressing and control cells through extracellular matrix was investigated using Matrigel invasion chambers. The 10% FBS was used as a chemoattractant in lower chambers. Cells migrated through the Matrigel matrix were counted, and representative fields were photographed under bright field microscopy. Quantitation of cells invaded through the Matrigel matrix in the above experiments is presented as a bar diagram. The mini-MUC4-expressing cells showed higher invasive index than the control cells (*P < 0.05).

Table 3 Growth of pancreatic tumours developed by orthotopic implantation of Panc1 control (parental and vector-transfected) and mini-MUC4-expressing cells in immunodeficient mice

| Cell line          | Mice with tumour (%) | Median tumour weight (mg) | Median tumour volume (mm³) | Mice with metastases (%) |
|--------------------|----------------------|---------------------------|---------------------------|--------------------------|
| Panc1 mini-MUC4    | 11/11 (100%)         | 1050 ± 150*               | 770 ± 55*                 | 3/11 (27%)               |
| Panc1              | 9/12 (75%)           | 235 ± 35                  | 28 ± 5                    | 2/12 (17%)               |
| Panc1 pSecTag C    | 7/11 (64%)           | 145 ± 20                  | 20 ± 2                    | 2/11 (18%)               |

Mice were injected orthotopically with 2 x 10⁶ cells from Panc1, vector transfected and mini-MUC4-expressing cell lines in nude mice. Mice were killed at day 60 post-injection and tumours were resected, weighed, and their dimensions were measured. The mini-MUC4-expressing cells showed a significant increase in tumour weight, tumour volume but no significant difference in metastasis was observed. *P < 0.0001 compared with Panc1 pSecTag C.
cells transfected with vector in comparison with mini-MUC4-transfected cells. Necrosis is a way of cell death in the absence of significant pools of cellular ATP. Despite having apoptotic signals, cells would still undergo necrosis if the ATP levels are low. Presence of mini-MUC4 by itself might be providing survival signals and preventing the apoptosis; however, due to lack of survival signals in the absence of mini-MUC4 and due to reduced levels of ATP in the vector containing cells, these cells might undergo necrosis (Bernardi et al, 1999; Denecker et al, 2001). The mechanism and the pathways involved in this phenomenon still need to be determined. Thus, results suggest that MUC4, by increasing mitochondrial mass, most probably contributes to the redox status of the cells, which in turn provides the cancer cells with a survival advantage in pre-angiogenesis, leading to increased tumour progression. This advantage was observed when the cells were grown in medium containing low serum concentration. Control cells were dying over time, whereas the MUC4-expressing cells survived and expanded.

Microarray analysis performed using the mini-MUC4 cellular model revealed overexpression of numerous genes coding for mitochondrial proteins in mini-MUC4-expressing cells (Table 1). These upregulated genes code for various enzymes that regulate different mitochondrial functions, such as citric acid cycle, ketoacidosis, glycolysis, amino acid, and lipid metabolism. None of the deregulated genes code for proteins implicated in other mitochondrial functions, such as apoptosis. Therefore, it appears that the mini-MUC4-mediated increase in mitochondrial mass is directly associated with an increase in metabolic activity.

Mitochondrial mass regulation is a key point of numerous physiologic processes, including embryonic development, fat metabolism, and ageing. Our results present MUC4 as a partner in the regulation of these vital physiological steps. MUC4, at the surface of the epithelial cells, might act as an active sensor for a differentiation pathway. Some of the genes downregulated in mini-MUC4-overexpressing cells were annexin I, apolipoprotein, collagen type XIV, CD53, CD9 antigen, metastasis associated (pta 1) and vascular cell adhesion molecule 1 (Table 2). The pta 1 and vascular cell adhesion molecule are known to be involved in the metastasis processes (Langley et al, 2001; Hofer et al, 2004). The nonsignificant changes in metastasis may be explained in part by downregulation of these genes in mini-MUC4-transfected cells.

One of the hypotheses that would need to be further investigated is the resistance to hypoxia-induced apoptosis driven by the increase in mitochondrial mass after mini-MUC4 overexpression. Cancer cells overexpressing MUC4 might use this strategy to survive and establish themselves before angiogenesis takes place. Some evidence is provided by the fact that only tumorigenicity was increased by mini-MUC4 overexpression and not the metastatic index. This observation suggests that an increase in tumorigenicity is associated to the unique sequence of MUC4 (MUC4b for instance) and the increase in metastatic index is related to the size of the large repetitive domain present in MUC4a.

In conclusion, our data provide evidence supporting the role of MUC4 mucin in the progression of pancreatic cancer. We demonstrate that MUC4 enhances the growth, motility, and invasive properties of pancreatic cancer cells in vitro. Over-expression of MUC4 mucin also induces cellular changes and tumour progression of pancreatic cancer cells. Hence, MUC4 can be a useful target in the development of novel therapeutic strategies for the treatment of pancreatic cancer.

