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## ORIGINAL PAPER

**A role for human MUC4 mucin gene, the ErbB2 ligand, as a target of TGF- $\beta$  in pancreatic carcinogenesis**Nicolas Jonckheere<sup>1</sup>, Michaël Perrais<sup>1</sup>, Christophe Mariette<sup>1</sup>, Surinder K Batra<sup>2</sup>, Jean-Pierre Aubert<sup>1</sup>, Pascal Pigny<sup>1</sup> and Isabelle Van Seuning<sup>\*.1</sup><sup>1</sup>Unité INSERM 560, Place de Verdun, 59045 Lille Cedex, France; <sup>2</sup>Department of Biochemistry and Molecular Biology, UNMCI Eppley Cancer Center, University of Nebraska Medical Center, 984525 Nebraska Medical Center, Omaha, NE, USA

**MUC4 encodes a large transmembrane mucin that is overexpressed in pancreatic adenocarcinomas. The molecular mechanisms responsible for that altered pattern of expression are unknown. TGF- $\beta$ , a pleiotropic cytokine, regulates numerous genes involved in pancreatic carcinogenesis via activation of the Smads proteins and MUC4 promoter is rich in Smad-binding elements. Our aim was to study whether the regulation of MUC4 expression by TGF- $\beta$  in pancreatic cancer cells was strictly dependent on Smad4 activity. Three pancreatic cancer cell lines, CAPAN-1 (MUC4+/Smad4-), CAPAN-2 (MUC4+/Smad4+) and PANC-1 (MUC4-/Smad4+), were used. By RT-PCR, transfection assays and immunohistochemistry, we show that (i) both MUC4 mRNA and apomucin expression are upregulated by TGF- $\beta$ , (ii) Smad2 positively cooperates with Smad4 to activate the promoter, (iii) activation of Smad4 by exogenous TGF- $\beta$  induces Smad4 binding to the promoter, (iv) Smad7 and c-ski both inhibit activation by Smad4. When Smad4 is mutated and inactive, TGF- $\beta$  activates MUC4 expression via MAPK, PI3K and PKA signaling pathways. Absence of expression in PANC-1 cells is due to histone deacetylation. Altogether, these results indicate that upregulation of MUC4 by TGF- $\beta$  is restricted to well-differentiated pancreatic cancer cells, and point out a novel mechanism for TGF- $\beta$  as a key molecule in targeting MUC4 overexpression in pancreatic adenocarcinomas.**

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**Keywords:** MUC4; pancreatic cancer; TGF- $\beta$ ; transcription; mucin; Smad4; acetylation

**Introduction**

MUC4 is a high molecular weight transmembrane mucin that is expressed by various epithelial cells (trachea, lung, stomach, colon and cervix) in normal tissues (Audié *et al.*, 1993). In the pancreas, MUC4 is not expressed in normal situation, whereas it is over-

expressed in pancreatic tumors (Balagué *et al.*, 1995; Swartz *et al.*, 2002; Park *et al.*, 2003). Lately, much attention has focused on MUC4 for it could represent a new therapeutic target in epithelial diseases as it is a transmembrane protein that was shown to interfere with tumor cell properties (Singh *et al.*, 2004) and participate in ErbB2 signaling (Carraway *et al.*, 2000). Altogether, these data establish a strong association between MUC4 overexpression and pancreatic carcinogenesis.

Pancreas cancer is one of the most deadly cancers in the world with a very low (5%) survival rate at 5 years (Huguier and Mason, 1999), and mucinous carcinomas of the pancreas are correlated with poor prognosis (Lichtenstein and Carr-Locke, 1995). Moreover, inability to detect cancer at an early stage and the lack of effective therapeutic treatment are largely responsible for the low survival of the patients (Hruban, 2001). It is thus mandatory to better understand gene regulation in pancreas cancer in order to identify new therapeutic targets and propose new treatments. Besides being overexpressed in human epithelial pancreatic carcinomas (Balagué *et al.*, 1995), MUC4 has been found highly expressed in several differentiated pancreatic tumor cell lines (Hollingsworth *et al.*, 1994; Choudhury *et al.*, 2000a,b; Perrais *et al.*, 2001). The dysregulation of MUC4 expression, often dramatic, together with the homology to ratMuc4, that is considered to promote tumor cell metastasis (Carraway *et al.*, 2000), points out an important role for MUC4 in human tumor biology and more particularly in pancreatic tumors (Kim *et al.*, 1999).

However, despite extensive data showing overexpression of MUC4 in pancreatic adenocarcinomas, the molecular mechanisms responsible for the upregulation of MUC4 mucin gene expression are still unknown. In order to understand the role and contribution of MUC4 altered expression during development and progression of pancreatic adenocarcinoma, we have recently characterized the 5'-flanking region of MUC4 and shown that, in pancreatic cancer cells, MUC4 is activated in a synergistic fashion by interferon- $\gamma$  and transforming growth factor- $\alpha$  or interferon- $\gamma$  and tumor necrosis factor- $\alpha$  (Perrais *et al.*, 2001). Moreover, the promoter sequence showed a high content in putative Smad-binding elements (SBE) (CAGAC), which could be in

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favor of a regulation by the transforming growth factor- $\beta$  (TGF- $\beta$ ), another growth factor/cytokine responsible for the dysregulation of numerous genes in cancer (de Caestecker *et al.*, 2000). TGF- $\beta$  possesses both tumor-suppressive and oncogenic activities (Akhurst and Derynck, 2001). After binding to its receptor, TGF- $\beta$  signals *via* activation of several pathways. The first identified was the Smad pathway (Massagué and Wotton, 2000), but lately it was shown that activation of MAPKs, PI3K (Roberts, 2002) or small GTPases (Derynck *et al.*, 2001) pathways could also mediate TGF- $\beta$  effects.

