



The MUC1 oncomucin regulates pancreatic cancer cell biological properties and chemoresistance. Implication of p42-44 MAPK, Akt, Bcl-2 and MMP13 pathways

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Highlights

- Loss of MUC1 decreases proliferation and tumor growth via β -catenin and p42–44 MAPK.
 - Inhibition of MUC1 decreases cell migration and invasion through MMP13.
 - Loss of MUC1 decreases survival and increases apoptosis via Akt and Bcl-2 pathways.
 - Loss of MUC1 sensitizes cells to gemcitabine and 5-Fluorouracil chemotherapeutic drugs.
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The MUC1 oncomucin regulates pancreatic cancer cell biological properties and chemoresistance. Implication of p42–44 MAPK, Akt, Bcl-2 and MMP13 pathways

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ABSTRACT

MUC1 is an oncogenic mucin overexpressed in several epithelial cancers, including pancreatic ductal adenocarcinoma, and is considered as a potent target for cancer therapy. To this aim, we undertook to study MUC1 biological effects on pancreatic cancer cells and identify pathways mediating these effects. Our *in vitro* experiments indicate that inhibiting MUC1 expression decreases cell proliferation, cell migration and invasion, cell survival and increases cell apoptosis. Moreover, lack of MUC1 in these cells profoundly altered their sensitivity to gemcitabine and 5-Fluorouracil chemotherapeutic drugs. *In vivo* MUC1-KD cell xenografts in SCID mice grew slower. Altogether, we show that MUC1 oncogenic mucin alters proliferation, migration, and invasion properties of pancreatic cancer cells and that these effects are mediated by p42–44 MAPK, Akt, Bcl-2 and MMP13 pathways.

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1. Introduction

The mucins are a family of large O-glycoproteins divided into two groups: secreted mucins and membrane-bound mucins [1]. Secreted mucins are involved in the protection of epithelial cells to external influences such as inflammation, pathogens or pH variations by forming the mucus gel which is a physical protective barrier of epithelia. Membrane-bound mucins are involved in cell interactions and cell signaling by transmitting intracellular signals of cell survival and cell growth under stress condition. The mucin MUC1 is a membrane-bound mucin expressed at the apical pole of normal epithelial polarized cells [2,3]. In cancer cells, MUC1 is frequently overexpressed, internalized, circumferentially delocalized around the tumor cell surface when cells lose polarity. MUC1 becomes oncogenic as it is involved in several oncogenic pathways leading to altered cell properties such as cell growth, cell migration and invasion [4]. These profound modifications result from the interaction of MUC1 with tyrosine kinase receptors such

as EGFR and the activation of oncogenic signaling pathways like MAPK, Akt or Wnt/ β -catenin [5–7].

Pancreatic Ductal Adenocarcinoma (PDAC) is the fourth leading cause of death by cancer in Western countries and has a very poor prognosis due to a late diagnosis and a lack of efficient treatment. PDAC have a dramatic outcome with the 5 years survival rate lower than 5% [8]. Less than 20% of patients are entitled to surgical resection and the remaining 80% of patients present a locally advanced metastatic PDAC [9]. They may benefit from palliative chemotherapy based either on gemcitabine or FOLFIRINOX composed of 5-Fluorouracil (5-Fu), oxaliplatin and SN-38 [10]. In 90% of PDAC, the mucin MUC1 is overexpressed and MUC1 was proposed as one of the most robust predictive marker of PDAC survival [11,12]. It was shown that MUC1 induced EMT [13] and resistance to gemcitabine [14] and also increased cell invasion through Stat3 [15] and PDGFR- β [16]. For these reasons MUC1 appears as an attractive target to moderate pancreatic cancer progression.

To better understand MUC1 biological effects on PDAC cells, we undertook to study its effects both *in vitro* and *in vivo* and the underlying cellular mechanisms mediating these effects. We generated stable cell lines knocked-down for MUC1 and showed *in vitro* that inhibiting MUC1 decreased cell proliferation via p42–44 MAPK, cell migration and invasion via MMP13, cell survival and apoptosis via Akt and Bcl2, and tumor growth *in vivo*. These

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results confirmed the potential of MUC1 as an attractive target to slow-down PDAC progression.

