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ORIGINAL ARTICLE

Galectin-3 regulates MUC1 and EGFR cellular distribution and EGFR downstream pathways in pancreatic cancer cells

J Merlin[1,2], L Stechly[1,2,3], S de Beaucé[1,2,4], D Monté[5], E Leteurtre[1,2,4], I van Seuningen[1,2], G Huet[1,2,3] and P Pigny[1,2,3]

[1]*INSERM, U837, Equipe 5, 'Mucines, Différenciation et cancérogenèse épithéliales', Place de Verdun, Lille Cedex, France;* [2]*Université de Lille 2, Centre de Recherche Jean-Pierre Aubert, Place de Verdun, Lille Cedex, France;* [3]*Laboratoire de Biochimie & Hormonologie, Centre de Biologie & Pathologie, CHRU, Lille Cedex, France;* [4]*Service d'anatomie-pathologique, Centre de Biologie & Pathologie, CHRU, Lille Cedex, France and* [5]*Institut de Recherche Interdisciplinaire, USR3078 CNRS, Villeneuve d'Ascq, France*

MUC1 is a transmembrane glycoprotein which is typically expressed at the apical membrane of normal epithelial cells. In cancer cells, the over-expression of MUC1 and its aberrant localization around the cell membrane and in the cytoplasm favours its interaction with different protein partners such as epidermal growth factor receptor (EGFR) and can promote tumour proliferation through the activation of oncogenic signalling pathways. Our aims were to study the mechanisms inducing MUC1 cytoplasmic localization in pancreatic cancer cells, and to decipher their impact on EGFR cellular localization and activation. Our results showed that galectin-3, an endogenous lectin, is co-expressed with MUC1 in human pancreatic ductal adenocarcinoma, and that it favours the endocytosis of MUC1 and EGFR. Depletion of galectin-3 by RNA interference increased the interaction between MUC1 and EGFR, EGFR and ERK-1,2 phosphorylation, and translocation of EGFR to the nucleus. On the contrary, silencing of galectin-3 led to a decrease of cyclin-D1 levels and of cell proliferation. The galectin-3-dependent regulation of MUC1/EGFR functions may represent an interesting mechanism modulating the EGFR-stimulated cell growth of pancreatic cancer cells.
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Keywords: pancreatic cancer; galectin-3; MUC1; EGFR; intracellular localization; endocytosis

Introduction

MUC1 is a membrane-bound epithelial mucin (Hattrup and Gendler, 2008) composed of two subunits assembled by a non-covalent link, MUC1 N-terminal and MUC1 C-terminal (Ligtenberg *et al*., 1992). The MUC1 N-terminal subunit is a large extracellular 'mucin' subunit (>250 kDa) consisting of variable numbers of 20 amino-acid tandem repeats (TRs) on which are linked hundreds of *O*-glycans. Besides, five putative *N*-glycosylation sites are distributed on the whole MUC1 apomucin. The MUC1 C-terminal is a transmembrane subunit composed of a short extracellular domain, a transmembrane domain and a 72 amino-acid cytoplasmic tail (CT) that can interact with several protein partners (Singh and Hollingsworth, 2006). Indeed MUC1 CT interacts directly with p53 and regulates *CDKN1A* transcription (Wei *et al*., 2005), or with β catenin and co-activates the transcription of Wnt target genes such as *CCND1* (Huang *et al*., 2003). The extent to which MUC1 CT interacts with β-catenin is regulated by phosphorylation of SPYEKV motif upstream of the β-catenin binding site (Singh and Hollingsworth, 2006). For example, epidermal growth factor receptor (EGFR) phosphorylates this peptide motif and promotes the binding of MUC1 CT to c-Src and β catenin (Li *et al*., 2001). MUC1 CT also co-localized with β-catenin in the nucleus thus suggesting that it could be cleaved/released from the membrane and traffic from the membrane to the cytoplasm and nucleus (Wen *et al*., 2003).

In a wide range of normal epithelial cells, MUC1 is expressed at the apical side where it forms a protective barrier, senses environmental stress and relays the information to the cell interior. In several adenocarcinomas such as those of the breast, lung, kidney or pancreas, MUC1 is frequently over-expressed, underglycosylated and loses its exclusive apical localization (Leroy *et al*., 2002; Hollingsworth and Swanson, 2004). Indeed, in the pancreas, MUC1 is expressed by normal ductal cell but is gradually over-expressed from the early steps of carcinogenesis to pancreatic ductal adenocarcinoma (PDAC) (Maitra *et al*., 2003). Moreover, pathologists have shown a strong and diffuse membrane and cytoplasmic staining for MUC1 on human PDAC samples, using different antibodies (Monges *et al*., 1999; Nassar *et al*., 2004). In the clinical practice, the MUC1 cytoplasmic staining is used as an indicator of malignancy on cytology obtained by fine-needle aspiration of the pancreas (Wang *et al*., 2007). Interestingly, EGFR is also aberrantly expressed in pancreatic cancer from intraepithelial neoplasia lesions to PDAC and exhibits membrane and cytoplasmic staining (Ozaki *et al*., 2009). Previous reports demonstrated that MUC1 physically interacts with EGFR at the cell membrane, promotes its endocytosis and recycling, and inhibits

Correspondence: Professor P Pigny, INSERM U837, Team 5 Place de Verdun, Lille Cedex 59045, France.
E-mail: pascal.pigny@inserm.fr
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its degradation (Li *et al*., 2001; Schroeder *et al*., 2001; Pochampalli *et al*., 2007; Ramasamy *et al*., 2007). These data raise evidence for a connection between MUC1 and EGFR trafficking, and downstream signalling pathways.

However, the mechanisms that govern the intracellular MUC1 sequestration in epithelial cancer cells are currently unknown. Different hypothesis have been put forward such as the role of mucin glycosylation. The alterations of the *O*-glycan structure of MUC1 that occurred in cancer cells, that is, presence of truncated chains such as Tn (GalNAcα-) or TF (Galβ1,3GalNAcα-) antigen(s) (Hollingsworth and Swanson, 2004), can promote MUC1 endocytosis in a clathrin-dependent manner, and its intracellular sequestration (Altschuler *et al*., 2000). Interestingly, galectin-3, an endogenous lectin with one carbohydrate recognition domain showing binding specificity for β galactosides present in N- and O-linked glycans (Dumic *et al*., 2006) and encoded by *LGALS3*, was recently shown to interact directly with the *N*-glycoprotein EGFR (Partridge *et al*., 2004) or MUC1 (Yu *et al*., 2007). The latter requires the presence of unsialylated TF antigen on MUC1 and leads to a redistribution of MUC1 on the cell surface (Yu *et al*., 2007), exposure of adhesion molecules such as E-cadherin and increase of the survival of tumour cells (Zhao *et al*., 2010). Moreover, galectin-3 was recently shown to facilitate MUC1–EGFR interaction in response to EGF in a breast cancer model, but this report provided no insights on the downstream pathways (Ramasamy *et al*., 2007). In this context, we suggested that the abnormality of EGFR and/or MUC1 trafficking in epithelial cancer cells may imply an alteration of the function or/and expression of galectin-3. Indeed, a previous report shows that galectin-3 regulated the apical delivery of glycoproteins through a raft-independent pathway in Madin-Darby canine kidney (MDCK) cells (Delacour *et al*., 2007). Galectin-3 is widely expressed in epithelial tissues mainly as a cytosolic protein but it can also be found in the nucleus, mitochondria or secreted outside the cell (Dumic *et al*., 2006). In the pancreas, galectin-3 is faintly expressed in normal ductal cells but strongly in most PDAC (Berberat *et al*., 2001). Therefore, the aims of this work were to determine whether galectin-3 has a role in the trafficking of MUC1 and EGFR in pancreatic cancer cells and whether this lectin could affect the oncogenic signalling pathways downstream of MUC1 and EGFR.