In this work, besides the Smad pathway, we focused our attention on p42<sup>MAPK</sup>, PKA, PKC and PI3K because we previously showed that they regulate *MUC4* expression (Perrais *et al.*, 2001; Mariette *et al.*, 2004). *MUC4* regulation by TGF- $\beta$  was studied in CAPAN-1 and CAPAN-2 cells that originate from two well-differentiated pancreatic carcinomas, and in PANC-1 cells that are undifferentiated (Sipos *et al.*, 2003). In these models, Smad4 is expressed in CAPAN-2 and PANC-1 cells, whereas it is mutated and inactive in CAPAN-1 cells (Schutte *et al.*, 1996). Our results indicate that TGF- $\beta$

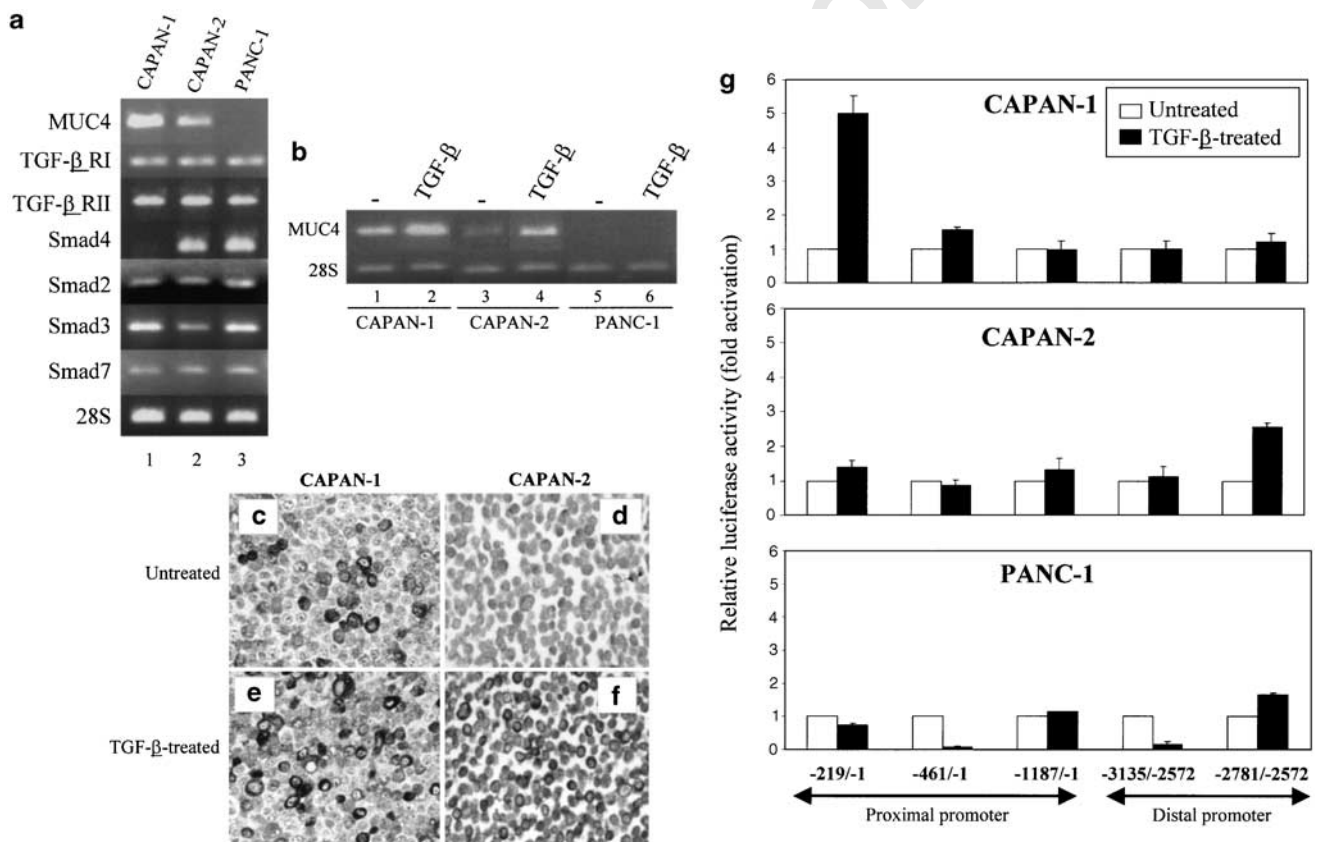
regulates *MUC4* at the transcriptional level via activation of the Smad pathway or by switching on MAPK, PI3K and PKA when the Smad pathway is inactive. Implications in pancreatic carcinogenesis are discussed.

## Results

### Expression of *MUC4* in the three pancreatic cancer cell lines and regulation by TGF- $\beta$

Expression of TGF- $\beta$ RII, TGF- $\beta$ RI, Smad2, Smad3, Smad4 and Smad7 is shown in Figure 1a. As expected, no Smad4 was found in CAPAN-1 cells that bear a mutated inactive form of Smad4 (Schutte *et al.*, 1996). All the other factors are expressed in the three cell lines. *MUC4* mRNA is strongly expressed in well-differentiated CAPAN-1 cancer cells (Figure 1a, lane 1). Its expression is lower in the well-differentiated pancreatic cancer cell line CAPAN-2 (lane 2). *MUC4* is not expressed in undifferentiated PANC-1 cells (lane 3).

To study the effect of TGF- $\beta$  on *MUC4* expression, cells were treated with TGF- $\beta$  and then processed for



**Figure 1** Effects of TGF- $\beta$  on *MUC4* mRNA and protein expression and on promoter activity in pancreatic cancer cells by RT-PCR, immunohistochemistry and transient transfection. (a) PCR products of the genes of interest were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide, CAPAN-1 (lane 1), CAPAN-2 (lane 2) and PANC-1 (lane 3). (b) *MUC4* (5  $\mu$ l) and 28S (2  $\mu$ l) PCR products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Untreated cells (lanes 1, 3, and 5), TGF- $\beta$ -treated cells (lanes 2, 4 and 6). (c–f) Immunostaining of CAPAN-1 (c and e) and CAPAN-2 (d and f) cells with *MUC4* monoclonal antibody. Untreated cells (*untreated*, c and d) and TGF- $\beta$ -treated cells (TGF- $\beta$ -treated, e and f). (g) Transfected cells were treated with TGF- $\beta$  (black bars) and processed as described in Materials and methods section. The values obtained in untreated cells were referred to as 1 (white bar). The results are means  $\pm$  s.d. and represent three separate experiments in triplicate for each construct

reverse transcriptase–polymerase chain reaction (RT–PCR), immunohistochemistry or transient transfection studies. As shown in Figure 1b, addition of exogenous TGF- $\beta$  to the cells led to a substantial increase of *MUC4* mRNA both in CAPAN-1 (lane 2) and CAPAN-2 (lane 4) cells. Increase of *MUC4* mRNA in CAPAN-1 and CAPAN-2 cells led to an increase in the protein expression, as shown by immunohistochemistry (Figure 1, compare (e) to (c) and (f) to (d), respectively). There was no induction of *MUC4* mRNA (Figure 1b, lane 6) or apomucin (not shown) expression in PANC-1 cells.

