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ONCOGENOMICS

Epigenetic regulation (DNA methylation, histone modifications) of the 11p15 mucin genes (*MUC2*, *MUC5AC*, *MUC5B*, *MUC6*) in epithelial cancer cells

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The human genes *MUC2*, *MUC5AC*, *MUC5B* and *MUC6* are clustered on chromosome 11 and encode large secreted gel-forming mucins. The frequent occurrence of their silencing in cancers and the GC-rich structure of their promoters led us to study the influence of epigenetics on their expression. Pre- and post-confluent cells were treated with demethylating agent 5-aza-2'-deoxycytidine and histone deacetylase (HDAC) inhibitor, trichostatin A. Mapping of methylated cytosines was performed by bisulfite-treated genomic DNA sequencing. Histone modification status at the promoters was assessed by chromatin immunoprecipitation assays. Our results indicate that *MUC2* was regulated by site-specific DNA methylation associated with establishment of a repressive histone code, whereas hypermethylation of *MUC5B* promoter was the major mechanism responsible for its silencing. DNA methyltransferase 1 was identified by small interfering RNA approach as a regulator of *MUC2* and *MUC5B* endogenous expression that was potentiated by HDAC2. *MUC2* and *MUC5B* epigenetic regulation was cell-specific, depended on cell differentiation status and inhibited their activation by Sp1. The expression of *MUC5AC* was rarely influenced by epigenetic mechanisms and methylation of *MUC6* promoter was not correlated to its silencing. In conclusion, this study demonstrates the important role for methylation and/or histone modifications in regulating the 11p15 mucin genes in epithelial cancer cells.

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Keywords: mucin; 11p15; methylation; acetylation; cancer; epithelial

Introduction

DNA methylation, associated with histone deacetylation, is a common mechanism used by cancer cells to inhibit the expression of tumour suppressor genes (Herman and Baylin, 2003) and genes involved in tumour formation (Mompalmer, 2003). Recent works aimed at studying the importance of epigenetics in cancer opened the way to a host of innovative diagnostic and therapeutic strategies, attesting that DNA methylation is a powerful tool in the clinic (Laird, 2003). Hence, discovery of new methylated genes in cancer will help both in the classification of tumours and the identification of genes influencing tumour progression.

MUC2, *MUC5AC*, *MUC5B* and *MUC6* mucin genes encode large secreted O-glycoproteins that participate in mucus formation and play an important role as a physiological barrier against various aggressions of the underlying epithelia (Hollingsworth and Swanson, 2004). These four genes are located within a 400-kb long cluster, on the p15.5 region of chromosome 11 (Pigny *et al.*, 1996) in an area known to be a hot spot of abnormal methylation in cancer (De Bustros *et al.*, 1988).

Having previously found that these genes were located in a hot spot of methylation in the genome (Pigny *et al.*, 1996), that their pattern of expression was altered in epithelial cancers (Copin *et al.*, 2001; Van Seuningen *et al.*, 2001) and that their promoters were GC-rich (Van Seuningen *et al.*, 2001), we undertook to investigate, in the same study, the importance of not only DNA methylation but also the influence of histone modifications for each gene of the cluster and propose key CpG sites that could be used as valuable markers in epithelial cancers. To our knowledge, this is the first time that such a question is investigated simultaneously for the four genes. So far the few studies conducted focused on *MUC2* methylation and no data are available regarding *MUC6*. Hanski *et al.* (1997) showed that repression of *MUC2* gene expression in non-mucinous colon carcinoma cells was associated with methylation of its promoter (Gratchev *et al.*, 1998, 2001). In pancreatic carcinoma cells (Siedow *et al.*, 2002) and in mucinous gastric carcinomas (Mesquita *et al.*, 2003), on the other hand, it was shown that *de novo* expression of *MUC2* was triggered by promoter

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Dedicated to Dr Jean-Pierre Aubert (DR1 INSERM) who passed away on 3 September, 2005. It is an immense loss for us, as the director of our laboratory, and for the mucin field scientific community, who appreciated him for his human qualities and as the discoverer of three mucin genes in the 1990s.

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demethylation or hypomethylation, respectively. We recently showed that repression of *MUC5B* in gastric cancer cells was due in part to the presence of methylated cytosines throughout its promoter (Perrais *et al.*, 2001a).

In this paper, we demonstrate that among the four 11p15 mucin genes, *MUC2* and *MUC5B* are highly submitted to DNA methylation and histone modifications, whereas *MUC5AC* is rarely influenced by epigenetic regulation and *MUC6* is not. *MUC2* repression by methylation is the result of site-specific methylation within its promoter associated with establishment of a repressive histone code. *MUC5B* silencing is the result of an extensive methylation of its distal promoter and site-specific methylation of its proximal promoter. *MUC2* and *MUC5B* repression by methyla-

tion is cell-specific, is controlled by DNA methyltransferase 1 (DNMT1) and dramatically impairs their activation by the transcription factor Sp1.

Results

Influence of DNA methylation and histone deacetylation on the expression of the 11p15 mucin genes

To study the role of methylation and histone acetylation on mucin gene expression, cells were treated with demethylating agent 5-aza-2'-deoxycytidine (5-aza, Figure 1a) or histone deacetylase (HDAC) inhibitor trichostatin A (TSA, Figure 1b). As 11p15 mucin genes have a cell- and tissue-specific pattern of expression in