Results

MUC1 and galectin-3 are expressed in human PDAC

MUC1 and galectin-3 expression were studied by immunohistochemistry on a series of 16 human PDAC. As expected, a strong MUC1 expression was observed in pancreatic cancer cells, together with an abnormal cellular localization, that is, delocalization both around the cell membrane and in the cytoplasm (Figure 1A). In contrast, normal ductal cells expressed lower levels of MUC1, mainly at the apex (inset a). Most of the pancreatic cancer cells also exhibited a strong cytoplasmic and nuclear galectin-3 immunostaining (Figure 1B). In contrast, normal ductal cells showed a weak galectin-3 immunostaining (inset b).

Silencing of galectin-3 is accompanied by a decrease of MUC1 and EGFR in CAPAN-1 cells

Three different small hairpin RNA (shRNA) (Sh1, Sh2 and Sh3) were subcloned into a pSuper.retro.puro vector, and pancreatic CAPAN-1 cells were stably transfected by retroviral infection. A scramble *sh*RNA (Sc) and an empty vector (Rs) were used as negative controls. *LGALS3* mRNA levels strongly decreased in galectin-3 knock-down (KD) Sh1 and Sh3 cells by comparison with control cells, whereas no apparent modification occurred in Sh2 cells (Figure 2a). Accordingly, at the protein level (Figure 2b), galectin-3 was highly decreased in Sh1 and Sh3 cells thus demonstrating the efficiency of the silencing in these two cell populations. Analysis of the transcriptome of Sh1 and Sc cells was carried out by micro-arrays on two independent RNA preparations. A ninefold decrease of *LGALS3* messenger was observed in Sh1 versus Sc control cells. No variation of the other galectins occurred in Sh1 cells thus demonstrating the specificity of the silencing (Supplementary Table S1).

Next, we evaluated whether galectin-3 silencing affected the levels of MUC1 and its membrane partner

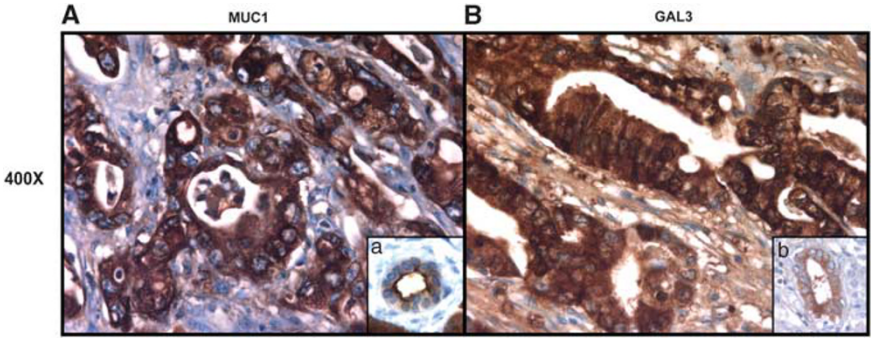

Figure 1 MUC1 and galectin-3 expression in human PDAC. (**A**) MUC1 immunostaining: most of pancreatic cancer cells exhibited a strong and diffuse cytoplasmic immunostaining. In contrast, normal pancreatic ductal cells (inset a) showed less intense staining that mainly concerns the cell apex. (**B**) Galectin-3 immunostaining: most of pancreatic cancer cells exhibited a strong cytoplasmic and nuclear galectin-3 immunostaining, whereas normal ductal cells showed a faint staining (inset b). Magnification ×400.

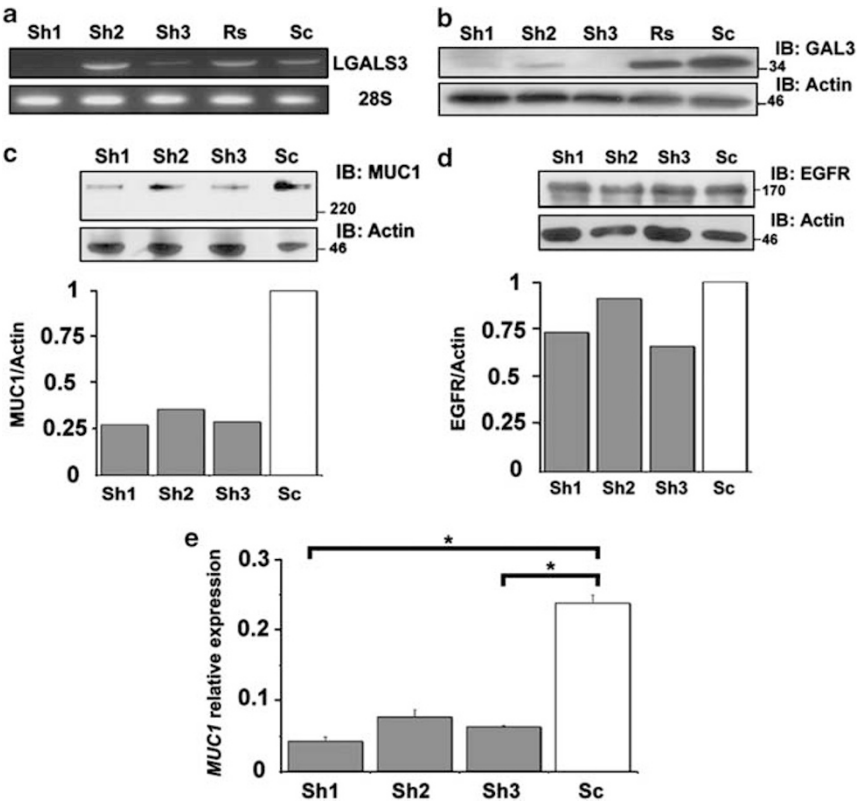

Figure 2 Characterization of galectin-3 KD cells. CAPAN-1 cells were stably transfected with a pSUPER.retro.puro vector containing either a shRNA directed against *LGALS3* (Sh1, Sh2 or Sh3) or a scramble shRNA with no homology to a human mRNA target (Sc) or an empty vector (Rs). (**a**) *LGALS3* mRNA levels were evaluated by semiquantitative reverse transcription–PCR using 28S as an internal control. (**b**–**d**) Whole cell lysates were prepared from subconfluent cells and proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), before being transferred onto nitrocellulose and immunoblotted (IB). β-actin was used as a loading control. Molecular masses of protein standard (kDa) are shown on the right. In all panels, results are representative of at least two independent experiments. (**b**) Representative analysis of galectin-3 protein levels. (**c**) Representative analysis of MUC1 protein levels. Densitometry analysis of MUC1 protein was normalized over β-actin levels in each sample (bottom). (**d**) Representative analysis of EGFR protein levels. Densitometry analysis of EGFR protein was normalized over β-actin levels in each sample (bottom). (**e**) *MUC1* mRNA levels in the different cell populations were measured by quantitative PCR and normalized against the amount of 18S messenger in each sample as described in Materials and methods. Graphs show mean ± s.e.m. of two independent experiments performed in triplicate. $*P = 0.05$ (Student's *t*-test).