2. Materials and methods

2.1. Cell culture and establishment of MUC1-KD stable cell lines

Capan-2 cell line was purchased from ATCC and was cultured as described previously [17]. Capan-2 stable cell lines knocked-down (KD) for MUC1 (Capan-2 MUC1-KD1/2) were generated by retroviral infection as previously described [18] using MUC1-Sh1 primers FP: GATCCCCACCTCCAGTTTAATTCCTCTTCAAGAGAGAGGAAT TAAACTGGAGGTTTTTTA and RP: GGGTGGAGGTGCAATTAAGGAGA AGTTCTCTCTCTTAATTCGACCTCCAAAAAATTCGA. MUC1-Sh2 primers FP: GATCCCCATGTTTTGCAGATTATATCAAGAGATATAAAT CTGCAAAAACATTTTTTA and RP: GGGTACAAAAACGT CTAATATA AGTTCTCTATATTAGACGTTTTGTAAAAAATTCGA.

2.2. Cell proliferation, migration and invasion assays

Stable cell lines were seeded at 10^5 cells per well in 6-well plates. Cells were counted daily using a Malassez counting chamber using Trypan Blue exclusion dye (Life Technologies) during 96 h. Invasion and migration assays were respectively performed using 24-well Boyden chambers (8 μ m pores) coated or not with MatrigelTM (Pharmingen, BD Biosciences). Briefly, 5×10^4 cells were seeded on the top chamber and FBS 10% (v/v) was used as a chemoattractant in the bottom chamber for 24 h. Staining were performed using Vectashield hard set mounting medium with DAPI H-1500 (4',6'-diamidino-2-phénylindole) (Vector labs). Three independent experiments were performed in triplicate.

2.3. Cytotoxicity assay

10^4 cells were seeded in 96-well plates during 24 h. Medium was refreshed with gemcitabine, 5-Fu, oxaliplatin or SN-38 for 72 h. The viability of cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay (MTT, Sigma–Aldrich, Saint Quentin Fallavier, France) as previously described [17]. Formazan crystals were solubilized in dimethylsulfoxide (Sigma–Aldrich) and analyzed at 570 nm with a microplate reader (Bio-Rad).

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using miRNeasy Mini Kit with Qiazol[®] (Qiagen) according to the manufacturer's instructions. cDNA was prepared as previously described. qPCR was performed using SsoFast Evagreen[®] Supermix (Bio-Rad) and the CFX96 real time PCR system (Bio-Rad). Expression levels of MUC1 (FP: TGCCGCCGAAAG AACTACG and RP: TGGGGTACTCGCTCATAGGAT) were normalized to GAPDH (FP: CCACATCGCTCAGACACCAT and RP: CCAGGCGCCC AATACG). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were performed.

2.5. Protein extraction and Western-blot analysis

Total proteins were extracted, electro-transferred, immunostained, and visualized as described before [19]. Antibodies used were MUC1 (M8, 1/250, from Pr. D. Swallow); β -actin (A5441 AC15, 1/5000) from Sigma–Aldrich; cyclin D1 (sc-718, 1/250), CDK6 (sc-177, 1/250) from Santa Cruz; Bcl-2 (#2872, 1/250), phospho p42–44 MAPK (#9101, 1/500), p42–44 MAPK (#9102, 1/500),

β -catenin (#8480s, 1/1000), phospho AKT (#4060, 1/500), AKT (#4691s, 1/500) from Cell Signaling, Ozyme; MMP13 (Ab39012, 1/1000) from Abcam. Bands were quantified with image J analysis software three independent experiments were performed.

2.6. Subcutaneous xenografts

Subcutaneous (SC) xenografts (2×10^6 cells in 100 μ l of RPMI 1640) of MUC1-Mock, MUC1-KD1 and MUC1-KD2 cell lines were carried out with 100 μ l of MatrigelTM (BD Biosciences) into the flank of severe-combined immunodeficient (SCID) male mice (CB-17, Charles Rivers) that were bred and maintained under pathogen-free conditions (6 mice/group). Tumor development was followed weekly. The tumor volume (mm^3) was determined by calculating $V = W^2 \times L$ in which W corresponds to the width (mm) and L to the tumor length (mm). Mice were sacrificed 28 days after inoculation. All procedures were performed in accordance with the guidelines and approved by the animal care ethical committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, CEEA #122012).