Identification of TGF- $\beta$ -responsive regions within *MUC4* promoters was studied by carrying out transfections in which transfected cells were treated with TGF- $\beta$  as above (Figure 1g). The five deletion mutants chosen were previously described and cover the proximal and distal promoters of *MUC4* (Perrais *et al.*, 2001). Five and eight putative SBE were found in the proximal and distal promoters, respectively. The luciferase diagrams indicate that TGF- $\beta$  transactivates the –219/–1 region of the proximal promoter (fivefold activation) in *MUC4*-expressing CAPAN-1 cells. In CAPAN-2 cells, the transactivation occurs on the distal promoter (–2781/–2572, 2.5-fold activation). In PANC-1 cells, there was inhibition of both the proximal (–461/–1) and distal (–3135/–2572) promoters. *MUC4* activation by TGF- $\beta$  was correlated to Smad4 translocation into the nucleus in CAPAN-2 cells as shown in Figure 2, whereas, as expected, no Smad4 translocation occurred in CAPAN-1 cells. In conclusion, these experiments indicate that activation of *MUC4* expression by TGF- $\beta$  (i) is correlated to Smad4 activation/translocation in CAPAN-2 cells and (ii) also occurs in Smad4-deficient CAPAN-1 cells (see Figure 1).

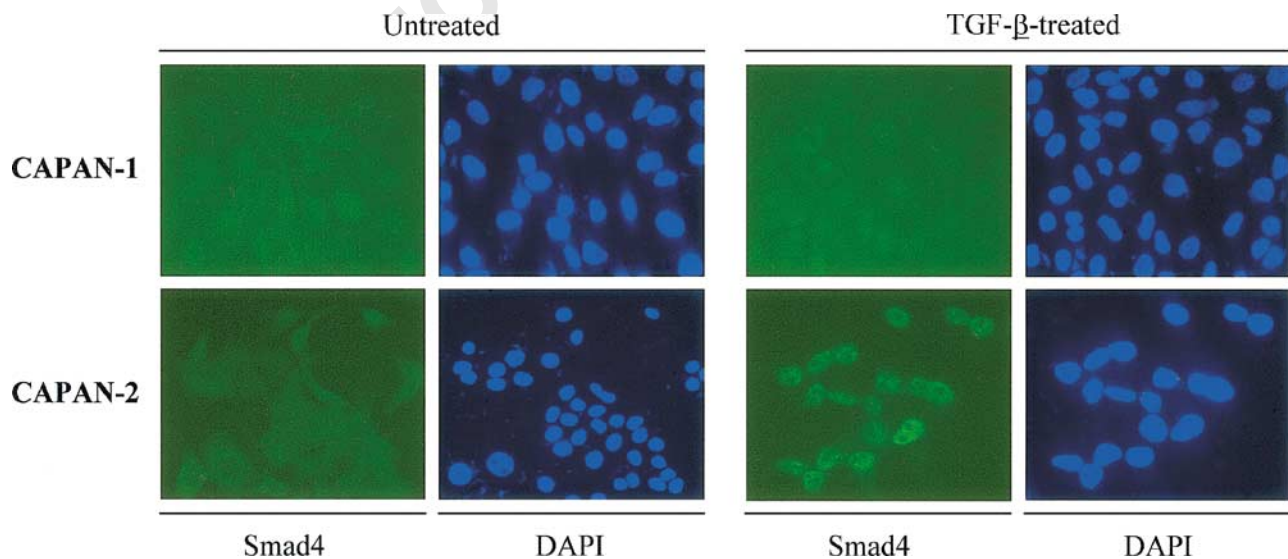
Activation of *MUC4* expression by TGF- $\beta$  in CAPAN-1 and CAPAN-2 cells occurs at the transcrip-

tional level since pretreatment of cells with actinomycin D led to a strong decrease in the amount of *MUC4* transcript (Figure 3a, lanes 4). This process does not require *de novo* protein synthesis since pretreatment of cells with cycloheximide did not modify the level of *MUC4* transcript (lanes 3). The absence of regulation of *MUC4* in PANC-1 undifferentiated cells led us to study the status of *MUC4* locus in these cells. Inhibition of methylation with 5-aza-2'-deoxycytidine did not lead to re-expression of *MUC4* (Figure 3b, lane 4). On the contrary, treatment with an inhibitor of histone deacetylation, trichostatin A, led to re-expression of *MUC4* (lane 2). From this result, we can conclude that *MUC4* is repressed in PANC-1 cells because of histone deacetylation. From then on, we focused our work on *MUC4* regulation by TGF- $\beta$  in CAPAN-1 and CAPAN-2 cells.

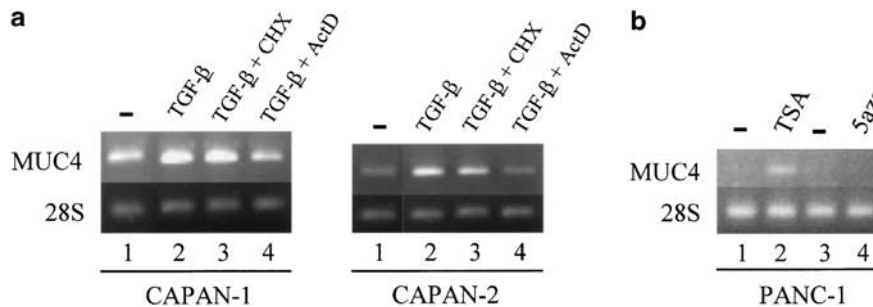
#### Regulation of *MUC4* expression by the Smad signaling pathway

Since Smad4 plays a pivotal role in mediating TGF- $\beta$  effects toward its target genes, we studied the effect of Smad4 on the expression of endogenous *MUC4* by transiently transfecting CAPAN-1 and CAPAN-2 cells with either empty pcDNA3.1. vector or a vector encoding Smad4 (pcDNA3.1-Smad4) (Figure 4a). RT–PCR analysis indicates that Smad4 overexpression in these cells leads to upregulation of *MUC4* expression (lanes 2 and 4).

Activation of Smad factors by TGF- $\beta$  leads to the formation of Smad2/Smad4 or Smad3/Smad4 complexes that translocate to the nucleus, where they activate transcription of the target gene. In order to characterize their role in *MUC4* regulation, we performed co-transfection experiments in the presence of expression vectors encoding either Smad2, Smad3 or



**Figure 2** Effect of TGF- $\beta$  on the translocation of Smad4 in CAPAN-1 and CAPAN-2 cells by immunofluorescence. Cells were treated with TGF- $\beta$  and processed for immunofluorescence as described in Materials and Methods section. Untreated cells (untreated) and TGF- $\beta$ -treated cells (TGF- $\beta$ -treated). Immunostaining with anti-Smad4 antibody (*Smad4*), DAPI nuclear staining (*DAPI*)



**Figure 3** Effect of cycloheximide and actinomycin D on *MUC4* expression in CAPAN-1 and CAPAN-2 cells. Influence of DNA methylation and histone deacetylation on *MUC4* expression in PANC-1 cells. (a) Cells were treated for 30 min with 20  $\mu$ g/ml of cycloheximide (CHX) or 1  $\mu$ g/ml of actinomycin D (ActD) before adding TGF- $\beta$  for 24 h. Cells were then lysed and processed for *MUC4* expression by RT-PCR as described in Materials and methods section. (b): Cells were treated for 24 h with 0.3  $\mu$ M trichostatin A (TSA) or for 72 h with 5  $\mu$ M 5-aza-2'-deoxycytidine (5-aza) before being processed for *MUC4* expression by RT-PCR as above. *MUC4* (10  $\mu$ l) and 28S (2  $\mu$ l) PCR products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide

Smad4, alone or in combination, along with pGL3-*MUC4* deletion mutants. The results indicate that Smad4 is able to transactivate *MUC4* promoters in a cell-specific manner (Figure 4b, black bars). In CAPAN-1, Smad4 overexpression led to a strong transactivation of the distal promoter (−3135/−2572 and −2781/−2572, 6- and 10-fold, respectively) and to a weaker activation of the proximal promoter (−219/−1, twofold). In CAPAN-2 cells, overexpression of Smad4 induced activation of both proximal (−219/−1, −461/−1, −1187/−1; six- to eightfold) and distal (−3135/−2572 and −2781/−2572, 10- and sixfold, respectively) promoters.

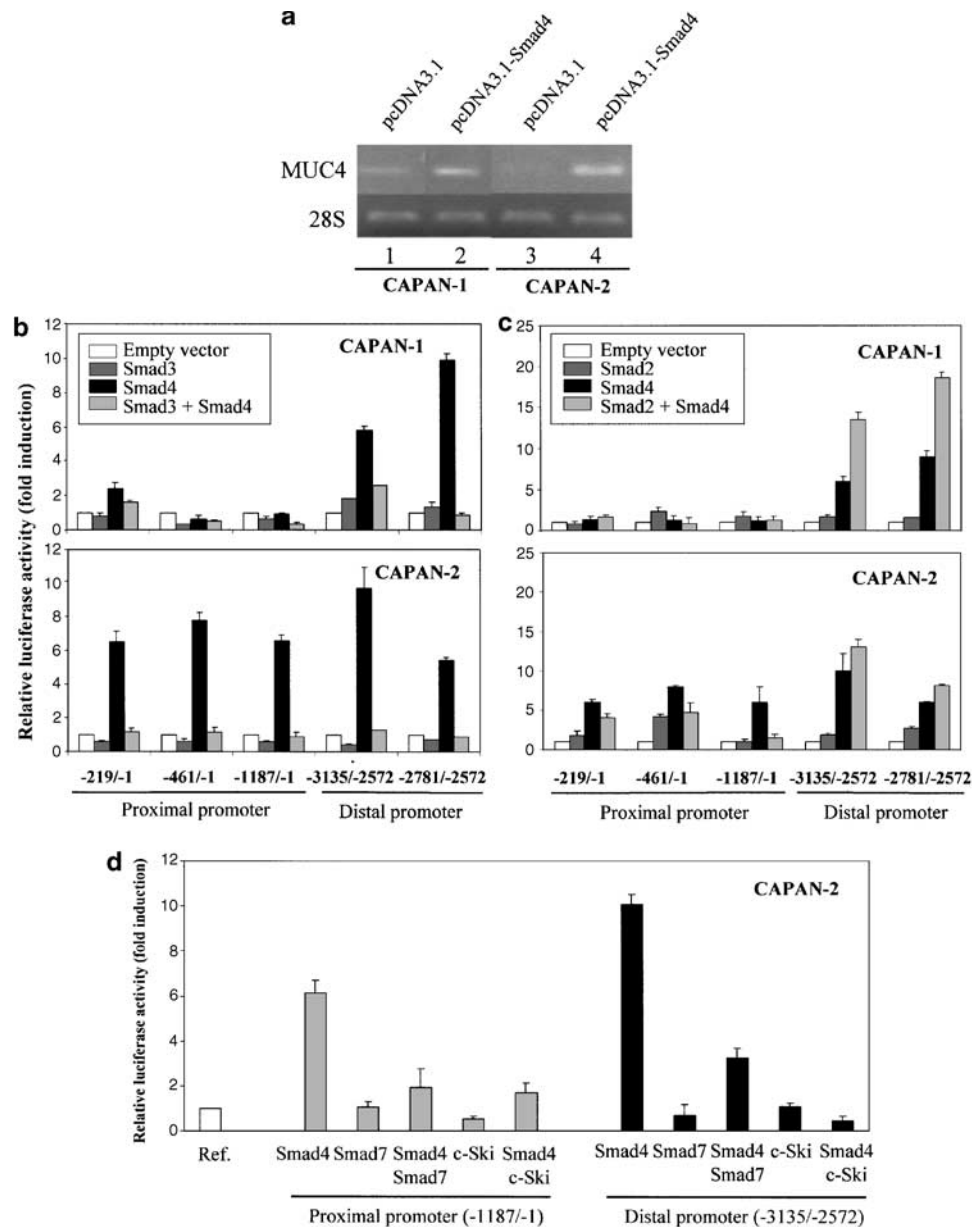
Overexpression of Smad3 did not induce any transactivation of *MUC4* promoters (Figure 4b, dark gray bars). It even showed inhibitory effect (50–80% inhibition) on the proximal promoter in both cell lines and on the distal promoter in CAPAN-2 cells (75% inhibition, −3135/−2572). When Smad3 was simultaneously co-transfected with Smad4, the transactivating effect of Smad4 previously seen in CAPAN-1 and CAPAN-2 cells was lost, indicating that Smad3 acts as a competitor (light gray bars).

Overexpression of Smad2 had no effect on *MUC4* promoter activity except for a fourfold activation of the −461/−1 proximal region and a 2.5–3-fold activation of the distal promoter (−2781/−2512) in CAPAN-2 cells (Figure 4c, dark gray bars). Simultaneous co-expression of Smad2 and Smad4 in CAPAN-1 cells led to a strong synergistic activation of the distal promoter (14–20-fold) (light gray bars). In CAPAN-2 cells, an additive effect was found on the distal promoter.

Smad signaling pathway is tightly controlled either by I-Smad (Smad7) or co-repressors like c-Ski (Miyazono *et al.*, 2003). Study of their effect on Smad4-mediated activation of *MUC4* promoters in CAPAN-2 cells is shown in Figure 4d. The results indicate that Smad7 and c-Ski completely inhibit the transactivating effect of Smad4 on both the proximal (gray bars) and distal (black bars) promoters.