EGFR. MUC1 protein levels were strongly decreased in the three Sh populations, by comparison with Sc cells (Figure 2c). *MUC1* mRNA levels assessed by quantitative reverse transcription–PCR were strongly decreased in the three cell populations by comparison with Sc cells (Figure 2e) thus demonstrating that galectin-3 positively controls *MUC1* expression at the transcriptional level in pancreatic cancer cells. In the same way, EGFR protein levels were slightly decreased in the three galectin-3 KD cells by comparison with Sc (Figure 2d). For the following experiments, Sh1 and Sc cells were selected as unique galectin-3 KD and control cell populations, respectively.

Influence of galectin-3 on MUC1–EGFR interaction and EGFR activation

As MUC1 physically interacts with EGFR at the cell membrane upon EGF treatment, we evaluated the role of galectin-3 in this process in pancreatic cancer cells. MUC1 and EGFR interaction was analysed by immunoprecipitation with an anti-MUC1 N-terminal antibody (see Supplementary Table S2). We observed that MUC1 interacted with EGFR only in EGF-treated cells and that galectin-3 silencing strongly increased the interaction (Figure 3a). Phosphorylation levels of EGFR following a 10-min EGF treatment (100 ng/ml) were determined. EGF treatment (Figure 3b) induced a $\approx$2-fold higher phosphorylation of the EGFR on Y_{1173} in Sh1 cells in comparison with the control Sc cells (mean of two separate experiments). As Y_{1173} serves as a docking site for adaptor proteins involved in the ERK activation, we evaluated ERK1,2 phosphorylation in the two cell populations in response to an EGF treatment (100 ng/ml EGF for 0–60 min; Figure 3c). In Sc cells, phosphoERK levels increased at 2 min to reach a ratio of 1.8, remained stable until 10 min and then declined to undetectable levels at 60 min. By contrast, in Sh1 cells the EGF-induced ERK phosphorylation was more intense with a maximal phosphoERK/ERK ratio of 3.8 at 5 min, and then declined to the basal values at 30 min.

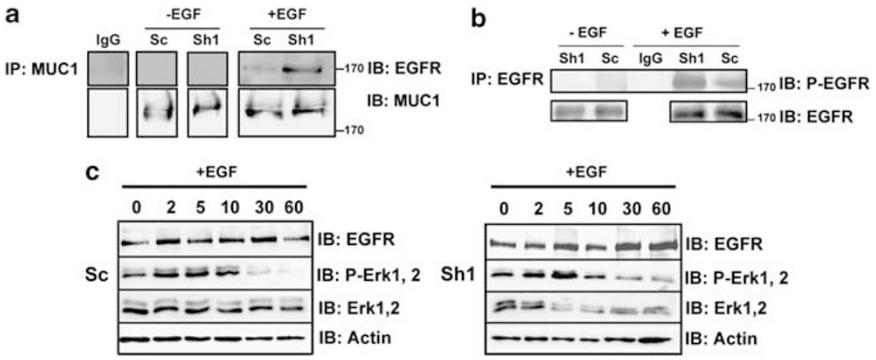

Figure 3 Influence of galectin-3 on MUC1–EGFR interaction and EGFR activation. Sh1 and Sc cells were serum-starved and treated or not with EGF (100 ng/ml) for 10 min. Whole cell protein lysates (300 or 150 μg) were used for immunoprecipitation (IP) with either an anti-MUC1 (**a**), anti-EGFR antibody (**b**) or irrelevant IgG as a control. Immunoprecipitates were separated by SDS–PAGE before being immunoblotted (IB) for EGFR, phosphoY_{1173} EGFR or MUC1 (m8 antibody). Molecular masses of the protein standards (kDa) are shown on the right side. White lines on the figures were used to indicate non-continuous samples run on the same gel. IP experiments were carried out once (set-up experiments excluded). (**c**) ERK1,2 activation in response to EGF treatment in Sc and Sh1 cells. Cells were serum-starved and either left untreated (0) or stimulated with 100 ng/ml EGF for 2, 5, 10, 30 or 60 min. Whole cell lysates were analysed by SDS–PAGE and IB with the indicated antibodies. A densitometry analysis of the bands and a Phospho ERK-1,2/ERK 1,2 ratio was calculated. Each blot is a representative example from two independent experiments.

Influence of galectin-3 on the subcellular localization of EGFR and MUC1

Besides its classical role as a membrane receptor that signals through the Ras/Raf/MAPK pathway, EGFR was also described in the nucleus of carcinoma cells where it regulates the transcription of several genes such as *CCND1* (Lin *et al*., 2001). Therefore, we evaluated whether galectin-3 could influence EGFR nuclear localization in pancreatic cancer cells. Nuclear and cytosolic extracts of Sc and Sh1 cells were prepared and EGFR expression levels were determined by western blotting (Figure 4a). Strikingly, EGFR levels were higher in the nucleus of Sh1 cells than in those of Sc cells both in absence (almost twofold) and in presence of EGF (almost 2.2-fold, mean of two independent experiments). As expected, EGF treatment promotes the nuclear translocation of EGFR in both clones. Of note, in Sc cells the EGF-induced EGFR translocation is accompanied by a decrease of nuclear galectin-3 level. By contrast, we observed that MUC1 CT expression did not vary between the Sh1 and Sc cells in absence or in presence of EGF (Figure 4a). In conclusion, the silencing of galectin-3 led to a marked increase of the nuclear translocation of EGFR in pancreatic cancer cells but did not affect that of MUC1 CT.

To evaluate whether galectin-3 directly modulates the nucleo-cytoplasmic shuttling of EGFR, we analysed galectin-3 and EGFR interaction by immunoprecipitation. We showed that EGFR physically interacts with galectin-3 in Sc cells and to a lesser extent in Sh1 cells that expressed residual levels of galectin-3 (Figure 4b). In conclusion, our results show that EGFR is an endogenous binding partner of galectin-3 in pancreatic cancer cells. Finally, we evaluated the levels of phosphoEGFR in the nucleus of Sh1 and Sc cells in response to EGF treatment. The phosphoEGFR/EGFR ratio was higher in EGF-treated Sh1 than Sc cells.