2.7. Immunohistochemistry

Tumor xenografts were fixed in 10% (w/v) buffered formaldehyde, paraffin-embedded, cut at 4 μ m thickness and applied on SuperFrost[®] slides (Menzel-Glaser, Thermo Scientific). Histology was assessed by staining tissues with Hematoxylin-Eosin. Automatic IHC was performed with an automated immunostainer (ES, Ventana Medical System, Strasbourg, France) as described [20]. Immunostainings in xenografts were carried out with anti-MUC1 M8 Mab (1/50) antibodies.

2.8. Gene Expression Omnibus microarray

Public PC microarrays were analyzed from the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). A set of gene-expression profiles from GEO containing both normal pancreas and PC tissue were used: 45 tumors and adjacent non-tumor tissues from PDAC cases (GSE28735). Data were analyzed using GEO2R software. The dataset GSE28735 used Affymetrix GeneChip Human Gene 1.0 ST array.

2.9. Statistical analyses

Statistical analyses were performed using Graphpad Prism 4.0 software (Graphpad softwares Inc.). Data are presented as mean $\pm$ SD or $\pm$ SEM. Differences in the mean of samples were analyzed by the student's t test or one way ANOVA test with selected comparison using Tukey's HSD post-hoc test. Differences less than 0.05 considered significant and were indicated with a *, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

3. Results

3.1. Silencing of MUC1 in Capan-2 PDAC cell line

In order to study the functional role of MUC1 in PDAC we generated two stable cell lines knocked-down for MUC1 by short-hairpin RNA (Sh-RNA) approach. The MUC1 decreased expression in MUC1-KD cells compared to control Mock cells was confirmed by qRT-PCR (Fig. 1A) ($p < 0.05$) and by Western-blot (Fig. 1B).

3.2. Inhibiting MUC1 decreased PDAC cell growth in vitro

Cells knocked-down for MUC1 showed a statistically significant decrease of cell proliferation (Fig. 1C), at 72 and 96 h ($p < 0.001$). To identify the mechanisms underlying the alterations of cell proliferation mediated by MUC1 knock-down, we measured the activation of the major signaling markers/pathways in the three cell lines. Accordingly to the decreased cell proliferation, we observed a decrease of phospho p42–44 MAPK, cyclin D1 and β -catenin that was more pronounced in the MUC1-KD2 cell line than in the MUC1-KD1 (Fig. 1D).

3.3. Inhibiting MUC1 decreased PDAC cell migration and invasion in vitro

Next to cell proliferation, we decided to evaluate MUC1 properties on migration and invasion capacity of PDAC cells. Using Boyden chambers and DAPI staining we showed a statistically significant decrease of cell migration in MUC1-KD2 cells ($p < 0.01$) (Fig. 2A). Invasive capacities of MUC1-KD cells were statistically decreased both in MUC1-KD1 ($p < 0.05$) and in MUC1-KD2 ($p < 0.01$) cells when compared to Mock cells (Fig. 2A). In order to identify the molecular mechanisms involved in the alteration of cell migration and invasion through MUC1 inhibition, we measured the expression level of metalloprotease MMP13. Accordingly to the inhibition of cell migration and invasion, we observed a decrease of MMP13 expression both in MUC1-KD1 and MUC1-KD2 cells compared to Mock cells (Fig. 2B).

3.4. Inhibiting MUC1 decreased PDAC cell survival, increased apoptosis and gemcitabine and 5-Fu sensitivity in vitro

Investigation of the implication of MUC1 in survival and apoptosis pathways using Western-blotting, indicated a decrease of phospho-Akt expression and a decrease of anti-apoptotic marker Bcl-2 in MUC1-KD cells compared to Mock cells (Fig. 2C). PDAC being an extremely chemoresistant cancer, we undertook to evaluate MUC1 effect on PDAC cell chemosensitivity to gemcitabine or FOLFIRINOX drugs (5-Fu, oxaliplatin and SN-38). Measurement of cell survival following drug treatment indicated that inhibition of MUC1 led to a mild (MUC1-KD1) and a strong (MUC1-KD2) decrease of cell viability following treatment with gemcitabine or 5-Fu ($p < 0.5$ and $p < 0.01$) (Fig. 2D). We did not observe variation on sensitivity after treatment with oxaliplatin or SN-38. Altogether, these results indicate that PDAC cells down-expressing MUC1 harbor altered cell survival and apoptosis *in vitro* and are more sensitive to gemcitabine and 5-Fu chemotherapeutic drugs.