#### Identification of Smad4 cis-elements within the promoters of *MUC4*

Computer analysis of *MUC4* promoters indicated that 13 putative SBE (CAGAC or GTCTG) were present. Eight of these sites are located in the distal promoter upstream of the TATA box and five in the proximal TATA-less region (Figure 5a). Oligonucleotides representative of these sites were synthesized, annealed, radiolabeled and used as probes in EMSA experiments performed with CAPAN-2 nuclear extracts (Figure 5b). As a positive control, an oligonucleotide containing a Smad3/4 consensus-binding site was used (lanes 1–5). With that probe, two shifted bands were observed (lane 2, arrows). A complete inhibition of the binding was produced when cold competition was performed (lane 3). Addition of anti-Smad2 antibody in the mixture did not modify the pattern (lane 4), whereas addition of anti-Smad4 led to an almost complete disappearance of the two shifted bands (lane 5). The same experiment was performed with T69 (lanes 6–10) and T78 (lanes 14–18) probes representative of SBE located in the distal and proximal promoters of *MUC4*, respectively (Figure 5a). In each case, one retarded band (lanes 7 and 15) that migrated to the same level as the upper band of the consensus Smad3/4 probe (lane 2) was obtained. The specificity of the binding was confirmed by cold competition that abolished the formation of the complex (lanes 8 and 16). Smad4 binding to T69 and T78 was lost when mutated oligonucleotides were used (lanes 12 and 20). Addition of Smad2 antibody to the mixture did not alter the pattern (lanes 9 and 17), whereas addition of anti-Smad4 antibody led to complete disappearance of the shifted band (lanes 10 and 18). Smad4 binding was also observed with proximal probes T72 and T89 and distal probes T70, T71, T87 and T88. No binding was seen with proximal T120 and T121 and distal T107 and T108 probes (not shown). Increase of Smad4 binding to T69 and T78 SBE was observed when cells were treated with TGF- $\beta$  (lane 23) when compared with nuclear extracts from untreated cells (lane 22). From these experiments, one can conclude that three proximal (−1498/−1494, −1310/−1306, −987/−983) and four



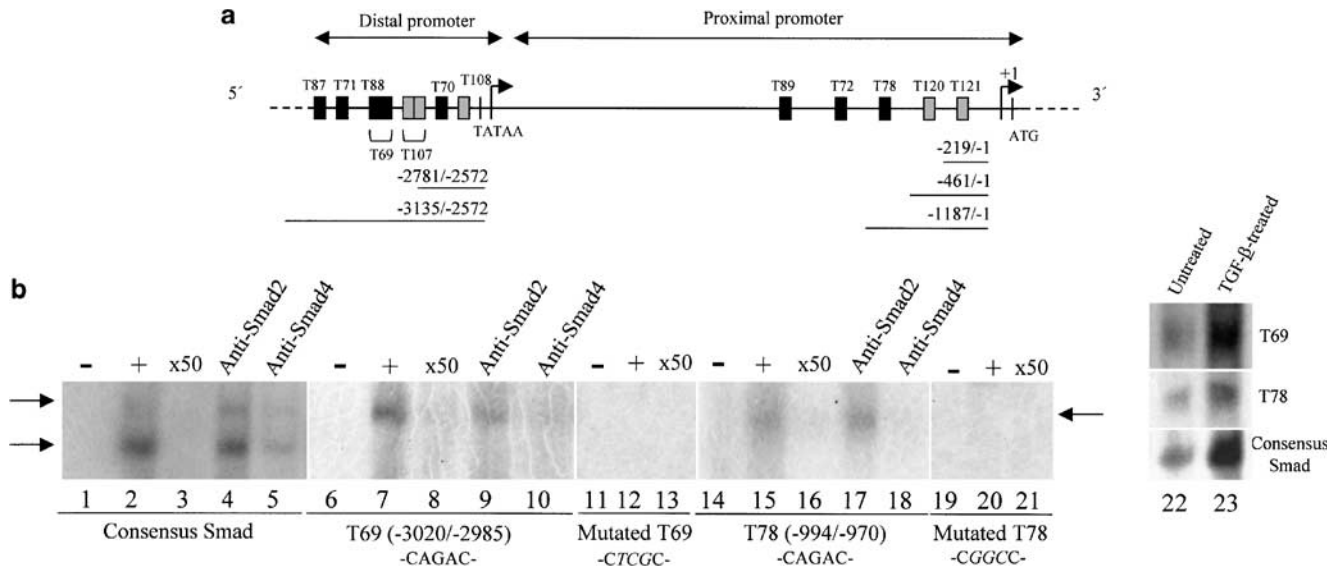
**Figure 4** Role of Smad4 in activation of *MUC4* expression and regulation of *MUC4* promoters by Smad2, Smad3, Smad4, Smad7 and c-Ski in CAPAN-1 and CAPAN-2 cells. (a) Cells were transfected with 2  $\mu$ g of the empty vector (*pcDNA3.1*) or the vector encoding Smad4 (*pcDNA3.1-Smad4*) as described in Materials and methods section. *MUC4* (5  $\mu$ l) and 28S (2  $\mu$ l) PCR products were, respectively, loaded on a 1.5% agarose gel containing ethidium bromide. (b) Effect of Smad3 (dark gray bars), Smad4 (black bars) or in combination (light gray bars). (c) Effect of Smad2 (dark gray bars), Smad4 (black bars) or in combination (light gray bars). (d) Effect of Smad7 and c-Ski on the Smad4-mediated transactivation of *MUC4* proximal (gray bars) and distal (black bars), respectively. The arbitrary unit of 1 corresponds to the luciferase activity obtained with the empty vector (white bars). The results are means  $\pm$  s.d. and represent three separate experiments in triplicate for each construct

distal (-3380/-3376, -3353/-3349, -3014/-3010, -2996/-2992) TGF- $\beta$ -responsive SBE bind Smad4 within the promoters of *MUC4*.

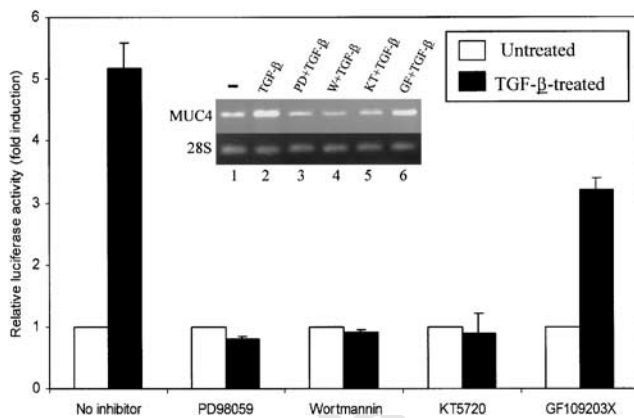
#### Regulation of *MUC4* expression by TGF- $\beta$ in Smad4-deficient CAPAN-1 cells