As MUC1 CT and galectin-3 in the nucleus promote cyclin-D1 expression through the accumulation and/or stabilization of nuclear β-catenin, respectively (Wen *et al*., 2003; Song *et al*., 2009), we studied the levels of cyclin D1 in total cell lysates before and after a 10-min EGF treatment (Figure 4c). Our results show that Sc cells expressed higher levels of cyclin D1 than Sh1 cells in absence or in presence of EGF. Moreover, Sc cells proliferate at a higher rate *in vitro* when compared with galectin-3 KD Sh1 cells (Figure 4d). MUC1 CT levels were also increased in Sc cells by comparison with Sh1 cells, in accordance with the results obtained for full-length MUC1 levels (Figure 2c). In conclusion, we confirm that variations of cyclin-D1 levels follow those of MUC1 CT as previously shown (Bitler *et al*., 2010) and demonstrate that in pancreatic cancer cells cyclin-D1 expression positively depends on galectin-3. Moreover, silencing of galectin-3 leads to a significant decrease of cell proliferation.

Silencing of galectin-3 inhibits MUC1 and EGFR endocytosis in pancreatic cancer cells

First, the analysis of Sc and Sh1 cells by confocal microscopy showed the presence of galectin-3 on the cell surface, in the cytoplasm and in the nucleus of Sc cells (Supplementary Figure S1). By contrast, the intensity of galectin-3 staining was very weak in Sh1 cells (Supplementary Figure S1) in agreement with western blotting data.

MUC1 localization was studied in both Sc and Sh1 cells using an antibody directed against the TR region. In Sc cells, MUC1 was expressed at the plasma membrane and in the cytoplasm (Figure 5A, panel a) but not in the nucleus (panel b), a pattern similar to that previously observed on human PDAC samples (Figure 1A). In galectin-3 KD cells, MUC1 was only expressed at the plasma membrane where it co-localized with cholera toxin-labelled GM1 ganglioside (Figure 5A, panel c).

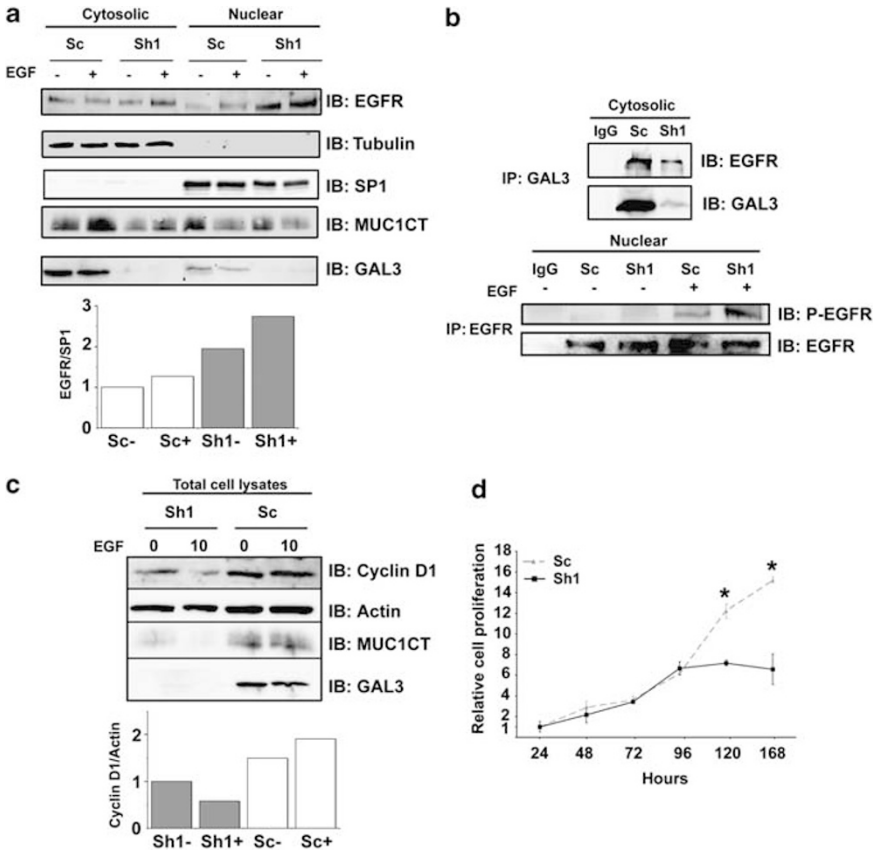

Figure 4 Galectin-3 interacts with EGFR and influences its nuclear localization. Sh1 and Sc cells were serum-starved, and then left untreated or treated with EGF (100 ng/ml) for 10 min. (**a**) Cytosolic and nuclear fractions were prepared and separated by SDS–PAGE and immunoblotted (IB) with anti-EGFR, anti-MUC1 CT or anti-galectin-3 antibodies. Fraction purity and loading were controlled by immunoblotting for Sp1 transcription factor (nucleus) or tubulin (cytosol). Each blot is a representative example from two independent experiments. Densitometry analysis of nuclear EGFR protein levels was normalized over Sp1 levels in each sample (bottom). (**b**) Cytosolic protein (300 μg) from cells grown in culture medium or nuclear protein (400 μg) from starved- or EGF-treated cells were used for immunoprecipitation (IP) with either an anti-galectin-3, anti-EGFR antibody or an irrelevant IgG (Ig). Immunoprecipitates were run on either an 8% (EGFR or phosphoEGFR) or 13% (galectin-3) SDS–PAGE before being IB with the indicated antibodies. IP experiments were carried out once (set-up experiments excluded). (**c**) Total cell lysates were prepared, resolved on SDS–PAGE and blotted with anti-cyclin D1, MUC1 CT or galectin-3 antibody, respectively. β actin was used as a loading control. A densitometry analysis of the bands was carried out as described in Materials and methods. Cyclin-D1 protein levels were normalized against actin levels in each sample (bottom). Results shown are representative of two independent experiments. (**d**) *In vitro* cell proliferation assay. Cells were seeded at 10^5 cells/well in 6-well plates and living cells were counted for 7 days using a cell counter (Invitrogen countess). Results are expressed as relative number of cells and normalized to the cell number at day 1 (mean ± s.d. of two independent experiments run in triplicate). $*P<0.05$.

Flow cytometry confirmed a strong cytoplasmic MUC1 expression in Sc cells, as compared with Sh1 (Supplementary Figure S2). In order to further address the role of galectin-3 in MUC1 endocytosis, and since extracellular galectin-3 is internalized (Furtak *et al*., 2001), Sh1 cells were incubated with recombinant galectin-3 (4 μM for 30 h) before being analysed by confocal microscopy. Results clearly showed that galectin-3 treatment induced the redistribution of MUC1 from the cell surface to the cytoplasm (Figure 5B, panel a). Thus our results suggest that galectin-3 promotes MUC1 endocytosis (Supplementary Figure S4) and is responsible for its intracellular retention in pancreatic cancer cells.