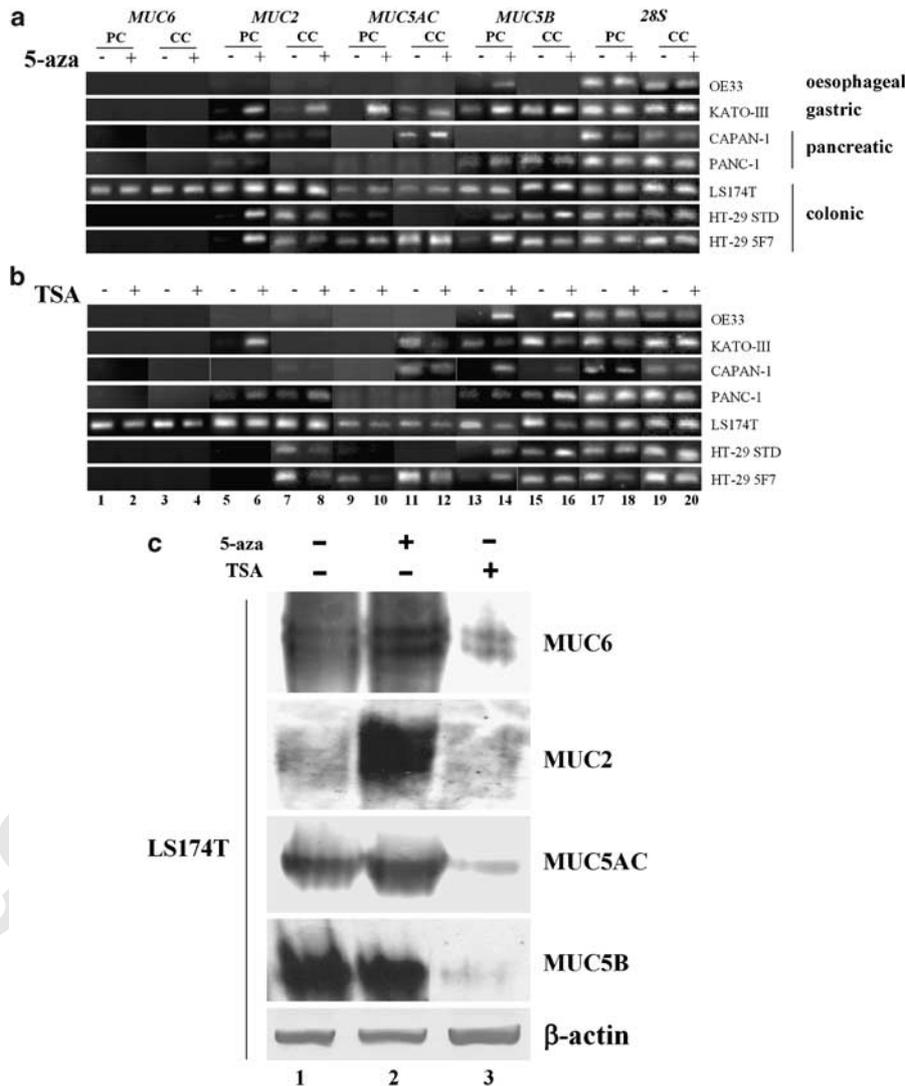


Figure 1 Influence of epigenetics on the expression of the 11p15 mucin genes in epithelial cancer cell lines. RT-PCR was performed as described in Materials and methods section. The expected size for PCR products of *MUC6* (lanes 1–4), *MUC2* (lanes 5–8), *MUC5AC* (lanes 9–12), *MUC5B* (lanes 13–16) and *28S* (lanes 17–20) are 421, 401, 409, 415 and 231 bp, respectively. Untreated (–) or treated (+) cells with (a) 5-aza or (b) TSA. PC: proliferating cells, CC: confluent cells. (c) Influence of 5-aza and trichostatin A (TSA) treatment on MUC6, MUC2, MUC5AC and MUC5B apomucins expression in LS174T PC. Untreated, 5-aza- or TSA-treated cells (lanes 1, 2 and 3, respectively). Total cellular extracts were prepared as described in Materials and methods section. 20 μ g of proteins were loaded on a 2% (w/v) SDS-agarose gel before being transferred onto nitrocellulose membrane and processed for immunohistochemical detection.

normal adult, we investigated the influence of epigenetics in seven adenocarcinomatous cell lines with different epithelial origins and phenotypes: oesophageal OE33, gastric KATO-III, pancreatic CAPAN-1 (well-differentiated) and PANC-1 (undifferentiated) and colonic LS174T (mucus-secreting), HT-29 STD (undifferentiated) and HT-29 5F7 (enterocyte-like).

MUC6 showed a very specific pattern of expression restricted to LS174T cells (Figure 1a). Cell treatment with 5-aza or TSA did not lead to re-expression of *MUC6* in the cell lines not expressing the gene (Figure 1a and b). Higher concentrations of 5-aza were used (20, 40, 80 μM) but remained ineffective (data not shown). A possible synergistic action of 5-aza and TSA was also assayed but had no effect (data not shown).

MUC2 was strongly expressed in LS174T cells (Figure 1a). It was very weakly expressed in CAPAN-1 and PANC-1 and not expressed in proliferative OE33, KATO-III, HT-29 STD and HT-29 5F7 cells (PC). Its expression was strongly induced after 5-aza treatment except for OE33 and PANC-1 (Figure 1a). Influence of methylation on *MUC2* expression was lost once HT-29 STD and HT-29 5F7 cells became confluent (CC) and differentiated as no increase of *MUC2* mRNA was visualized in 5-aza-treated CC. In KATO-III and PANC-1 PC, *MUC2* silencing was also because of histone deacetylation as TSA treatment induced its expression (Figure 1b). This was lost once KATO-III cells became confluent.

MUC5AC was only weakly expressed in LS174T and HT-29 5F7 PC. In HT-29 5F7, its expression increased once cells became confluent. 5-aza treatment induced *MUC5AC* expression in KATO-III PC and increased mRNA level in HT-29 5F7 PC and KATO-III CC. In CAPAN-1 CC, 5-aza treatment induced an increase of *MUC5AC* mRNA expression correlated to an increase of the protein amount (data not shown).

MUC5B was highly expressed in LS174T cells and weakly expressed in KATO-III, PANC-1 and HT-29 5F7 PC. Its repression in CAPAN-1 was not because of DNA methylation, whereas 5-aza treatment induced its expression in OE33 and HT-29 STD. In low-expressing KATO-III, PANC-1 and HT-29 5F7 PC, 5-aza treatment substantially increased the level of *MUC5B* mRNA (Figure 1a). In these three cell lines, expression of *MUC5B* increased substantially (threefold) when cells became confluent (lane 15) at which point demethylation by 5-aza became ineffective (lane 16). Histone deacetylation was also involved in *MUC5B* repression since TSA treatment induced its expression in all the cell lines studied (Figure 1b) except for high-expressing KATO-III and LS174T.