Since we observed activation of *MUC4* by TGF- $\beta$  in CAPAN-1 cells that do not express Smad4, we postulated that other signaling pathways may be

involved. We focused our attention onto pathways that were known to be both responsive to TGF- $\beta$  and activate *MUC4*, that is MAPK, PI3K, PKA and PKC (Perrais *et al.*, 2001; Mariette *et al.*, 2004). Inhibition of these pathways was performed by pretreating cells with specific pharmacological inhibitors before adding exogenous TGF- $\beta$ . The effect on *MUC4* endogenous mRNA expression was studied by RT-PCR (Figure 6, inset) and effect on promoter activity by transient transfection. RT-PCR results indicate that the increase



**Figure 5** Identification of Smad4 *cis*-elements within *MUC4* promoters by EMSA. (a) Cartoon representing *MUC4* promoters and location of the 13 putative SBE. Black boxes indicate true SBE identified by EMSA and gray boxes putative binding sites that did not show Smad4 binding. (b) CAPAN-2 nuclear proteins were incubated with Smad3/4 consensus (lanes 1–5), distal T69 (lanes 6–10), mutated T69 (lane 11–13), proximal T78 (lanes 14–18) and mutated T78 (lanes 19–21) radiolabeled probes. The DNA–protein complexes (+, lanes 2, 7, 12, 15, 20) were separated by electrophoresis on a 4% nondenaturing polyacrylamide gel. Probe alone (–, lanes 1, 6, 11, 14, 19), cold competitions ( $\times 50$ , lanes 3, 8, 13, 16, 21) and super-shift analyses with anti-Smad2 (lanes 4, 9, 17) and anti-Smad4 (lanes 5, 10, 18) antibodies. Smad4 binding with nuclear extracts from untreated cells (lane 22) and TGF- $\beta$ -treated cells (lane 23). Vacuum-dried gels were autoradiographed for 24 h at  $-80^{\circ}\text{C}$ . Arrows on each side of the autoradiograms indicate the position of the shifted bands



**Figure 6** Identification of the signaling pathways induced by TGF- $\beta$  to regulate *MUC4* mRNA expression and promoter activity in CAPAN-1 cells. Cells were transfected with the TGF- $\beta$ -responsive construct  $-219/-1$  and treated with pharmacological inhibitors as in the inset before adding exogenous TGF- $\beta$ . The arbitrary unit of 1 corresponds to the luciferase activity obtained in untreated cells (white bars). Black bars represent values in TGF- $\beta$ -treated cells with or without pharmacological inhibitor pretreatment as indicated. The results are means  $\pm$  s.d. and represent three separate experiments in triplicate for each inhibitor. (Inset): RT-PCR was performed as described in Materials and methods. Untreated cells (lane 1), TGF- $\beta$ -treated cells (lane 2). Cells were pretreated for 30 min with MAPK (PD, PD98059, lane 3), PI3K (W, wortmannin, lane 4), PKA (KT, KT5720, lane 5) and PKC (GF, GF109203X, lane 6) inhibitors before adding exogenous TGF- $\beta$ . *MUC4* (7  $\mu\text{l}$ ) and *28S* (2  $\mu\text{l}$ ) PCR products were loaded on a 1.5% agarose gel containing ethidium bromide

of *MUC4* mRNA levels in CAPAN-1 cells after TGF- $\beta$  treatment (lane 2) returned to basal level when cells were pretreated with PD98059 (MAPK inhibitor, lane 3),

KT5720 (PKA inhibitor, lane 4), or wortmannin (PI3K inhibitor, lane 5). Partial inhibition was observed when PKC inhibitor was used (GF109203X, lane 6). When we looked at the effect on *MUC4* promoter (construct  $-219/-1$ ), the same result was obtained. The strong transactivating effect of TGF- $\beta$  (no inhibitor) was lost when MAPK (PD), PI3K (W) and PKA (KT) signaling pathways were inhibited. Partial inhibition (30%) was obtained when PKC signaling pathway was inhibited (GF). In conclusion, these results indicate that, in Smad4-deficient CAPAN-1 cells, TGF- $\beta$  upregulates *MUC4* expression by activating MAPK, PI3K and PKA signaling cascades.

## Discussion

*MUC4* is a mucin gene that is not expressed in normal pancreas, whereas it is strongly overexpressed in pancreatic tumors (Balagué *et al.*, 1995; Swartz *et al.*, 2002; Park *et al.*, 2003). Lately, much attention has focused on *MUC4* for it could also represent a new therapeutic target in cancer as it is a transmembrane protein that was shown to interfere with tumor cell properties (Singh *et al.*, 2004) and participate in ErbB2 signaling (Carraway *et al.*, 2000). Altogether, these data establish a strong association between *MUC4* expression and pancreatic carcinogenesis. However, *MUC4* regulation at the transcriptional level remains largely unknown.

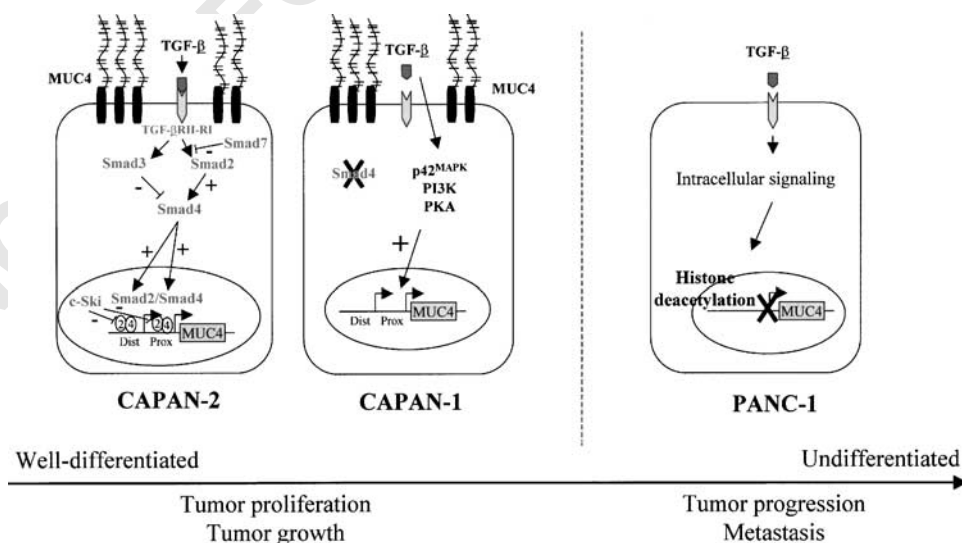
In this work, we show that *MUC4* is regulated by TGF- $\beta$  at the transcriptional level in pancreatic cancer

cells. We show that the regulation can be mediated via several signaling pathways and is not restricted to the activation of the Smad signaling cascade. This is particularly important with regard to pancreatic carcinogenesis in which inactivating mutations of Smad4 are often seen (55%), so that cancer cells escape regulation by TGF- $\beta$  (Schutte, 1999). TGF- $\beta$  indeed shows bifunctional mode of action on cancer cells. In the early phases of carcinogenesis it inhibits cellular growth, whereas in the late phases it participates in tumor progression, cell metastasis and dissemination (Akhurst and Derynck, 2001). TGF- $\beta$  signaling implies the activation of the Smad family of proteins that act both as signaling molecules and transcription factors, among which Smad4 plays a pivotal role as a co-factor of Smad2 and Smad3 (Massagué and Wotton, 2000; Ten Dijke *et al.*, 2000). In this work, we demonstrate the important role of Smad4 in *MUC4* regulation by TGF- $\beta$ , showing that activation of *MUC4* transcription implies cooperation between Smad2 and Smad4, whereas Smad3 acts as a competitor and that Smad7 and c-Ski are strong inhibitors of Smad4-mediated activation of *MUC4*. The activation of *MUC4* by the Smad factors is very efficient and is probably due to the high number of TGF- $\beta$ -responsive SBE identified within both promoters of *MUC4*.