3.5. In vivo biological properties of PDAC cells stably knocked-down for MUC1

Having shown *in vitro* that MUC1 plays a role in Capan-2 cell growth, we undertook to evaluate their activity *in vivo*. To this aim, subcutaneous (SC) xenografts of Capan-2 MUC1-KD cells were performed in SCID mice. A significant decrease of tumor growth

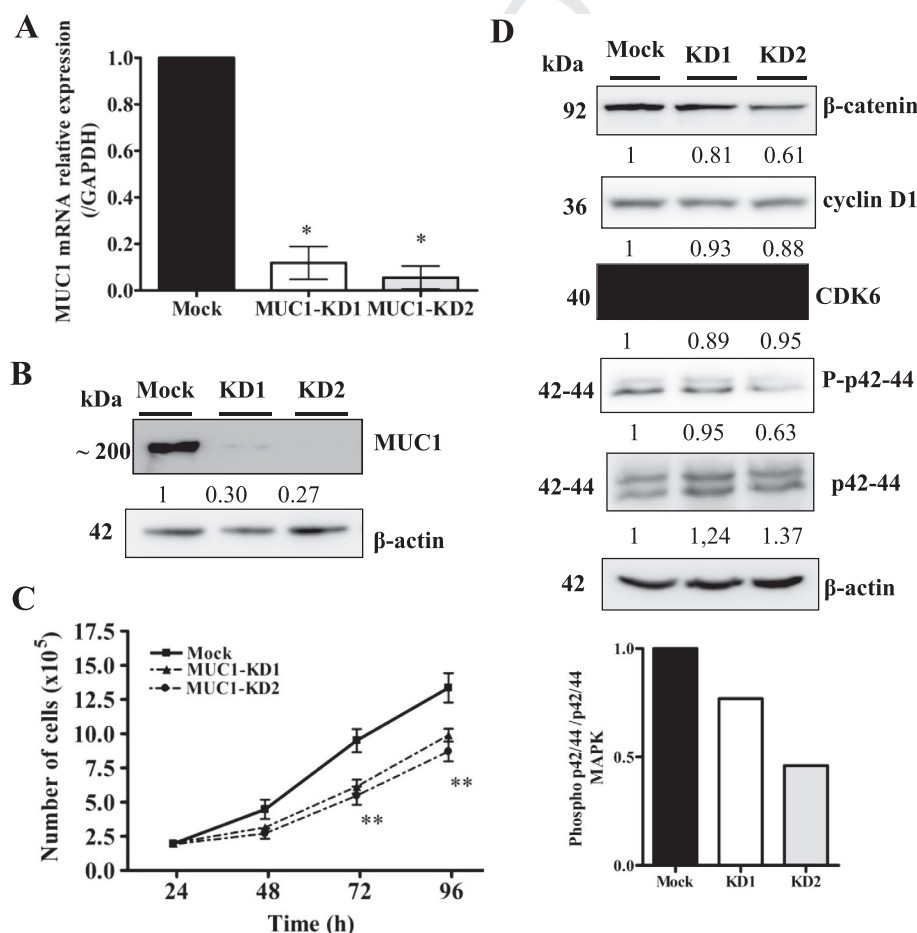


Fig. 1. Establishment of Capan-2 MUC1-KD cells. (A) Analysis of MUC1 mRNA expression in Capan-2 MUC1-KD1 and MUC1-KD2 cell lines by qRT-PCR. MUC1 expression was normalized to control GAPDH. MUC1 expression in Mock cells was arbitrarily set to 1 ($p < 0.05^*$). (B) Analysis of MUC1 protein expression by Western-blot. (C) Measurement of cell proliferation for 96 h by cell counting and trypan blue exclusion dye ($p < 0.01^{**}$). (D) Analysis of MUC1, β -catenin, cyclin D1, phospho-p42–44 MAPK, p42–44 MAPK, and CDK6 expression by Western-blot. β -Actin was used as internal control.

($p < 0.01$) was found 28 days post xenografts when compared to control Mock cells (Fig. 3A). Tumor weights were also significantly decreased for MUC1-KD1 ($p < 0.05$) and for MUC1-KD2 ($p < 0.01$) (Fig. 3B). We confirmed by IHC the down-expression of MUC1 in SC xenografts (Fig. 3C). Altogether these results indicate that inhibiting MUC1 *in vivo* slow-down pancreatic tumor growth.

