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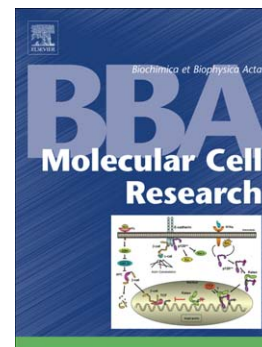
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**The MUC1 mucin regulates the tumorigenic properties of human esophageal
adenocarcinomatous cells**

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Abstract

MUC1 is a membrane-bound mucin known to participate in tumor proliferation. It has been shown that MUC1 pattern of expression is modified during esophageal carcinogenesis, with a progressive increase from metaplasia to adenocarcinoma. The principal cause of development of esophageal adenocarcinoma is gastro-esophageal reflux and MUC1 was previously shown to be upregulated by several bile acids present in reflux. In this report, our aim was thus to determine whether MUC1 plays a role in biological properties of human esophageal cancer cells. For that, a stable MUC1-deficient esophageal cancer cell line was established using a shRNA approach. *In vitro* (proliferation, migration and invasion) and *in vivo* (tumor growth following subcutaneous xenografts in SCID mice) biological properties of MUC1-deficient cells were analyzed. Our results show that esophageal cancer cells lacking MUC1 were less proliferative and had decreased migration and invasion properties. These alterations were accompanied by a decreased activity of NFκB p65, Akt and MAPK (p44/42, JNK and p38) pathways. MCM6 and TSG101 tumor-associated markers were also decreased. Subcutaneous xenografts showed a significant decrease in tumor size when cells did not express MUC1. Altogether, the data indicate that MUC1 plays a key role in proliferative, migrating and invasive properties of esophageal cancer cells as well as in tumor growth promotion. MUC1 mucin appears thus as a good therapeutic target to slow down esophageal tumor progression.

Keywords: Esophagus, adenocarcinoma, mucin, MUC1, proliferation, invasion.

Highlights:

- *In vitro* MUC1 deficiency leads to decreased proliferation, migration and invasion capacities of esophageal adenocarcinomatous cells.
- *In vivo* MUC1 deficiency leads to a decrease of tumor growth.
- MUC1 properties are mediated by MMP13, NFκB p65, Akt and MAPK pathways.

Introduction

Esophageal cancer is the 7th cause of death due to cancer worldwide and esophageal adenocarcinoma (EA) incidence has been increasing importantly for the last 40 years [1, 2]. Despite the use of a multimodal therapy with surgery more and less chemo- or radiotherapy to treat this cancer, the prognosis remains poor with 15 to 25 % of 5-year overall survival [2, 3]. It is mainly due to a late diagnosis with advanced stage of the disease and resistance to schemes of chemotherapy and/or radiotherapy [2, 4].

Duodeno-gastro-esophageal reflux responsible for chronic exposure of the distal esophagus has been demonstrated to have a major participation in EA initiation [5-7]. This exposure induces the initiation of early carcinogenic lesion called Barrett esophagus (BE). BE is characterized by the replacement of a normal epithelium by an intestinal and/or gastric type metaplasia and by the increase of the mucus-secreting cells [8]. Under the effects of this chronic exposure to the reflux, BE lesions may evolve to low-grade then high-grade dysplasia and finally to EA [9]. We previously showed in a rat surgical model of induced gastro-duodenal reflux that the duodenal component of the reflux plays a significant role in initiating the carcinogenic sequence, whereas duodenogastric juice is more involved in progression to EA [10].

In order to improve patient therapy and prognosis, there is an urgent need to better understand the molecular mechanisms and identify new actors involved during this carcinogenesis in order to further find new targets for early diagnosis or new molecules that could be used as therapeutic targets.

Among these potential targets, we previously identified the mucin MUC1 [11-13]. The mucin MUC1, which is a large transmembrane O-glycoprotein, is overexpressed in EA [12] whereas it is expressed at a very low level in normal esophageal epithelium [12, 14, 15]. This altered pattern of expression of MUC1 is often associated with protumorigenic activities of

MUC1 in many epithelial cancers that include activation of proliferative pathways, tumor progression and participation in metastasis formation [11, 16, 17].