Interestingly enough, when we looked at *MUC4* regulation by TGF- $\beta$  in Smad4-deficient CAPAN-1 cells, we could still observe upregulation of *MUC4* expression, which was in favor of the activation of other pathways. Recent data on other target genes of TGF- $\beta$  have shown that TGF- $\beta$  is able to transduce signal by activating other kinases such as MAPKs or PI3K (Roberts, 2002). By using specific pharmacological inhibitors of PKA, PKC, PI3K and MAPK, we show that TGF- $\beta$  is able to induce *MUC4* expression in CAPAN-1 cells via PKA, PI3K and MAPK, and to a lower extent via PKC. In CAPAN-1, TGF- $\beta$ -responsive

elements were located to the proximal part of the promoter. Since TGF- $\beta$  effect is mediated by PKA, PI3K, MAPK and PKC, one can hypothesize that responsive elements to those signaling pathways are present within this region. This correlates well with previous data in which we found PKA- and PKC-responsive elements within the proximal promoter of *MUC4* (Perrais *et al.*, 2001). In the previous work, we had identified responsive elements to EGF and TGF- $\alpha$ , two growth factors also involved in pancreatic carcinogenesis (Sakorafas *et al.*, 2000), in the -219/-1 region of *MUC4* proximal promoter. EGF signals through the MAPK pathway, so one can hypothesize that the same *cis*-elements are activated in response to either EGF (Perrais *et al.*, 2001) or TGF- $\beta$  (this report) depending on the cellular situation. In CAPAN-2 cells, TGF- $\beta$  responsive elements were located in the distal promoter. This indicates that the SBE identified in this region will play a major role in conveying Smad4-mediated *MUC4* activation by TGF- $\beta$  in these cells.

Our results indicate that *MUC4* can still be upregulated in pancreatic cancer cells that have a deficient Smad signaling pathway. Studies in patient tissues have shown that Smad4 mutations are correlated with histopathological grades (Wilentz *et al.*, 2000). Moreover, it was also shown that Smad4 is still expressed in low-grade pancreatic neoplasias, while its expression is lost in high-grade neoplasias. Thus, it was proposed that Smad4 mutations occur late in the progression of cancer after K-Ras mutation, p16 alteration and p53 genetic alteration (Miyaki and Kuroki, 2003). The fact that *MUC4* expression is still induced in pancreatic cancer cells despite Smad4 mutation is in favor of a role for *MUC4* in tumor growth. As depicted in Figure 7, TGF- $\beta$ -mediated overexpression of *MUC4* in well-differentiated pancreatic cancer cells will go through activation of Smad signaling pathway, when active, or compensated by switching on p42<sup>MAPK</sup>, PI3K and PKA when



**Figure 7** Schematic representation of the regulation of *MUC4* expression by TGF- $\beta$  in pancreatic cancer cells. Identification of the signaling pathways involved in relation with the degree of differentiation of the cells



Smad pathway is inactive. As a transmembrane mucin that protrudes over 2  $\mu$ m from the cell surface (Moniaux *et al.*, 2001) and as a ligand of ErbB2 oncogene (Carraway *et al.*, 2000), it is tempting to postulate that constant overexpression of MUC4 may interfere with biological properties of the pancreatic cancer cell, its ability to escape immune system or metastasize. Those important biological roles in tumorigenesis have already been described for another transmembrane mucin, MUC1, overexpressed by most carcinomas (Gendler, 2001; Hollingsworth and Swanson, 2004) and whose extracellular domain is not as long as MUC4 (Carraway *et al.*, 2000; Choudhury *et al.*, 2000a, b). MUC4 regulation by TGF- $\beta$  seems to be under the control of different regulatory mechanisms in pancreatic and mammary cancer cells. In pancreatic cancer cells, the regulation occurs at the transcriptional level (this report; Choudhury *et al.*, 2000a, b), whereas in mammary cancer cells it is due to post-translational mechanisms (Soto *et al.*, 2003).

Compared with CAPAN-1 and CAPAN-2 cells, PANC-1 cells are undifferentiated and are characterized by fewer cell organelles, specialized membrane structure and mucin granules (Sipos *et al.*, 2003). MUC4 is not expressed by PANC-1 cells and we were not able to induce its expression by addition of exogenous TGF- $\beta$  despite expression of TGF- $\beta$ RII, TGF- $\beta$ RI and Smads in these cells (Baldwin *et al.*, 1996; Schutte *et al.*, 1996). We thus looked at MUC4 status in PANC-1 cells and found that it is repressed by histone deacetylation. This is the first time that this epigenetic mechanism is described to repress a mucin gene. Grade 3 cell lines that are undifferentiated like PANC-1 cells are representative of a later stage in carcinogenesis, with a high metastasizing potential, and thus the absence of MUC4 expression/regulation in these cells is not in favor of a role for MUC4 in metastasis. We thus hypothesize that MUC4 overexpression in pancreatic cancer cells and its upregulation by TGF- $\beta$  independently of Smad4 functionality is in favor of a biological role for MUC4 in cancer proliferation and tumor growth.

## Materials and methods

### Cell culture

Pancreatic cancer cell lines CAPAN-1 and CAPAN-2 were a kind gift of Dr MA Hollingsworth (University of Nebraska Medical center, NE) and PANC-1 cells were purchased from the European Cell Culture Collection (ECACC). CAPAN-1 and CAPAN-2 cells were cultured in RPMI 1640 medium supplemented with 15 and 10% fetal calf serum (Roche diagnostics), respectively. PANC-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 2mM glutamine and 10% fetal calf serum. All cells were maintained in a 37°C incubator with 5% CO<sub>2</sub>. In TGF- $\beta$  studies, cells were treated with TGF- $\beta$  (10 ng/ml) for 24 h. In inhibition studies, pharmacological inhibitors were added to the cells for 30 min, prior to the addition of TGF- $\beta$ , at the following final concentrations: PD98059 (30  $\mu$ M, inhibitor of MAPK, Calbiochem), KT5720 (1 ng/ml, inhibitor of PKA, Calbiochem), GF109203X (10  $\mu$ M, inhibitor of PKC, Calbiochem) and wortmannin (2.5 nM, inhibitor of PI3K). To inhibit protein synthesis or transcription, cells were treated for 30 min with cycloheximide (20  $\mu$ g/ml) or actinomycin D (1  $\mu$ g/ml), respectively, before adding TGF- $\beta$ . DNA methylation and histone deacetylation were studied by treating cells with 5-aza-2'-deoxycytidine (5  $\mu$ M, 72 h) and trichostatin A (0.3  $\mu$ M, 24 h), respectively. All reagents were from Sigma unless otherwise indicated.