In parallel, we studied EGFR localization in EGF-starved or EGF-treated cells. In absence of EGF (Figure 6a), EGFR is mainly expressed at the cell membrane of Sh1 cells whereas in Sc cells, EGFR is found in the cytoplasm, the perinuclear region and the nucleus. EGF treatment in Sh1 cells induces EGFR endocytosis, thus explaining the cytoplasmic, perinuclear and nuclear punctuate staining observed with the anti-EGFR antibody (Figure 6b). In Sc cells, EGF treatment led to a diffuse nuclear EGFR labelling. In conclusion, galectin-3 induces EGFR endocytosis in absence of EGF treatment in pancreatic cancer cells. To further document the role of galectin-3 on MUC1 and EGFR endocytosis, double labelling experiments were carried out with the marker of early endosome EEA1. MUC1 showed a higher co-localization with EEA1 in Sc than in Sh1 cells (Supplementary Figure S3A, panel a versus b), in agreement with the galectin-3-mediated MUC1 endocytosis shown above (Figure 5). Next, we showed (Supplementary Figure S3B) that EGFR co-localized with EEA1 in untreated Sc cells (panel a) whereas co-localization occurred only in EGF-treated Sh1

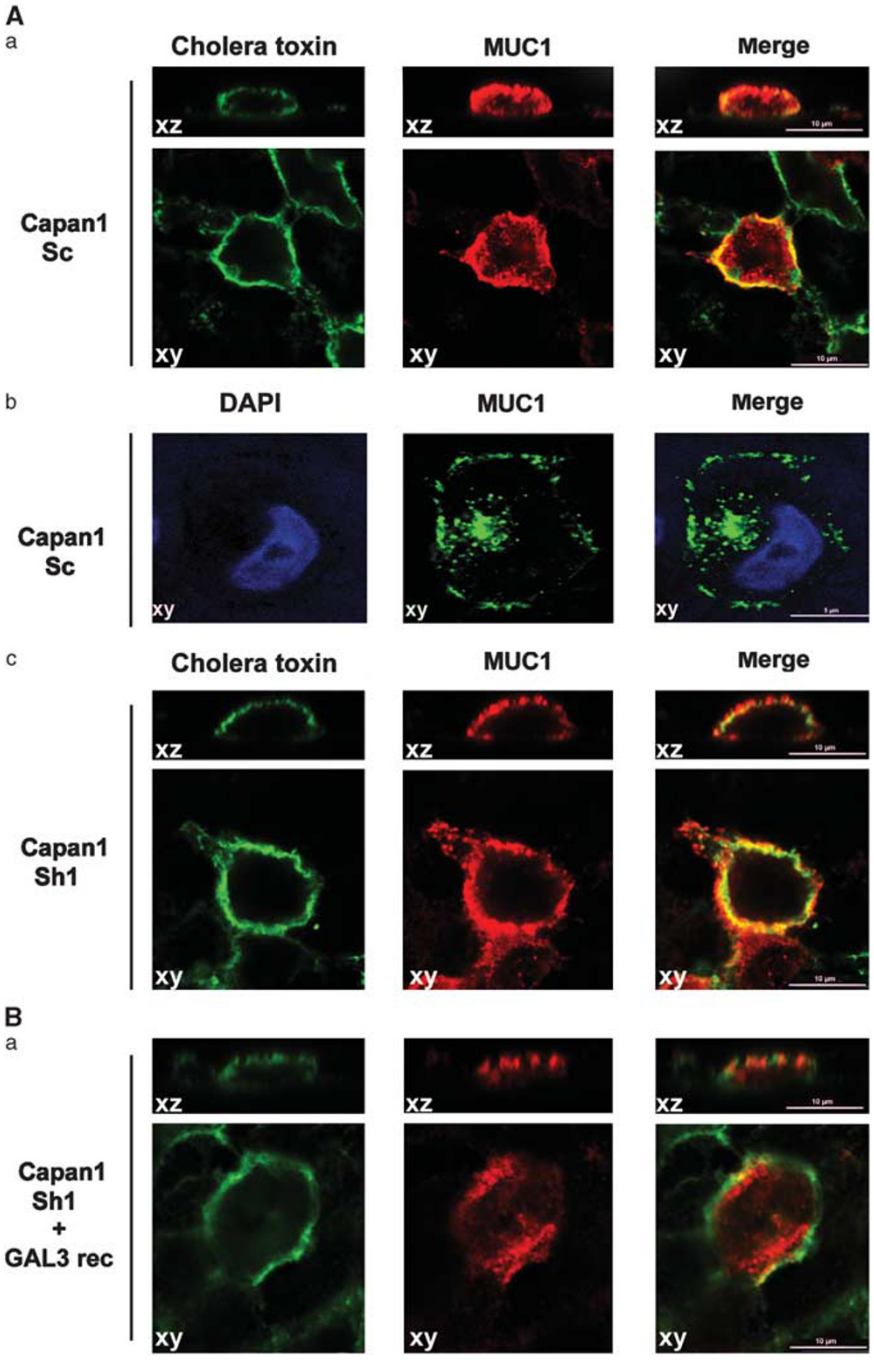

Figure 5 Galectin-3 induced MUC1 endocytosis in pancreatic cancer cells. Sh1 and Sc cells were fixed, permeabilized, immunostained and analysed by confocal microscopy, as described in Materials and methods. xy or xz sections were performed. Scale bar throughout this figure: 10 μm, except for **A**, panel b: 5 μm. (**A**) MUC1 expression (Texas Red, red) was evaluated in Sc (panels a and b) and Sh1 cells (panel c) using an antibody directed against MUC1 TR (N-terminal subunit). Nuclei are stained in blue (diamidino-2-phenylindole (DAPI)). Alexa-488 conjugated cholera toxin was used to stain the cell membrane (Fluoresceine IsoThioCyanate (FITC), green). Images were merged to study co-localization (in yellow colour). (**B**) Rescue experiment. Sh1 cells were left untreated (**A**, panel c) or treated with 4 μM recombinant galectin-3 for 30 h (**B**, panel a) before being stained for MUC1 (Texas Red, red). Alexa-488 conjugated cholera toxin (FITC, green).

cells (panel d). Finally, we studied MUC1 and EGFR co-localization. In Sh1 cells, MUC1 is co-localized with EGFR at the cell membrane in absence of EGF (Figure 7c). As previously shown, EGF treatment induced EGFR endocytosis and a strong decrease of its co-localization with MUC1 at the cell membrane (Figure 7d). In Sc cells (starved conditions), we confirmed that most of the EGFR is in the cytoplasm where it strongly co-localized with MUC1 (Figure 7a); EGF treatment seems to reinforce this co-localization (Figure 7b).