Interestingly, histone deacetylation following TSA treatment systematically resulted in the decrease of mucin gene expression when they were highly expressed in the cells (*MUC2* in HT-29 STD and HT-29 5F7, *MUC5B* in KATO-III, *MUC5AC*, *MUC5B* and *MUC6* in LS174T) (Figure 1b). To investigate whether these RNA variations translated into a decreased amount of proteins, western blotting was carried out in LS174T cells, which express the four secreted mucins (Figure 1c,

lane 1). The decrease of *MUC5AC*, *MUC5B* and *MUC6* expression observed at the mRNA level after TSA treatment (Figure 1b) indeed led to a substantial decrease of apomucins (lane 3).

Methylation patterns of the 11p15 mucin gene promoters

To map methylated cytosines within the promoters, we used methylation-specific PCR and bisulfite-treated DNA sequencing (Figure 2). Primer information is given in Supplementary Tables. To study simultaneously the methylation status of the four genes of the cluster, we had to identify and characterize the promoter of *MUC6*, which was not known at the time this study was initiated. The 5' end of *MUC6* was characterized by 5' rapid amplification cDNA end PCR (Supplementary methods) and identified as a guanine residue located 61 nucleotides upstream of the first ATG (Supplementary data 1).

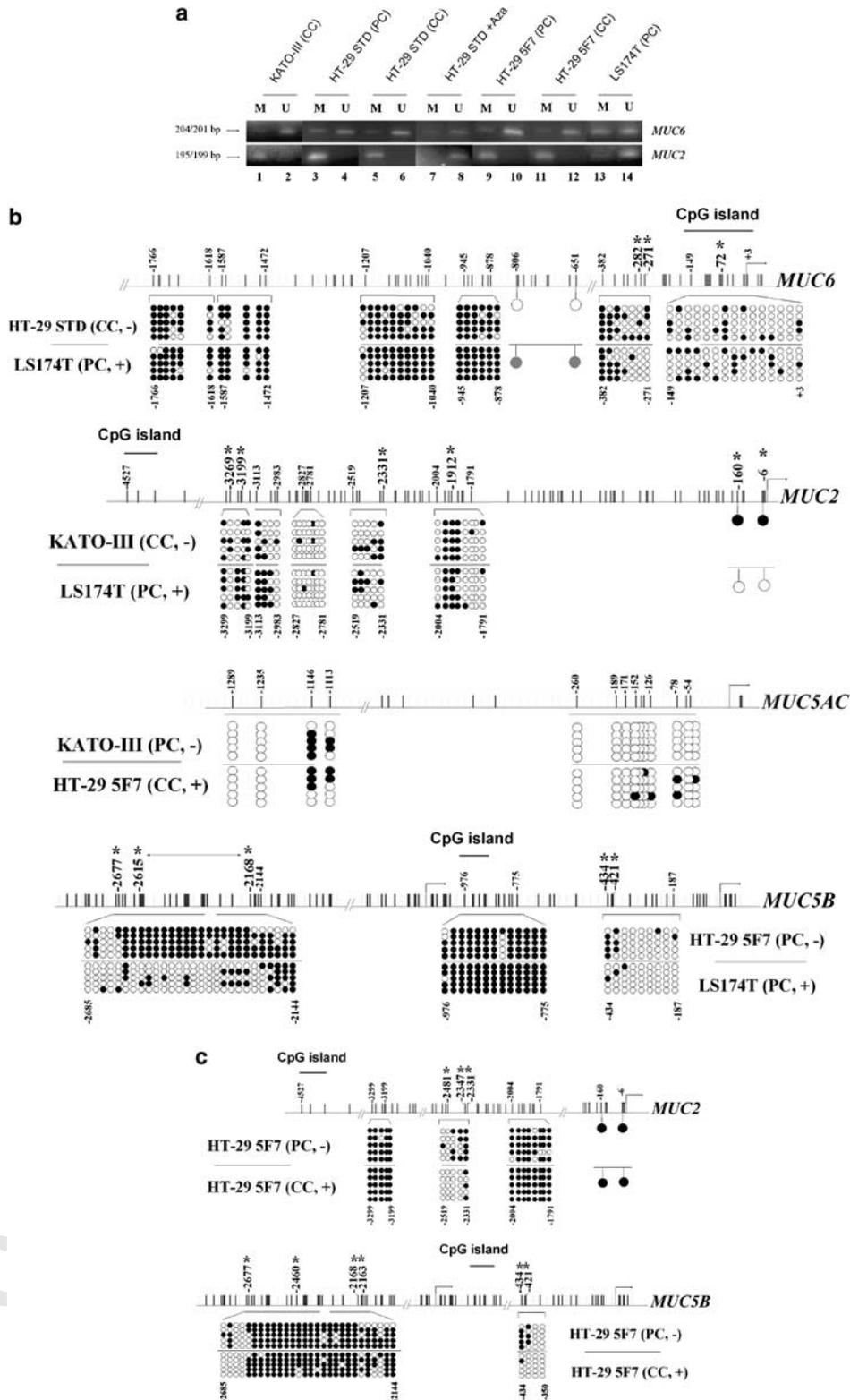
Computer analysis of the four promoters indicated that the 5'-flanking regions of *MUC6*, *MUC2* and *MUC5B* contained a high percentage of CpG dinucleotides (up to 75, 70 and 80%, respectively) and CpG islands of lengths 163, 115 and 104 bp, respectively (Figure 2). The promoter of *MUC5AC* was characterized by a lower percentage of CpG dinucleotides (55%) and no CpG island.

MS-PCR studies of *MUC6* promoter methylation (Figure 2a) indicated that cytosines at -806 and -651 were partially methylated in HT-29 STD PC and LS174T PC and unmethylated in the other cell lines studied. The studies on *MUC2* promoter indicated that the cytosines at -160 and -6 were methylated in all the cell lines studied except for the *MUC2*-expressing LS174T cells and for 5-aza-treated HT-29 STD cells (Figure 2a).