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REFERENCES

Alos L, Lujan B, Castillo M, Nadal A, Carreras M, Caballero M, de BC, Cardesa A (2005) Expression of membrane-bound mucins (MUC1 and MUC4) and secreted mucins (MUC2, MUCSAC, MUC5B, MUC6 and MUC7) in mucosaeipidermoid carcinomas of salivary glands. Am J Surg Pathol 29: 806–813

Andrianfahanana M, Agraval A, Singh AP, Moniaux N, Van SL, Aubert JP, Meza J, Batra SK (2005) Synergistic induction of the MUC4 mucin gene by interferon-gamma and retinoic acid in human pancreatic tumour cells involves a reprogramming of signalling pathways. Oncogene 24: 6143–6154

Andrianfahanana M, Moniaux N, Schmied BM, Ringel J, Fries H, Hollingsworth MA, Buchler MW, Aubert JP, Batra SK (2001) Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumour marker of diagnostic significance. Clin Cancer Res 7: 4035–4040

Audie JP, Janin A, Pochet N, Copin MC, Gosselin B, Aubert JP (1993) Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by in situ hybridization. J Histochem Cytochem 41: 1479–1485

Audie JP, Tetaert D, Pigny P, Buisine MP, Janin A, Aubert JP, Pochet N, Boersma A (1995) Mucin gene expression in the human endocervix. Hum Reprod 10: 98–102

Balshaw C, Azzam E, Carrato C, Pochet N, Aubert JP, Kim YS, Real FX (1994) Altered expression of MUC2, MUC4, and MUC5 mucin genes in pancreas tissues and cancer cell lines. Gastroenterology 106: 1054–1061

Bax DA, Haringsma J, Einerhand AW, van DH, Blok P, Siersema PD, Kuipers EJ, Kusters JG (2004) MUC4 is increased in high grade intraepithelial neoplasia in Barrett’s oesophagus and is associated with a proapoptotic Bax to Bcl-2 ratio. J Clin Pathol 57: 1267–1272

Bernardi P, Scorrano L, Colonna R, Petronilli V, Di Lisa F (1999) Mitochondria and cell death Eur. J Biochem 264: 687–701

Brand KA, Hermfisse U (1997) Aerobic glycolysis by proliferating cells: a protective strategy against reactive oxygen species. FASEB J 11: 388–395

Buisine MP, Devisme L, Copin MC, Durand-Reville M, Gosselin B, Aubert JP, Porchet N (1999) Developmental mucin gene expression in the human respiratory tract. Am J Respir Cell Mol Biol 20: 209–218

Buisine MP, Devisme L, Savidge TC, Gepach C, Gosselin B, Porchet N, Aubert JP (1998) Mucin gene expression in human embryonic and fetal intestine. Gut 43: 519–524

Carraway KL, Price-Schiavi SA, Komatsu M, Idris N, Perez A, Li P, Jepson S, Zhu X, Carvalaj ME, Carraway CA (2000) Multiple facets of sialomucin complex/MUC4, a membrane mucin and erbb2 ligand, in tumors and tissues (Y2K update). Front Biosci 5: D95–D107

Chaturvedi P, Singh AP, Moniaux N, Senapati S, Chakraborty S, Meza JI, Batra SK (2007) MUC4 mucin potentiates pancreatic tumor cell proliferation, survival, and invasive properties with its interaction to extracellular matrix proteins. Mol Cancer Res 5: 309–320

Choudhury A, Moniaux N, Winpenny JP, Hollingsworth MA, Aubert JP, Batra SK (2000a) Human MUC4 mucin cDNA and its variants in pancreatic carcinoma. J Biochem (Tokyo) 128: 233–243

Choudhury A, Singh RK, Moniaux N, El-Metwally TH, Aubert JP, Batra SK (2000b) Retinoic acid dependent transforming growth factor-beta2-mediated induction of MUC4 mucin expression in human pancreatic tumor cells follows retinoic acid receptor-alpha signaling pathway. J Biol Chem 275: 33929–33936

Denecker G, Vercammen D, Steemans M, Bergha TV, Brouckaert G, Loo GV, Zhivetovsky B, Fiers W, Grooten J, Declercq W, Vandenberghe P (2001) Death receptor-induced apoptotic and necrotic cell...
death; differential role of caspases and mitochondria. *Cell Death Differ* 8: 829 – 840

Dietze EC, Caldwell LE, Grupin SL, Mancini M, Seewaldt VL (2001) Tamoxifen but not 4-hydroxytamoxifen initiates apoptosis in p53(-) normal human mammary epithelial cells by inducing mitochondrial depolarization. *J Biol Chem* 276: 5384 – 5394

Escande F, Lemaître L, Moniaux N, Batra SK, Aubert JP, Buisine MP (2002) Genomic organization of MUC4 mucin gene. Towards the characterization of splice variants. *Eur J Biochem* 269: 3637 – 3644

Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* 281: 1309 – 1312

Handra-Luca A, Lamas G, Bertrand JC, Fouret P (2005) MUC1, MUC2, MUC4, and MUC5AC expression in salivary gland mucoepidermoid carcinoma: diagnostic and prognostic implications. *Am J Surg Pathol* 29: 881 – 889

Hofer MD, Kuefer R, Varambally S, Li H, Shapiro GI, Gschwend JF, Hockenbery DM (1997) Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis. *Cell Biol* 86: 1 – 32

Komatsu M, Jepson S, Arango ME, Carothers Carraway CA, Carraway KL (2004) Endothelial expression of vascular adhesion molecule-1 correlates with monocytic infiltration in pancreatic ductal adenocarcinoma. *Biochem Biophys Res Commun* 317: 1573 – 1577

Khorrami AM, Choudhury A, Andrianifahanana M, Varshney GC, Aubert JP (1991) Molecular cloning and characterization of a human pancreatic adenocarcinoma mucin. *J Biochem (Tokyo)* 131: 21 – 29

Kluczynski C, Bossy-Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275: 1132 – 1136

Komatsu M, Jepson S, Arango ME, Carothers Carraway CA, Carraway KL (2001) Muc4/sialomucin complex, an intramembrane modulator of ErbB2/HER2/Neu, potentiates primary tumor growth and suppresses apoptosis in a xenotransplanted tumor. *OncoGene* 20: 461 – 463

Kroemer G (2006) Mitochondria in cancer. *Oncogene* 25: 4630 – 4632

Liang SY, Carlisle R, Ma L, Specian RD, Gerritsen ME, Granger DN (2001) Endothelial expression of vascular adhesion molecule-1 correlates with metastatic pattern in spontaneous melanoma. *Microcirculation* 8: 335 – 345

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147 – 157

Mancini M, Anderson BO, Caldwell E, Sedghinasab M, Paty PB, Hockenbery DM (1997) Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis in a human colon carcinoma cell line. *J Cell Biol* 138: 449 – 469

Nolley S, Moniaux N, Maury J, Petiprez D, Degand P, Laine A, Porchet N, Aubert JP (1998) Human mucin gene MUC4: organization of its 5'-region and polymorphism of its central tandem repeat array. *Biochem J* 332: 739 – 748

Ogata S, Uehara H, Chen A, Itzkowitz SH (1992) Mucin gene expression in colonic tissues and cell lines. *Cancer Res* 52: 5971 – 5978

Pagliacci MC, Spinozzi F, Migliorati G, Fumi G, Smacchia M, Grignani F, Riccardi C, Nicoletti I (1993) Genistein inhibits tumour cell growth in vitro but enhances mitochondrial reduction of tetrazolium salts: a further pitfall in the use of the MTT assay for evaluating cell growth and survival. *Eur J Cancer* 29A: 1573 – 1577

Park HU, Kim JW, Kim GE, Bae HJ, Cowley SC, Yang SC, Gum JR, Batra SK, Rousseau K, Swallow DM, Lesiseng MH, Kim YS (2003) Aberrant Expression of MUC3 and MUC4 membrane-associated mucins and sialyl lea antigen in pancreatic intraepithelial neoplasia. *Pancreas* 26: 48 – 54

Porchet N, Nguyen VC, Dufosse J, Audie JP, Guyonnet-Duperat V, Gross MS, Denis C, Degand P, Bernheim A, Aubert JP (1991) Molecular cloning and chromosomal localization of a novel human tracheo-bronchial mucin cDNA containing tandemly repeated sequences of 48 base pairs. *Biochem Biophys Res Commun* 175: 414 – 422

Remis M, Cantatore P, Loguercio PP, Fracasso F, Gadala-Maria N (1998) Content of mitochondrial DNA and of three mitochondrial RNAs in developing and adult rat cerebellum. *J Neurochem* 52: 750 – 754

Robin ED, Wong R (1988) Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J Cell Physiol* 136: 507 – 513

Sazer S, Sherwood SW (1990) Mitochondrial growth and DNA synthesis occur in the absence of nuclear DNA replication in fission yeast. *J Cell Sci* 97: 509 – 516

Shay JW, Pierce DJ, Werbin H (1990) Mitochondrial DNA copy number is proportional to total cell DNA under a variety of growth conditions. *J Biol Chem* 265: 14802 – 14807

Singh AP, Moniaux N, chauhan SC, Meza JL, Batra SK (2004) Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis. *Cancer Res* 64: 622 – 630

Swart MJ, Batra SK, Varshney GC, Hollingsworth MA, Yeo CJ, Cameron JL, Willentz RE, Hruban RH, Argani P (2002) MUC4 expression increases progressively in pancreatic intraepithelial neoplasia (PanIN). *Am J Clin Pathol* 117: 791 – 796

von Wangenheim KH, Peterson HP (1998) Control of cell proliferation by p53: differential regulation of cell death by proapoptotic and antiapoptotic members of the Bcl-2 family. *Oncogene* 17: 197 – 203

Wang HY, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* 30: e15