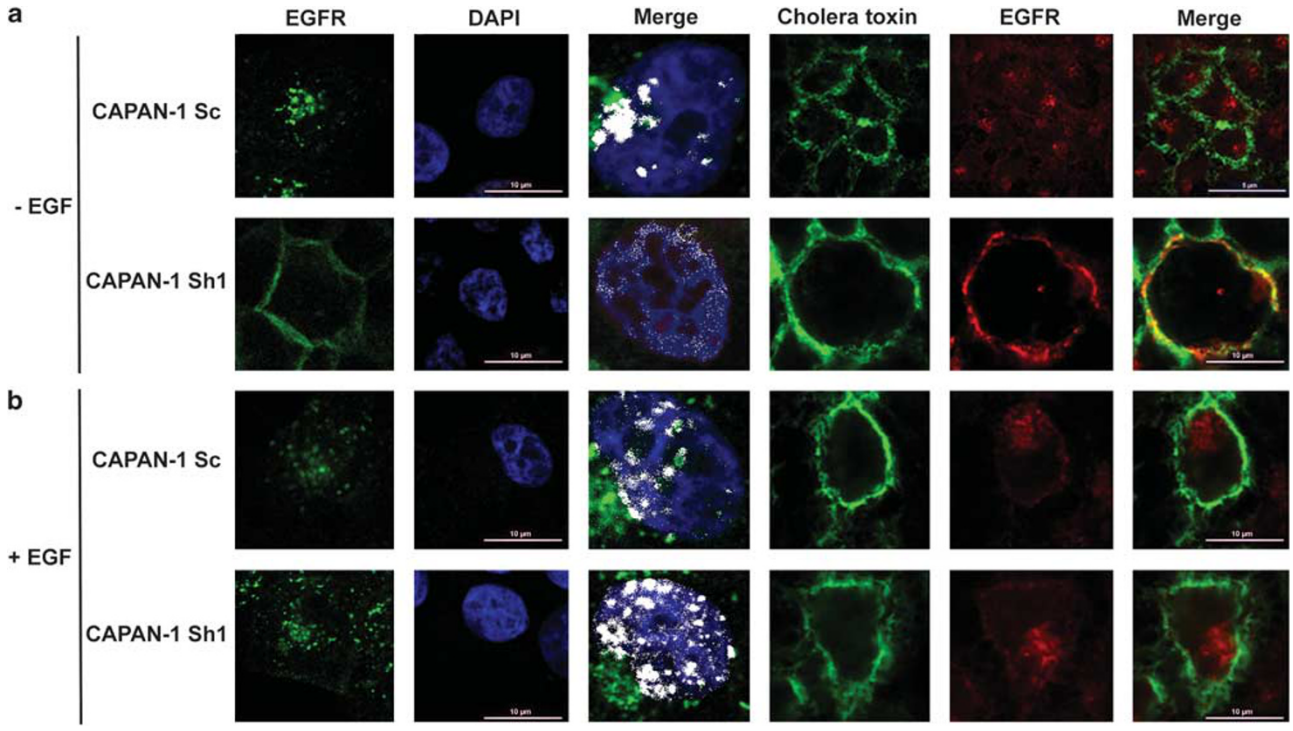

Figure 6 Galectin-3 controls both EGFR membrane and nuclear localization. Sh1 and Sc cells were serum-starved overnight and then left untreated (**a**) or stimulated with EGF (100 ng/ml for 10 min) when indicated (**b**). Cells were fixed, permeabilized and stained for EGFR (right panel: Texas Red, red; left panel: FITC, green) and/or with Alexa-488 conjugated cholera toxin (FITC, green). Nucleus is stained by diamidino-2-phenylindole (DAPI) (blue). Images were merged to study EGFR co-localization with the nucleus (in white colour) or cell membrane (in yellow colour), respectively. Scale bar throughout this figure: 10 μm, except for merged images of the nucleus.

Discussion

In normal epithelial cells, the physical interactions between the transmembrane mucin MUC1 and EGFR are prevented by their respective expression at the apical and basolateral side (Hattrup and Gendler, 2008). In contrast, in epithelial cancers MUC1 is often over-expressed and delocalized to the whole cell membrane and/or the cytoplasm thus allowing an interaction with EGFR. These interactions modulated the EGFR pathways at different levels, that is, inhibition of EGFR degradation, stimulation of EGFR recycling to the cell membrane and promotion of EGFR localization in the nucleus (Pochampalli *et al*., 2007; Bitler *et al*., 2010). Reciprocally, EGF-activated EGFR phosphorylates MUC1 CT on tyrosine, and facilitates its interaction with β-catenin (Singh and Hollingsworth, 2006). Of note, MUC1 induces the expression of galectin-3 which mediates the interaction between MUC1 and EGFR in breast cancer cell lines (Ramasamy *et al*., 2007). Galectin-3 is a cytosolic protein, but it can be translocated to the nucleus or externalized in the extracellular environment (Hughes, 1999; Nakahara *et al*., 2006), where it binds to polylactosamine glycans and core type 1 *O*-glycans. Altogether, these data suggested that interactions between MUC1, EGFR and galectin-3 had an important role in the regulation of MUC1 and EGFR trafficking, and activation of downstream pathways in epithelial cells.

In this study, we confirmed by using an anti-TR peptide antibody that in PDAC MUC1 is over-expressed and delocalized to the cell cytoplasm, and we demonstrated that galectin-3 is co-expressed with MUC1. However, since the influence of glycosylation on MUC1 immunodetection is not understood fully (Reddish *et al*., 1998; Reis *et al*., 1998), the possibility that changes of glycosylation may explain changes in MUC1 immunoreactivity could not be excluded. Anyway, our data strengthen the clinical relevance of studying the impact of galectin-3 on MUC1 localization. Our results showed that galectin-3 silencing led to a decrease of MUC1 expression, a re-localization of MUC1 to the plasma membrane and a complete disappearance of its intracellular distribution. Reciprocally, treatment of galectin-3 KD cells with recombinant galectin-3 triggered the endocytosis of cell surface MUC1, demonstrating that galectin-3 has a functional role in MUC1 internalization in pancreatic cancer cells. Whether there is a link between the decrease of MUC1 protein levels and the increase of its membrane expression in absence of galectin-3 remains an open question. Furthermore, silencing of galectin-3 strongly increased the expression of EGFR at the cell surface membrane.

As mentioned earlier, galectins can be externalized through non-conventional secretory pathway in the extracellular environment (Hughes, 1999), where they rapidly bind to cell surface glycoprotein or glycolipid ligands and are then endocytosed (Furtak *et al*., 2001;