To map precisely the methylated cytosines within mucin gene promoters, bisulfite-treated DNA sequencing approach was conducted by comparing methylation patterns in an expressing versus a non-expressing cell line. Analysis of the distal part of *MUC6* promoter (Figure 2b, -1766/-878) indicated that most of the CpG dinucleotides were methylated both in *MUC6*-expressing LS174T and *MUC6* non-expressing HT-29 STD cells. In the proximal part of the promoter (-382/+5), which contains the CpG island, low methylation was observed except for two sites (-382 and -350). Three cytosines (-282, -271 and -72) were more methylated in HT-29 STD non-expressing cell line compared to LS174T.

As the proximal region of *MUC2* promoter (-1989/+288) had already been studied by Hamada *et al.* (2005), we focused our studies on the distal region (Figure 2b, -3299/-2983, -2827/-2781, -2519/-2331 and -2004/-1791). In the -3299/-2983 region, the cytosines at -3269 and -3199 showed lower methylation levels in *MUC2*-expressing LS174T cells compared to KATO-III. In the -2519/-2331 and -2004/-1791 regions, the cytosines at -2331 and -1912 were much less methylated in LS174T cells.

Q2



Analysis of *MUC5AC* promoter over 1.29 kb did not lead to identification of key CpG sites that would be more methylated in *MUC5AC* non-expressing KATO-III PC compared to expressing HT-29 5F7 cells.

Analysis of the distal promoter of *MUC5B* (−2685/−2144) indicated that most of the CpG dinucleotides were totally methylated in *MUC5B* non-expressing HT-29 5F7 cells, whereas they were not (or partially) methylated in LS174T cells. The same result was observed for the cytosines at −434 and −421 in the proximal promoter. In the −976/−775 region containing the CpG island, all but two cytosines (−976 and −854) were methylated in both cell lines.

Results obtained by reverse transcriptase (RT)–PCR in Figure 1a indicated that *MUC2* and *MUC5B* repression by methylation in HT-29 5F7 PC was lost once these cells became CC and differentiated. To confirm this result at the promoter level, we undertook to study the methylation status of the key CpG sites identified in Figure 2b in HT-29 5F7 PC and CC. Studies on *MUC2* promoter (Figure 2c) indicated that cytosines at −2481, −2347 and −2331 showed lower methylation levels in HT-29 5F7 CC compared to PC. The same result was observed for the cytosine residues at −2677, −2460, −2168, −2163, −434 and −421 of *MUC5B* promoter (Figure 2c).

Influence of methylation on *MUC2* and *MUC5B*-promoter activity and on their regulation by Sp1

In vitro methylation of *MUC2* and *MUC5B* promoters by mSssI (Figure 3a) resulted in a strong decrease of their activity (up to 80 and 90% inhibition, respectively). We then studied the influence of that methylation on their regulation by Sp1, an important regulator of the promoter of *MUC2* and of the proximal promoter of *MUC5B* (Van Seuning *et al.*, 2001), by performing cotransfections. The result indicated that methylation of *MUC2* and *MUC5B* promoters dramatically impaired their activation by Sp1 (50–100% loss, Figure 3b).

Histone H3 and H4 tail lysine modifications at 11p15 mucin gene promoters

Chromatin immunoprecipitation (ChIP) assays indicated that, in PANC-1 cells, *MUC2* and *MUC5B* repression was associated with histone H3 deacetylation and K9H3 methylation as well as with K27H3 trimethylation for *MUC5B* (Figure 4a). In LS174T cells, histones H3 and H4 were hyperacetylated at *MUC2* and *MUC5B* promoters (Figure 4b). Chromatin modification studies of *MUC2* also indicated absence of K27H3 trimethylation and only a weak methylation of K9H3. Having shown that silencing of *MUC2* and *MUC5B* only occurred in HT-29 STD PC and was influenced by histone deacetylation, we undertook to compare the chromatin status of these genes in HT-29 STD PC and CC (Figure 4b). The results showed decreased K9H3 and K27H3 methylation both at *MUC2* and *MUC5B* promoters and increased K4H3 methylation at *MUC5B* promoter in HT-29 STD CC compared to PC. No significant differences were observed for H3 and H4 acetylation between PC and CC cells. The same study in HT-29 5F7 PC and CC (Figure 4c) showed increased K9H3 acetylation at *MUC2* promoter and decreased K27H3 methylation at *MUC5B* promoter in HT-29 5F7 CC compared to PC.

Role of chromatin-modifier enzymes in the regulation of *MUC2* and *MUC5B* endogenous expression

To investigate whether DNMT1 participates in *MUC2* and *MUC5B* silencing, we performed knockdown assays with specific small interfering (siRNA) in HT-29 5F7 PC (Figure 5). DNMT1 knockdown led to a significant increase of *MUC2* (1.4-fold, $P \leq 0.05$, Figure 5a) and *MUC5B* (6.5-fold, $P = 0.0005$, Figure 5b) mRNA levels. As DNA methylation and histone deacetylation are partners in transcriptional repression and DNMT1 associates with class I HDACs, knockdown assays were performed with DNMT1 siRNA combined with either HDAC1, HDAC2 or HDAC3 siRNA. The results

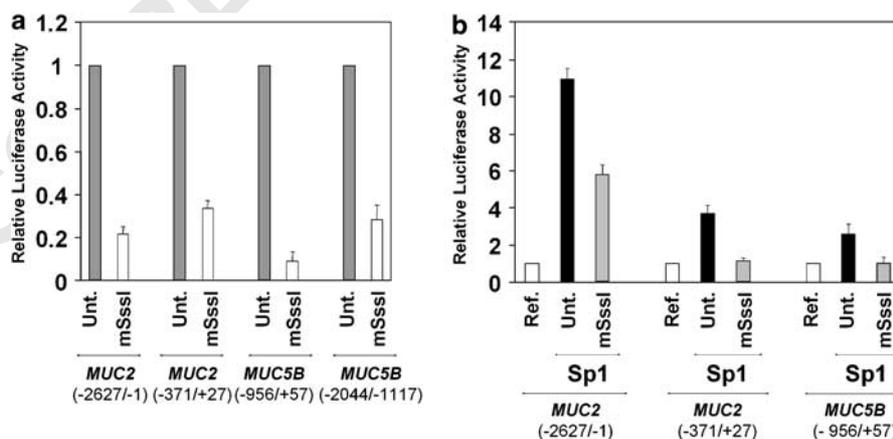


Figure 3 Influence of methylation on *MUC2* and *MUC5B* promoter activity and regulation by Sp1. (a) Luciferase diagram showing the effect of DNA methylation by mSssI on *MUC2* and *MUC5B* promoter activity in LS174T cells. Untreated (Unt., grey bars) and methylated constructs (mSssI, white bars). (b) Study of the effect of promoter methylation on *MUC2* and *MUC5B* transactivation by Sp1. Results are expressed as fold activation of luciferase activity in samples cotransfected with Sp1 (Unt., black bars; mSssI, grey bars) compared with that cotransfected with the empty vector (Ref., white bars).