3.6. Expression of MUC1 and MMP13 in human PDAC samples

To correlate our MUC1/MMP13 data obtained in PDAC cell lines with human PDAC samples we used PDAC datasets of the NCBI Gene Expression Omnibus (GEO, GSE28735) database. MUC1 and MMP13 mRNA expression was measured in 45 tumors (PDAC) and adjacent non tumor tissues (Control). We observed that MUC1 and MMP13 expression was significantly increased in PDAC tumors ($p < 0.001$) compared to control samples (Fig. 3D)

indicating a correlation between MUC1 and MMP13 overexpression in human PDAC.

4. Discussion

The oncomucin MUC1 is overexpressed in several epithelial cancers [12] including PDAC in which MUC1 overexpression is associated with a short survival rate [11]. In this report we have shown that stable inhibition of MUC1 in Capan-2 human pancreatic cancer cells decreased both their *in vitro* and *in vivo* behavior including cell proliferation, migration and invasion, increased apoptosis and gemcitabine sensitivity, and decreased tumor growth.

Our results suggest that the inhibition of cell migration and invasion may be mediated through the down-expression of MMP13 (collagenase 3) observed in PDAC cells. It was previously

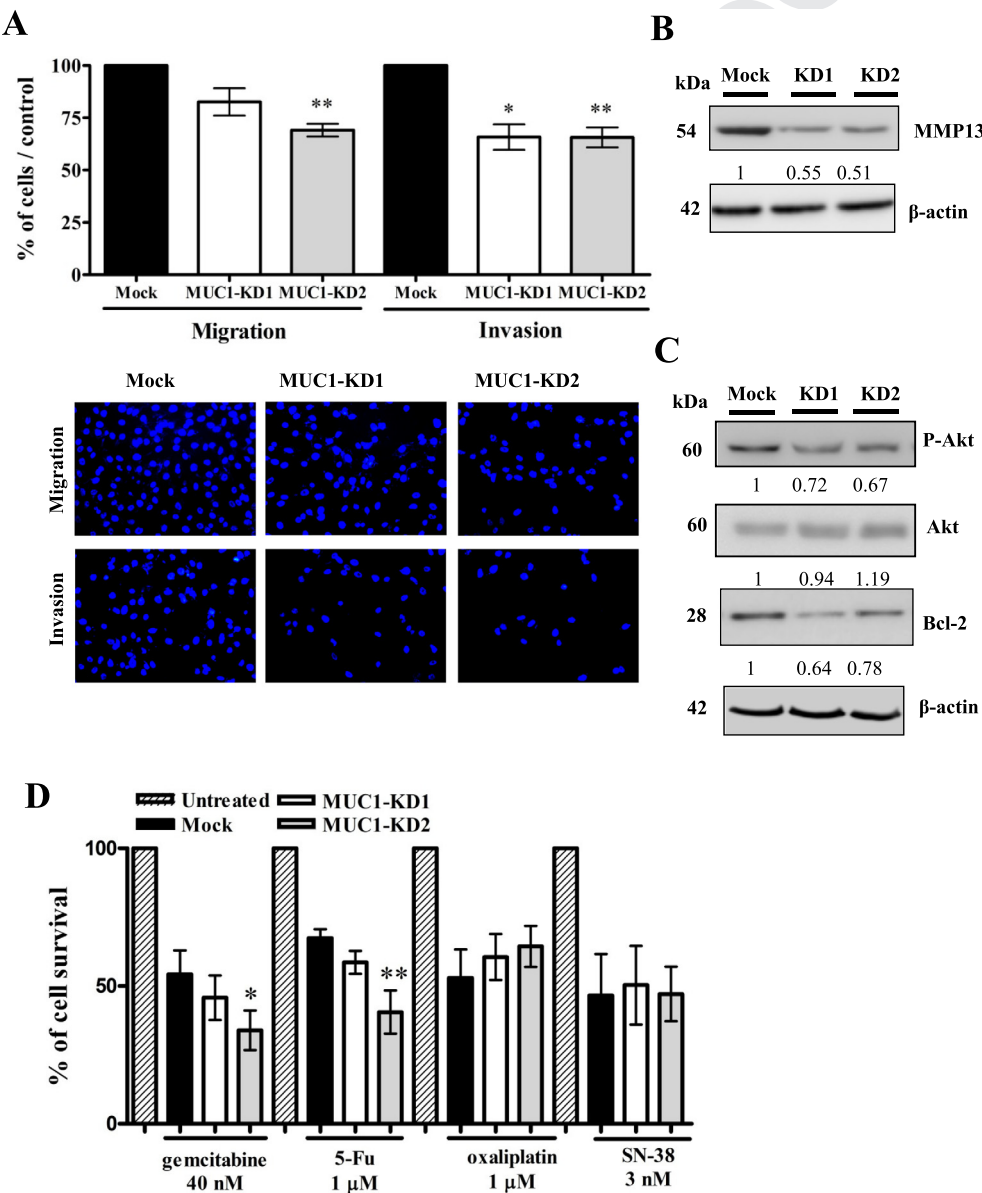


Fig. 2. *In vitro* biological properties of Capan-2 MUC1-KD cells. (A) Results are expressed as a percentage of migration or invasion of cells compared to the control Mock cells (100%). The DAPI staining is shown ($p < 0.05^*$ or $p < 0.01^{**}$). (B) Analysis of MMP13 protein expression by Western-blot. (C) Analysis of P-Akt, Akt and Bcl-2 protein expression by Western-blot. (D) Measurement of cell survival after 72 h of treatment of Capan-2 MUC1-KD cells with 40 nM of gemcitabine or 1 μM of 5-Fu, 1 μM of oxaliplatin and 3 nM of SN-38 ($p < 0.05^*$ or $p < 0.01^{**}$).

shown that the inhibition of MUC1 in esophageal carcinoma decreased MMP13 expression and cell proliferation and migration [18]. MUC1 is composed of two subunits MUC1-N and MUC1-C. MUC1-C is able in a tumorigenic context to be internalized and translocated to the nucleus and act as a co-factor of gene expression. Moreover, it was demonstrated that the MUC1-C subunit is able to bind with Runx-2 to the promoter of MMP13 and increases its expression in esophageal