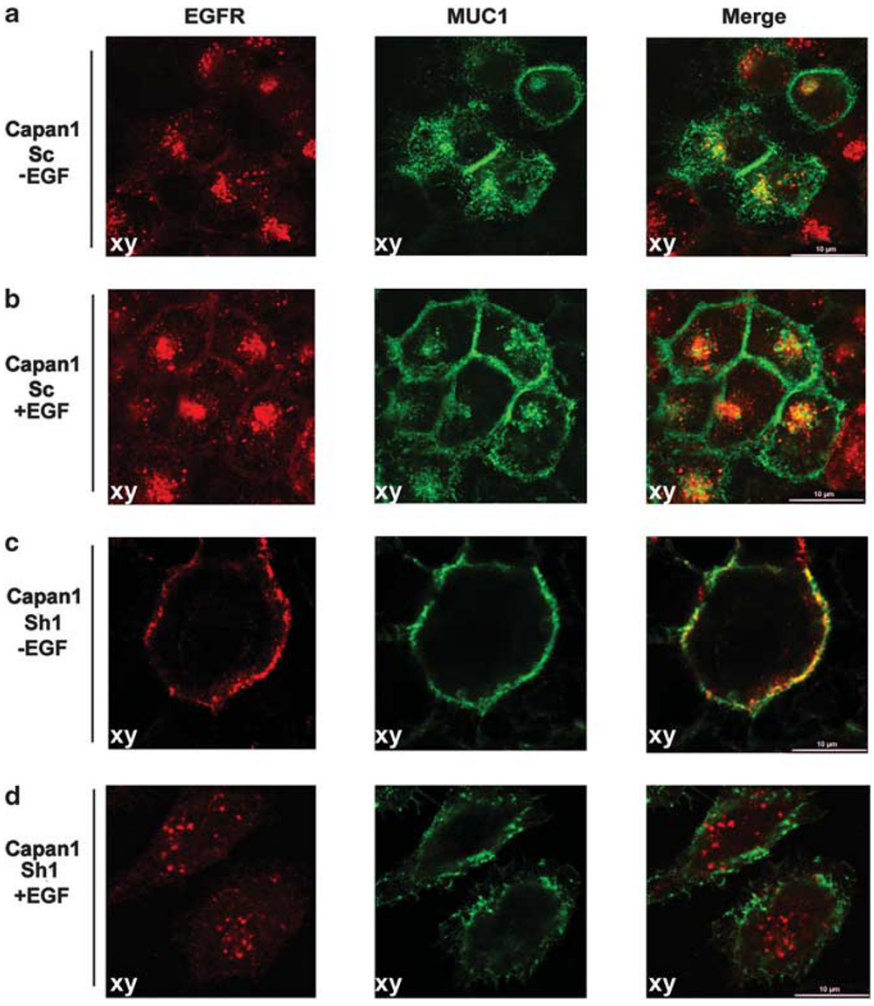

Figure 7 MUC1 and EGFR co-localization was studied in Sc and Sh1 cells in the absence (**a**, **c**) or presence of EGF (**b** and **d**, respectively) using anti-EGFR (Texas Red, red) and anti-MUC1 (FITC, green) antibodies. Images were merged to study co-localization (in yellow color). Scale bar: 10 μm.

Stechly *et al*., 2009). In the case of MUC1, galectin-3 can interact either with *O*-glycans branched on the TR domain (Yu *et al*., 2007) or with an *N*-glycan branched on Asn 36 of the C-terminal domain (Ramasamy *et al*., 2007). Our data suggest that galectin-3 could induce the endocytosis and intracellular accumulation of MUC1 and EGFR without stimulating their degradation. MUC1 is constitutively internalized and recycled via the trans-Golgi network (Litvinov and Hilkens, 1993). Inhibition of the *O*-glycosylation of MUC1 was shown to stimulate the endocytosis of MUC1 via clathrin-coated pits without enhancing its degradation (Altschuler *et al*., 2000). Therefore, the immature glycosylation of MUC1 in pancreatic cancer cells may favour its endocytosis via galectin-3 and its recycling to the cell surface.

In absence of stimulation, EGFR is constitutively internalized and recycled back to the cell surface (Resat *et al*., 2003), explaining the predominant membrane localization of EGFR in untreated Sh1 cells. In contrast, in case of EGF treatment, EGFR is internalized mostly through clathrin-mediated endocytosis and sorted to early endosomes. During the endosome maturation process, the EGF–EGFR complex is either recycled back to the cell membrane or sorted to the lysosomes for degradation (Sorkin and Goh, 2009). Our data suggest that galectin-3 may stimulate the endocytosis of EGFR in absence of EGF stimulation, and its sequestration in early endosomes and other(s) unidentified compartment(s), where it co-localized with MUC1. Whether EGFR and MUC1 are simultaneously internalized via galectin-3 or whether intracellular EGFR- and MUC1-enriched vesicles fused together is currently unknown.

Silencing of galectin-3 also increased EGF-induced EGFR phosphorylation and p42/44 ERK activation, in accordance to the higher cell surface expression of EGFR and MUC1 which has been shown to increase EGF-dependent signalling pathways (Schroeder *et al*., 2001). Actually, the implication of MUC1 in EGFR activation following silencing of galectin-3 is further reinforced by the marked increase in MUC1–EGFR interaction in EGF stimulated galectin-3 KD cells.

Moreover, galectin-3 was shown to bind to activated K-ras and to attenuate the ERK activation downstream of EGFR in cells expressing a *K-ras* G12V mutant (Elad-Sfadia *et al*., 2004). As CAPAN-1 cells expressed this mutant (Loukopoulos *et al*., 2004), we proposed that galectin-3 may affect the EGFR-MUC1-Ras-Raf-ERK pathway at different levels in pancreatic cancer cells by controlling either MUC1 and EGFR expression at the cell membrane, their interaction and/or K-ras activity.

Previous studies demonstrated that EGF treatment promotes the translocation of EGFR from the cell membrane to the perinuclear membrane and nucleus (Emlet *et al*., 1997; Hsu and Hung, 2007). This nuclear shuttling depends on a novel tripartite type of nuclear localization signal localized in the juxta-membrane domain of EGFR (Hsu and Hung, 2007). The EGFR-nuclear localization signal is recognized by importin α, that forms a complex with importin β1 to favour the nuclear EGFR import (Lo *et al*., 2006; Wang *et al*., 2010). Moreover, MUC1 promotes the nuclear accumulation of EGFR in breast cancer cells and its interaction with DNA (Bitler *et al*., 2010). Here, we showed that EGF treatment promotes EGFR internalization and subsequent translocation to the perinuclear membrane and nucleus in pancreatic cancer cells. Moreover, galectin-3 silencing increases the EGFR nuclear translocation and phosphorylation. Interestingly, galectin-3 shuttles between the cytoplasm and the nucleus (Davidson *et al*., 2006) and its nuclear import also involved the importin α/β1 complex (Nakahara *et al*., 2006). Here, we provide evidence that EGFR is a new endogenous glycoprotein partner of galectin-3 in pancreatic cancer cells, and that the nuclear content of EGFR negatively depends on galectin-3 presence. The mechanisms by which galectin-3 controls EGFR shuttling between cytoplasm and nucleus remain unknown. Our data suggest that galectin-3 directly binds to EGFR to sequestrate it in the cytoplasm and prevents its nuclear shuttling but other (s) mechanism(s) are not excluded. Finally, we showed, in agreement with previous data (Song *et al*., 2009), that cyclin D1 is upregulated in galectin-3-expressing cells and that its levels are correlated with total MUC1 CT levels as recently described (Bitler *et al*., 2010). Cyclin-D1 upregulation in Sc control cells may contribute to their higher proliferative rate *in vitro*.

In conclusion, we provide strong evidence that galectin-3 regulates MUC1 and EGFR internalization and subcellular localization, ERK1,2 activation downstream of the EGFR and EGFR nuclear translocation in pancreatic cancer cells. Clearly, elucidation of the mechanisms by which galectin-3 controls EGFR and MUC1 internalization and intracellular trafficking requires further analysis.