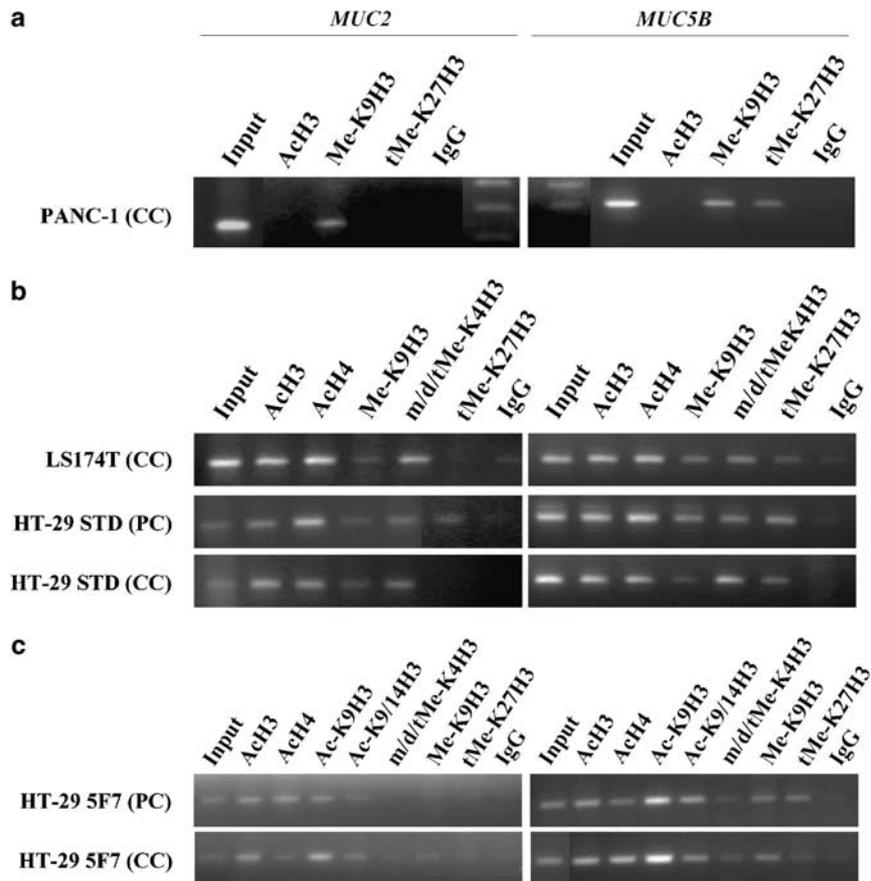


Figure 4 Histone modification status at *MUC2* and *MUC5B* promoters in PANC-1, LS174T, HT-29 STD and HT-29 5F7 cancer cells. Chromatin immunoprecipitation (ChIP) assays were performed as described in Materials and Methods. Acetylated histone H4 (AcH4) and six histone H3 modifications were analysed: acetylated lysine (AcH3), acetylated lysine 9 (Ac-K9H3), acetylated lysines 9 and 14 (Ac-K9/14H3), monomethylated lysine 9 (Me-K9H3), mono/di/trimethylated lysine 4 (m/d/tMe-K4H3) and trimethylated lysine 27 (tMe-K27H3). Input DNA was used as a positive amplification control; IgG indicates ChIP performed using rabbit IgGs as an isotypic antibody control.

indicated that HDAC2 knockdown potentiated the activating effect of DNMT1 silencing on both *MUC2* (1.9-fold, $P=0.0005$) and *MUC5B* (9.5-fold, $P=0.0039$) expression levels. HDAC1 and HDAC3 knockdown combined with that of DNMT1 had no additive effect.

Discussion

The studies performed in this work demonstrate that epigenetic regulation of the 11p15 mucin genes is complex, gene- and cell-specific. Our data showed that DNA methylation and/or histone deacetylation greatly influenced the level of expression of *MUC2* and *MUC5B* to a lower extent than that of *MUC5AC* and had no effect on *MUC6* (Figure 6). Different situations were observed; unique involvement of methylation in repressing the gene, which would suggest a probable hypermethylated state of the mucin gene that did not allow the inhibitors of HDACs to induce its expression or the opposite situation, with only the ability to restore mucin gene expression by inhibiting histone deacetyla-

tion (*MUC5B*). To our knowledge, this is the first time that such a regulatory mechanism, leading to stable repression by a histone modification-dependent mechanism, is described for a mucin gene. Recently, Yamada *et al.* (2006) showed that neither DNA methylation nor histone modification alone fully determined expression of *MUC2* in pancreatic cancer cell lines. Our results in pancreatic and colonic cancer cell lines are in agreement with this hypothesis since we showed that *MUC2* pattern of expression was the result of a cell-specific combination of site-specific methylation and chromatin modifications. This suggests that the same epigenetic mechanisms drive *MUC2* expression in epithelial cells with different tissue origins.