### RT-PCR

Total RNAs from pancreatic cancer cells were prepared using the RNeasy mini-kit from Qiagen. cDNAs and PCR were prepared and performed as previously described (Van Seuningen *et al.*, 2000; Mesquita *et al.*, 2003). Primer information is given in Table 1.

### Transient transfections

pGL3-MUC4 deletion mutant preparations and transient transfection experiments using Effectene<sup>®</sup> were performed as previously described (Perrais *et al.*, 2002). In TGF- $\beta$  experiments, relative luciferase activity was expressed as fold activation of luciferase activity in TGF- $\beta$ -treated cells compared with untreated cells. Each plasmid was assayed in triplicate in three separate experiments. In co-transfection

**Table 1** Sequence of the primers used to study MUC4, TGF- $\beta$  RI, TGF- $\beta$  RII, Smad2, Smad3, Smad4 and Smad7 expression in pancreatic cancer cells. 28S subunit was used as the internal control

Gene	Orientation	Sequences of primers (5' $\rightarrow$ 3')	T <sup>o</sup> m	PCR product size
MUC4	Sense	CGCGGTGGTGGAGGCGTTCTT	60°C	596 bp
	Antisense	GAAGAATCCTGACAGCCTTCA		
28S	Sense	GCAGGGCGAAGCAGAAGGAAACT	59°C	231 bp
	Antisense	TGAGATCGTTTCGGCCCCAA		
Smad2	Sense	GTCCATCTTGCCATTACG	55°C	192 bp
	Antisense	TGGTGATGGCTTTCTCAAGC		
Smad3	Sense	GGGCTCCCTCATGTCATCTA	60°C	443 bp
	Antisense	GGCTCGCAGTAGGTAACCTGG		
Smad4	Sense	CTCCTGAGTATTGGTGTTC	56°C	796 bp
	Antisense	CTAAAGGTTGTGGGTCTGC		
Smad7	Sense	TCCTGCTGTGCAAAGTGTTT	55°C	448 bp
	Antisense	TTGTTGTCCGAATTGAGCTG		
TGF- $\beta$ RI	Sense	CTCTCCTTTTTCTTCAGATCTGC	55°C	328 bp
	Antisense	AATCCAACCTCTTTGCCCTT		
TGF- $\beta$ RII	Sense	GCCAACAACATCAACCACAACACA	61°C	1003 bp
	Antisense	TAGTGTTTAGGGAGCCGTCTTCA		

studies, 1  $\mu$ g of the pGL3-MUC4 deletion mutant was transfected with 0.25  $\mu$ g of either the expression vector pCMV-Smad2, pcDNA3/Myc-Smad3, pcDNA3/HA-Smad4, pcDNA3-Smad7, pcDNA3-c-Ski, or the corresponding empty vector as the reference. Results were then expressed as fold induction of luciferase activity in cells transfected with expression vector encoding the transcription factor of interest compared with that obtained with the empty vector.

#### Nuclear extract preparation

Nuclear extracts were prepared as described in Van Seuingen *et al.* (1995), and kept at  $-80^{\circ}\text{C}$  until use. Protein content (2  $\mu$ l of nuclear extracts) was measured in 96-well plates using the bicinchoninic acid method as described in the manufacturer's instruction manual (Pierce).

#### Electrophoretic mobility shift assays (EMSAs)

EMSAs and supershift analyses were performed as previously described (Mesquita *et al.*, 2003). Identification of putative Smad4-binding sites was done using transfac 4.0 database (Quandt *et al.*, 1995), T69 SBE (TGACTTCA-GACTCCTGCCCTATGCAGACCCAGCAG,  $-3020/-2085$ ), T78 SBE (GTCTCCAGACTGTCCCTGGCTCC,  $-994/-970$ ), mutated T69 (TGACTTCTCGCTCCTGCCCTATGCTCGCCAGCAG) and mutated T78 (GTCTCCGGCCTGTCCCTGGCTCC). A commercial oligonucleotide containing the consensus-binding site for Smad3/4 transcription factors (Santacruz Laboratories) was used as a positive control. For supershift analyses, 1  $\mu$ l of anti-Smad4 (sc-7966) or anti-Smad2 (sc-6200) (Santacruz Laboratories) was used.

#### Immunohistochemistry

Immunohistochemistry on pancreatic cancer cells was performed as described before (Mariette *et al.*, 2004). The primary antibody directed against MUC4 (monoclonal) (Swartz *et al.*, 2002) was used at a 1:5000, 1:1000 and 1:1000 dilution in CAPAN-1, CAPAN-2 and PANC-1, respectively.

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#### Immunofluorescence

Cells were grown on a sterile glass coverslip (Lab-tek<sup>®</sup> chamber slide, Nalge Nunc) overnight at  $37^{\circ}\text{C}$  and washed once with PBS before being treated or not with TGF- $\beta$  as described above. Cells were fixed with 4% paraformaldehyde for 10 min, then washed three times for 5 min with PBS. Slides were incubated in PBS containing 0.1 M glycine for 10 min and washed three times for 5 min with PBS. Slides were then incubated in  $1 \times$  PBS containing 0.1% Triton X-100 for 10 min, washed three times, incubated in  $1 \times$  PBS containing 5% BSA for 20 min, then washed three times with PBS before adding the primary antibody diluted in PBS containing 1% BSA for 1 h. Anti-Smad4 antibody (sc-7966) was used at a 1:200 dilution. Slides were washed three times for 5 min with PBS. Fluorescein-conjugated antibody (Santacruz Laboratories) diluted in PBS containing 1% BSA was added at a 1:250 dilution and left for 45 min in the dark. Slides were washed three times for 5 min with PBS. Nuclear staining was performed using DAPI (1  $\mu$ g/ml, Sigma) for 5 min. Slides were washed three times for 5 min with PBS and mounted with Vectashield (Vector laboratories, Biovalley, France). Fluorescence was read on a LEICA DMRB microscope using the QFLUORO software (LEICA). Negative controls were run by omission of the primary antibody.

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