cancer [21]. In human PDAC samples, we observed a similar expression profile of both MUC1 and MMP13 as observed in esophageal carcinoma suggesting that in PDAC MUC1 could also be an activator of MMP13 expression. Indeed, like MUC1, MMP13 overexpression was linked to increased metastasis capacity of several cancers. We thus propose that the simultaneous overexpression of both MUC1 and MMP13 would increase PDAC aggressiveness.

The results of our study also suggest that inhibiting MUC1 decreases both *in vitro* cell proliferation and *in vivo* tumor growth. Additionally, we observed a decrease of cell proliferation markers such as cyclin D1, β -catenin and p42–44 MAPK. It was previously identified that MUC1 is able to co-activate the expression of cyclin D1 through its interaction with β -catenin on the promoter of *cyclin D1* gene thus favoring cell proliferation [22]. Moreover, MUC1 is

able to activate the p42–44 MAPK pathway through its interaction with tyrosine kinase receptor [5]. Thus inhibiting MUC1 leads to decreased cell proliferation through the Wnt/ β -catenin and the p42–44 MAPK pathways.

The mucin MUC1 is able to activate the PI3K/Akt survival pathway [7]. Thus the activation of this pathway may result from an increase of cell chemoresistance through the up-regulation of MDR-related Protein (MRP) detoxifying channels by the co-binding of MUC1 on the *MRP1* promoter with an unknown factor [14]. Thus the inhibition of MUC1 and the decrease of both Akt and gemcitabine resistance in MUC1-KD cells confirms this hypothesis. We also observed an increase of cell sensitivity to 5-Fu in MUC1-KD cells. It was previously shown that inhibition of mucin O-glycosylation by benzyl- α -GalNAc is important for 5-Fu sensitization [23,24]. However, for the first time we show a direct implication of MUC1 protein in cancer cell sensitivity to 5-Fu. Additionally, our results showed that the down-expression of MUC1 is able to increase cell apoptosis through the inhibition of anti-apoptotic Bcl-2 protein. Interestingly, it was previously shown that MUC1 is able to interact with Bax to block its pro-apoptotic function [25] or to increase Bcl-x_L anti-apoptosis protein level to increase cell survival and apoptosis [7]. Thus, we propose that MUC1