Studies at two different stages of cell growth gave us new information regarding the impact of methylation and histone modifications on mucin gene expression during cell proliferation and cell differentiation. In the majority of the cases, strong methylation of *MUC2* and *MUC5B* promoters and associated histones was observed when cells were proliferating and thus still not differentiated. In colonic HT-29 STD and HT-29 5F7 cell lines, loss of methylation was observed once cells

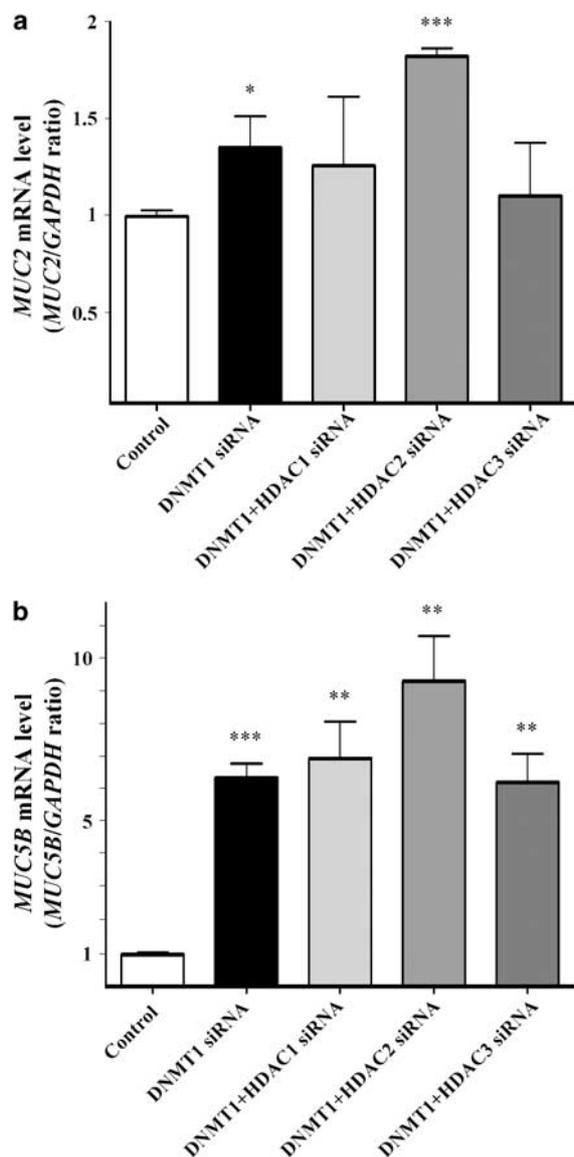


Figure 5 Role of chromatin modifier enzymes (DNMT1, HDAC1, HDAC2 and HDAC3) in the silencing of *MUC2* and *MUC5B*. siRNA experiments were carried out as described in the Materials and methods section. *MUC2*, *MUC5B* and *GAPDH* mRNA levels were assessed by RT-PCR. PCR products (10 μ l for *MUC2* and *MUC5B*, 6 μ l for *GAPDH*) were analysed on a 1.5% agarose gel. (a) Diagram showing data expressed as *MUC2*/*GAPDH* ratio. (b) Diagram showing data expressed as *MUC5B*/*GAPDH* ratio. Control corresponds to the means value from mock cells and cells transfected with non-targeting siRNA. * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.0005$.

became confluent and differentiated and this was correlated to demethylation of three specific cytosines at -2481, -2347 and -2331 in *MUC2* promoter and six cytosines at -2677, -2460, -2168, -2163, -434 and -421 in *MUC5B* promoter. This mechanism may explain the pattern of expression of these mucins described previously in intestinal (Sylvester *et al.*, 2001) and respiratory (Bernacki *et al.*, 1999) tracts that showed increase of expression concomitant to cell differentiation. We also found differences in the histone

modification pattern of *MUC2* promoter in PANC-1 non-expressing cells compared to HT-29 STD PC, which only expressed *MUC2* when confluent. This indicates that definitive and transient silencing of *MUC2* involves different epigenetic combinations. Therefore, from our studies it can be hypothesized that epigenetic silencing of *MUC2* and *MUC5B* would be a specific mechanism used by cancer cells to maintain their undifferentiated state or by normal cells not yet engaged in a differentiation process. These findings have important ramifications for digestive and respiratory cancer diagnosis and prognosis and are in agreement with previous studies that showed expression of the 11p15 mucins when goblet cells reached terminal differentiation (Koo *et al.*, 1999; Blache *et al.*, 2004) or in differentiated tumours (Copin *et al.*, 2001; Sylvester *et al.*, 2001). As such, *MUC2* and *MUC5B* may be considered as markers of differentiated mucus-secreting cells and screening for methylation of key CpG sites identified in this report may serve as a useful diagnostic/prognostic tool to identify cancer cells undergoing dedifferentiation.

Interestingly, we also observed that inhibition of histone deacetylation might induce a decrease of both mucin gene and apomucin expression. Recently, Ferguson *et al.* (2003) have shown that occupancy levels of Sp1 and Sp3 factors on the promoter of *Hmga2*, a gene involved in transcription, replication and control of chromatin structure, decreased significantly following TSA treatment. As Sp1 is an important activator of the 11p15 mucin genes (Van Seuning *et al.*, 2001), the TSA-mediated inhibition of Sp1 DNA binding may explain the decrease of expression observed in this report. This hypothesis was confirmed by RT-PCR in which we found strong inhibition of Sp1 expression in TSA-treated cells (Supplementary data 2). Interestingly, we observed bigger changes in apomucin expression than can be seen at the mRNA level. This could be explained either by sensitivity of transcription factors known to regulate mucin genes to TSA treatment, which we found for Sp1 and c-fos (Supplementary data 2), or by post-transcriptional mechanisms (mRNA sequestration in the cell).

Search for relevant regions or CpG sites confirmed that cytosine -6 and -160 were methylated in *MUC2* non-expressing cancer cells. This result is in agreement with previous data performed in colonic and gastric cancer cells (Gratchev *et al.*, 2001; Mesquita *et al.*, 2003). Although in these studies, the authors focused on the nine CpG sites immediately upstream of the transcription start site of *MUC2* promoter, we also studied CpG dinucleotides situated in the distal region of the promoter allowing identification of four more methylated cytosines at -3269, -3199, -2331 and -1912. These results support the hypothesis of site-specific methylation in *MUC2* silencing. Our data also indicate that histone deacetylation plays an important role in silencing *MUC2* and most probably is under the dependence of methylation as it is known that methylation can control the methylation and/or acetylation