Materials and methods

Cell culture, RNA interference and transfection experiments
CAPAN-1 cells were cultured as described before by Perrais *et al*. (2001). Three shRNA targeting *LGALS3* (target sequences (1) 5′-GAAGAAAGACAGTCGGTTT-3′ (Henderson *et al*., 2006); (2) 5′-GAGAGTCATTGTTTGCAAT-3′; (3) 5′-TACAGATATCAACCTACCT-3′ (Peng *et al*., 2008)) were cloned into the pSUPER.retro.puro vector system (Oligo Engine, Seattle, WA). A scrambled sequence (5′-AAGTC AATCAACACGGTAACA-3′) with no similarity with any other human mRNA was used as negative control. CAPAN-1 were transfected by retroviruses-encoding *sh*RNA. Selection of stably transfected CAPAN-1 cells was carried out by treatment with puromycin (9 μM).

Immunohistochemistry
Immunohistochemical studies were conducted on formalin-fixed, paraffin-embedded tissues sections (5 μm) using an automated immunostainer (Benchmark.XT, Ventana, Strasbourg, France). Primary antibodies used were diluted as follows: 214 D4 (1/50) and galectin-3 (1/100). Following deparaffinization, cell-conditioning solution was used as a pre-treatment for 30 min for MUC1 and 8 min for galectin-3. The sections were incubated for 32 min with each primary antibody. Revelation used the *ultra*VIEW Universal DAB Detection Kit technology (Ventana Medical System, Illkirch, France). Slides were counterstained with haematoxylin. Positive controls (pancreatic ductal acinar cancer for MUC1 and thyroid adenoma for galectin-3) and negative controls (slides without the primary antibody) were added for each automated immunohistochemical run. Tissue sections were evaluated by two pathologists in a blinded manner. Subcellular localization, percentage of positively stained cells and degree of reactivity were assessed.

Semiquantitative reverse transcription–PCR
Total RNA were prepared 24 h after cell confluence using the Nucleospin RNA II kit (Macherey Nagel, Hoerdt, France). cDNA was synthesized as described in Perrais *et al*. (2001). *LGALS3* cDNA was amplified as previously described (Lahm *et al*., 2000). 28S was used as an internal control. PCR products were analysed on a 1.5% agarose gel. A densitometry analysis of the bands was performed using the Gel Smart-Gel analysis software (Claravision, Orsay, France).

MUC1 mRNA quantification by qPCR
Absolute quantification of *MUC1* was carried out as previously described (Leroy *et al*., 2003) using a standard curve prepared from a dilution series of a pCEP.4 plasmid encoding a mini-MUC1 construct (MUC1 M1 generously given by Dr F Hanisch). For *MUC1*, the specific primers (target size: 104 bp) and probe were as follows: MUC1, forward primer: 5′-CAGACGTCAGCGTGAGTGATG-3′; MUC1, reverse primer: 5′-CTGACAGACAGCCAAGGCA AT-3′; MUC1 probe (FAM Tamra): 5′-TGCTGGTCTGTG TTCTGGTTGCGCT-3′. 18S was used as an internal standard and was co-amplified with the target in the same PCR. The cycle threshold values of all samples were measured with the Taqman Applied Biosystems ABI 7900 (Applied Biosystems Division, Villebon sur Yvette, France) and were transformed to nanogram of the target gene and 18S. A ratio between the nanogram of the target gene and nanogram of 18S internal control was calculated for each sample. Each sample was run in triplicate.

Whole cell lysates preparation and western blotting
Whole cell extract preparation and western blotting were performed as previously described (Jonckheere *et al*., 2009). Membranes were probed with specific primaries antibodies

as detailed in Supplementary Table S1. Secondary antibodies consisted of horseradish peroxidase-conjugated IgGs (Pierce, Perbio Sciences, Brebières, France). For antigen detection, blots were processed with West Pico chemiluminescent substrate (Perbio Sciences) and the signal was detected using the Fujifilm Las4000 analyzer (Fujifilm Medical Systems, Courbevoie, France).

Nuclear and cytosolic fractions
Nuclear and cytosolic extracts were prepared according to a previously published protocol by Dignam *et al*. (1983) which was modified by Van Seuningen *et al*. (1995). Briefly, cell membranes were lysed using a lysis buffer containing 0.1% NP-40 in order to retrieve the cytosolic proteins after centrifugation. The nuclear pellet was resuspended, nuclear membranes were lysed and proteins were extracted using a specific buffer. Proteins were stored at −70 °C.

Immunoprecipitation
Technical set-up experiments were required to define the appropriate amount of proteins to be immunoprecipitated. In total, 150 or 300 μg of whole cell proteins was immunoprecipitated for 12 h at 4 °C with a 1 μg of an anti-EGFR or an anti-MUC1 antibody (see Supplementary Table S1), respectively, and EZ view Red protein A Affinity Gel (Sigma, St Quentin Fallavier, France), according to the manufacturer's protocol. In case of nuclear extracts, 400 μg proteins were used. Irrelevant rabbit or mouse IgG was used as a negative control. Western blotting was performed as described in Jonckheere *et al*. (2009). The membrane was probed with an anti-phospho-EGFR, anti-EGFR or anti-MUC1 (m8) antibody. For galectin-3 immunoprecipitation, 300 μg of cytosolic proteins was analysed by the same protocol using 2 μg of a rabbit polyclonal antibody.

Confocal microscopy
Confocal microscopy was performed on Sc or Sh1 cell monolayers grown on Lab-Tek Chamber Slide permanox (Nunc, Brumath, France). For EGFR staining, cells were either serum-starved overnight (BSA 0.01%) or left untreated or treated with EGF (100 ng/ml, 10 min). The cells were fixed with 3% paraformaldehyde at 4 °C, quenched for 20 min with 50 mM NH_4Cl in D-PBS + Mg^{2+} + Ca^{2+} (Gibco, Cergy-Pontoise, France) and permeabilized with 0.2% saponin in D-PBS + Mg^{2+} + Ca^{2+} for 20 min. The saturation step was performed for 20 min with D-PBS + Mg^{2+} + Ca^{2+} containing 1% BSA and 0.2% saponin. For single labelling, cells were incubated overnight with specific antibodies whereas for double labelling each antibody was incubated sequentially during 90 min. The following antibodies were used: anti-galectin-3 (rabbit polyclonal antibodies) rigorously checked to exclude cross-reactivity against other galectins (Langbein *et al*., 2007) and raised against human galectin-3 obtained by recombinant production and controlled by mass spectrometry as well as 1D and 2D gel electrophoresis (André *et al*., 2006), anti-MUC1 or anti-EGFR. Membrane and nucleus staining were obtained with Alexa-488 conjugated cholera toxin (Molecular Probes, Invitrogen, Cergy-Pontoise, France) and diamidino-2-phenylindole (Invitrogen), respectively. The labelling of intracellular compartments was performed using antibodies directed against EEA1. DyLight 594 and DyLight 488 (Jackson, West Grove, PA, USA) were used as secondary antibodies. Rescue experiment was carried out on Sh1 cells with trade recombinant galectin-3 (R&D Systems Europe, Lille, France). The slides were visualized with a Leica TCS SP5 confocal microscope (Leica Geosystems, Le Pecq, France); images were captured and analysed with the LAS AF software (Leica microsystems, Mannheim, Germany).

Statistical analysis
Statistical analysis of the data was performed using a Student's two-tailed *t*-test. *P*-values <0.05 were considered as significant.

Conflict of interest

The authors declare no conflict of interest.

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