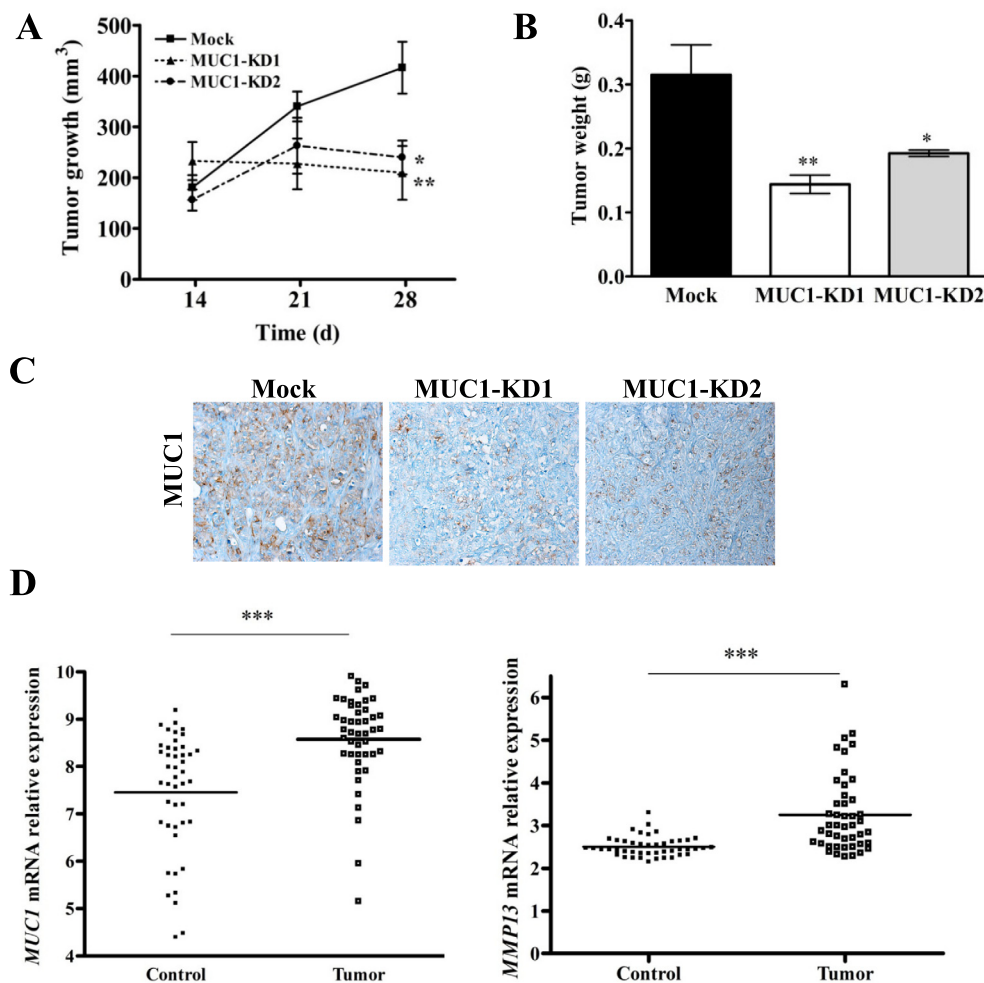


Fig. 3. *In vivo* effects of MUC1 on PDAC tumor properties. (A) SC xenografts of Capan-2 cells stably knocked-down for MUC1 in SCID mice. Tumor growth (mm³) was evaluated during 28 days ($p < 0.05^*$ and $p < 0.01^{**}$). (B) Tumor weight (g) was measured at the sacrifice ($p < 0.05^*$ and $p < 0.01^{**}$). (C) Analysis of MUC1 expression in SC tumors was carried out by IHC. Magnification $\times 20$. (D) MUC1 and MMP13 mRNA expression was evaluated in PDAC datasets of the NCBI Gene Expression Omnibus (GEO, GSE28735) database ($p < 0.001^{***}$).

deficiency in Capan-2 pancreatic cancer cells leads to decreased cell survival, increased cell apoptosis and gemcitabine and 5-Fu sensitivity through Akt and Bcl-2 pathways.

In conclusion, our results show that inhibiting MUC1 in human pancreatic cancer cells profoundly alters their behavior: (i) decreased cell proliferation and tumor growth through the Wnt/ β -catenin and the p42–44 MAPK pathways; (ii) decreased cell migration and invasion through MMP13; (iii) decreased cell survival and increased apoptosis and gemcitabine and 5-Fu sensitivity through the Akt and Bcl-2 pathways. Altogether, these results confirm the interest in inhibiting MUC1 expression in pancreatic cancer and to use MUC1 as a therapeutic target to slow-down PDAC progression.

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Conflicts of interest

Authors declare no conflict of interest.

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