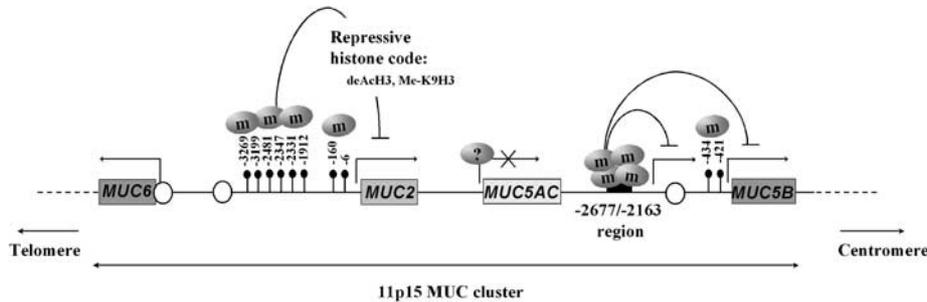


Figure 6 Schematic representation of the 11p15 mucin gene cluster regulation by epigenetics. Sense of transcription is indicated by the broken arrow situated at the transcriptional start site of each mucin gene. CpG islands are represented by open circles. Relevant methylated (m) CpG sites (black circles) or region (black square for *MUC5B*) implicated in the silencing are indicated. For *MUC2*, site-specific methylation is associated with the establishment of a repressive histone code. Silencing of *MUC5AC* is most probably due to indirect mechanisms or methylation of other regions. *MUC6* regulation is not influenced by epigenetics.

status of key lysines of the histone code (Fahrner *et al.*, 2002).

The methylation status of the two promoters of *MUC5B* was different. The distal promoter was heavily methylated throughout a 447-long nucleotide domain (−2615/−2168), whereas site-specific methylation of the proximal promoter was found at cytosines −434 and −421. We also found that the cytosines present in the CpG island were constitutively methylated regardless of *MUC5B* expression levels. Although significant differences were found in the histone acetylation status at *MUC5B* promoter in *MUC5B*-expressing versus *MUC5B* non-expressing cells, only slight changes were observed when we compared chromatin status in *MUC5B*-negative proliferating cells with *MUC5B*-positive CCs. Altogether these results suggest that, during dedifferentiation process, DNA methylation is the major epigenetic mechanism determining *MUC5B* repression before any modification of the chromatin status that would then lead to *MUC5B* complete silencing. This hypothesis is also supported by the fact that DNMT1 knockdown had a significantly more important effect on *MUC5B* endogenous expression when compared to its effect on *MUC2*.

Knocking down DNMT1 in combination with HDAC1, HDAC2 and HDAC3 indicated that among the class I HDACs, despite their high similarities at the protein level (de Ruijter *et al.*, 2003), only HDAC2 was able to potentiate the effect of DNMT1 on endogenous *MUC2* and *MUC5B* expression. It has already been shown that HDAC2 could serve specific function, particularly in the prevention of apoptosis during development of colonic cancer (Zhu *et al.*, 2004). This biological activity of HDAC2 could thus interfere with the protective function played by *MUC2* and *MUC5B* mucins as essential components of mucus.

Site-specific methylation of cytosines is known to impair regulation of target genes of Sp1, a transcription factor that recognizes GC-rich DNA elements and which is an important regulator of the 11p15 mucin genes (Van Seuning *et al.*, 2001). That inhibitory mechanism was confirmed in our studies for *MUC2* and *MUC5B* promoters in which we found site-specific methylation and inhibition of Sp1-mediated activation

of their promoters once methylated. These studies indicate that *MUC2* and *MUC5B* regulation by transcription factors known to interact with GC-rich DNA sequences, that represent potential methylation sites, will be altered whenever their promoters will be methylated.

Despite the presence of a CpG island, a high number of CpG sites throughout *MUC6* promoter and the identification of three key methylated cytosines at −282, −271 and −72, we were not able to reactivate the gene in any of the cancer cell lines studied. Moreover, expression of *MUC6* was sustained in LS174T cells even when the promoter was shown to be heavily methylated in these cells. Altogether these results indicate that repression of *MUC6* in cancer cells is rather because of the absence of the necessary transcription factors or by an unknown repressive mechanism than to the methylation of its promoter. Another explanation to the peculiar behaviour of *MUC6* promoter may be its early divergence from the progenitor gene giving rise to three other genes of the cluster, according to the evolution scheme we previously proposed (Desseyn *et al.*, 2000).

The promoter of *MUC5AC* is characterized by a lower number of CpG dinucleotides compared to the other genes of the cluster. Our studies on 1.3 kb of the promoter did not allow identification of specific methylated sites. The few methylated cytosines that we identified were either equally methylated in *MUC5AC*-expressing and *MUC5AC* non-expressing cells or more methylated in the *MUC5AC*-expressing cell line. The increase of *MUC5AC* expression in 5-aza-treated KATO-III PC may thus be the consequence of an indirect mechanism. This is in agreement with a previous study in which Ho *et al.* (2003) suggested that methylation of the CpG site at −152 in pancreatic cancer cells could not explain the silencing of *MUC5AC* and might involve the methylation of additional regions or other mechanisms.

In conclusion, this work demonstrates that, despite their genomic organization as a cluster and their localization in the close vicinity of an imprinted domain, hot spot of aberrant methylation in cancers, the 11p15 mucin genes possess a very specific pattern of methylation-dependent regulation. We observed a very high

correlation between the silencing of *MUC2* and *MUC5B*, promoter hypermethylation and establishment of repressive histone code. Moreover, in some cases, activation of *MUC2* and *MUC5B* expression during cell differentiation coincided with loss of methylation of their promoters. To our knowledge, this is the first study connecting promoter demethylation of *MUC2* and *MUC5B* and silent chromatin to cell differentiation. Finally, for these two genes we identified new key methylated cytosines that should provide useful tools, easy to detect, to screen mucin gene repression by methylation in epithelial cancers and identify tumours in which their repression may indicate good or poor prognosis.

Materials and methods

Cell lines and cell culture

The oesophageal (OE33), gastric (KATO-III), pancreatic (CAPAN-1, PANC-1) and colonic (LS174T, HT-29 STD, HT-29 5F7) epithelial cancer cell lines used in this study were cultured as described previously (Van Seuningen *et al.*, 2000; Perrais *et al.*, 2001a, b; Leteurtre *et al.*, 2004; Mariette *et al.*, 2004). The inhibitor of methylation, 5-aza (5 μ M) and the inhibitor of HDAC, TSA (0.3 μ M) (Sigma, Saint-Quentin Fallavier, France) were added to proliferating cells or CCs for 72 and 24 h, respectively. Cells were then lysed and processed for total RNA extraction or whole cellular extract preparation.

RNA extraction and RT-PCR

Total RNA was prepared using the QIAamp RNA blood and cell reaction kit (Qiagen, Courtaboeuf, France). Total RNA (1 μ g) was used to prepare cDNA using oligod(T) (1 μ l) and recombinant RT M-MLV (1 μ l) (Promega, Charbonnières, France). PCR was performed on cDNA (5 μ l), using specific pairs of primers for the 11p15 mucin genes (Perrais *et al.*, 2002). Single-stranded oligonucleotides were synthesized by MWG-Biotech (Germany). The ribosomal RNA 28S subunit was used as the internal control. PCR products (10 μ l) were separated on a 1.5% agarose gel containing ethidium bromide run in 1 \times TBE. The mucin/28S gene ratio was calculated after scanning DNA bands with GelAnalyst-GelSmart software (Claravision, Orsay, France).

Whole-cell extract preparation and western blotting

Preparation of total cellular extracts from proliferating LS174T cells, western blotting and apomucins and β -actin expression analysis were performed as described in Piessen *et al.* (2007). Specific antibodies were used as follows: monoclonal anti-human β -actin (1/1000, Sigma A5441), monoclonal anti-MUC2 (1/25, PMH1), monoclonal anti-MUC5AC (1/500, Novocastra 45M1, Le Perray, France), monoclonal anti-MUC5B (1/250, European consortium, BMH4-CT98-3222) (Rousseau *et al.*, 2003) and monoclonal anti-MUC6 (1/10, CLH5). Secondary antibodies consisted of alkaline phosphatase-conjugated IgGs (Promega, for β -actin) or horseradish peroxidase-conjugated goat anti-mouse IgGs (Pierce, for mucins). To detect MUC2, the nitrocellulose membrane was pretreated with neuraminidase (0.1 U, Sigma) in 0.1 M acetate buffer (pH 5.5) as described previously by Reis *et al.* (1998).

MS-PCR and sodium bisulfite-treated genomic DNA sequencing

Genomic DNA was prepared with the blood and cell culture DNA maxi kit (Qiagen). DNA content was quantified at 260 nm and stored at +4°C until use. Sodium bisulfite conversion was performed as described by Ghoshal *et al.* (2000). The promoter sequences of interest were amplified by PCR using AmpliTaq gold (Applied Biosystems, Courtaboeuf, France) as described previously (Ghoshal *et al.*, 2000). Primer sequences were designed using the MethPrimer software (<http://www.urogene.org/methprimer/>, Supplementary Tables). PCR products were separated on a 1.5% agarose gel containing ethidium bromide run in 1 \times TBE or cloned into pCR2.1 vector (Invitrogen). Positive clones were selected for plasmid preparation (QIaprep 8 Miniprep kit, Qiagen) and sequenced on both strands on an infra-red-based 4000L LICOR sequencer (ScienceTech, Les Ulis, France) using T7 and RM13 universal primers.

In vitro methylation of MUC2 and MUC5B promoters

DNA fragments were cut out of the pGL3 vector using *SacI* (*MUC2*, -2627/-1 and -371/+27), *KpnI-MluI* (*MUC5B*, -956/+57) and *MluI* (*MUC5B*, -2044/-1117) restriction enzymes. Fragments were gel-purified as in Perrais *et al.* (2001a) before being methylated with mSssI for 3 h at 37°C (New England Biolabs, OZYME, France). The degree of methylation of the fragment was confirmed by testing its resistance to *HpaII* digestion. The methylated fragments were then ligated into pGL3 basic vector. DNA concentration was measured at 260 nm before being used in transfection experiments as described in Perrais *et al.* (2001a). Influence of methylation on Sp1 transactivation of the promoters was studied by carrying out cotransfections in triplicate in three separate experiments in the presence of pCMV-Sp1 expression vector as described in Van Seuningen *et al.* (2000).

Chromatin immunoprecipitation assay

Cells (1.0×10^7) were fixed for 10 min at room temperature in 1% (v/v) formaldehyde and processed for ChIP analysis as described in Piessen *et al.* (2007). Specific antibodies (5 μ g) against histone H4 and H3 (anti-acetylated lysine, methylated lysine 9, mono/di/trimethylated lysine 4 and trimethylated lysine 27 were from Upstate and anti-acetylated lysines 9 and 14 from Diagenode. Immunoprecipitated chromatin (20 ng) was used as a template for PCR using the following primers: 5'-TTGGCATTACAGGCTACAGGG-3' and 5'-GGCTGGCAGGGGCGGTG-3', covering the -236/+24 region of *MUC2* promoter; 5'-TGACGGGGACTGTGACG-3' and 5'-CTTCCTGGGGGCTATGTG-3' covering the -1159/-861 region of *MUC5B* promoter. PCR was performed using AmpliTaq gold polymerase (Applied Biosystems). PCR products (15 μ l) were separated on a 2% agarose gel.

siRNA assays

HT-29 5F7 cell seeding (2×10^5 cells/well) and transfections were performed as described in Piessen *et al.* (2007) with 100 nM of DNMT1 ON-TARGETplus SMARTpool siRNA alone and in combination with either HDAC1, HDAC2 or HDAC3 ON-TARGETplus SMARTpool siRNA, using 1 μ l of DharmaFECT1 transfection reagent (Dharmacon, Brebières, France). Controls included mock-transfected cells, cells transfected with siCONTROL non-targeting siRNA or siCONTROL GAPD siRNA. Total RNA from two independent experiments in quadruplicate was isolated 48 h after transfection and RT-PCR was performed as described above.

Primers used for amplification of the internal control GAPDH were: 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The mucin/GAPDH gene ratio was calculated as described above.

Statistics

All values are means values \pm s.d. When indicated, data were analysed by Student's *t*-test using GraphPad Prism 4 software with differences $P \leq 0.05$ considered significant.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).