Having previously shown *in vitro* that MUC1 is positively regulated at the transcriptional level by bile acids responsible for the reflux leading to EA [13] and *in vivo* that MUC1 overexpression follows esophageal carcinogenesis [10], we undertook in this work to study the roles of MUC1 on the biological properties of human EA cells. Our results indicate that MUC1 is a therapeutic target of interest to slow down esophageal tumorigenesis and to propose new therapeutic approaches to better treat this cancer.

1. Material and Methods

2.1. Cell culture

The OE33 esophageal adenocarcinomatous cell line was cultured as described previously [18]. To study the role of MUC1, a stable cell line deficient in MUC1 (shMUC1) was established following a retro-viral infection using a pRetroSuper plasmid (SABiosciences™) containing a sequence targeting MUC1 (forward primer (FP):GATCCCCGGTACCATCAATGTCCACG-TTCAAGAGACGTGGACATTGATGGTACC-TTTTGGAAA; reverse primer (RP):AGCTTTTCCAAAAA-GGTACCATCAATGTCCACG-TCTCTTGAA-CGTGGACATTGATGGTACC-GGG) [19]. The control cells (Mock) were infected with the corresponding empty vector. All the results presented in this study correspond to the mean of two representative clones, shMUC1-1 and shMUC1-2 compared to two representative control clones, Mock1 and Mock2.

2.2. Small Interfering RNA (siRNA) assays

OE33 cells were transfected with specific NFκB p65, PI3K, MMP3 and MMP13 siRNA (ON-TARGET plus SMART pool siNFκB2, ON-TARGET plus SMART pool siPI3KCG, ON-TARGET plus SMART pool siMMP3, ON-TARGET plus SMART pool siMMP13) from Dharmacon (Epsom, Surrey, UK) following the protocol described previously ([20]). Controls were included using either a Non-Targeting siRNA (NT) or the transfection agent alone (Dharmafect 1, Dharmacon).

2.3. RNA extraction and Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated and purified from tissues using the NucleoSpin RNA L kit (Macherey-Nagel, Duren, Germany) as described in the manufacturer's protocol. The RNA quantity was determined by measuring the optical density at 260 nm with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Pittsburgh, Penn). Total RNA (1µg) was used to prepare cDNA using oligodT (1µl) and recombinant Retro-Transcriptase Moloney Murine Leukemia Virus (M-MLV) (1µl) (Promega, Charbonnières, France). PCR was performed on cDNA (5µl), using specific pairs of primers: 28S, PI3K (FP: GAAGCCATTGAGAAGAAAGGA, RP: GAGGTGTTTCAGTATTATCAGAGC), NFκB p65 (FP: GAAGAAGCGAGACCTGGAG, RP: TCCGGAACACAATGGCCAC) (MWG-Biotech (Ebersberg, Germany). For each gene, analyses were performed three times in triplicate. 28S was used as an internal control. PCR products (20 µl) were separated on a 1.5% (w/v) agarose gel containing ethidium bromide run in 1X Tris Borate EDTA. The gene of interest/28S ratio was calculated after scanning DNA bands with the Gel Analyst-gel Smart software (Claravision, Orsay, France).

2.4. Protein extraction and expression

Total protein extraction and expression using Western-Blotting were realized as described in [20]. Antibodies used were purchased from Sigma [β actin (A-5441 at 1/5000)], Abcam [TSG101 (ab83 at 1/500), MMP13 (ab39012 at 1/500) and MCM6 (ab4458 at 1/2000)], Cell Signaling Technology [PI3K (at 1/1000), Akt (clone C67E7 at 1/500), phospho-Akt (Ser473, clone D9E at 1/500), NFκB p65 (clone E498 at 1/500), phospho-NFκB p65 (Ser536, clone 93H1 at 1/500)], p44/42 (clone I37F5, 1/500), phospho-p44/42 (Thr202/Tyr204, clone

20G11, 1/500), JNK (clone 56G8, 1/500), phospho-JNK (Thr183/Tyr185, #9251, dilution 1/500), p38 (#9212, dilution 1/1000), phospho-p38 (Thr180/Tyr182, clone D3F9, 1/1000). MUC1 antibody (M8 at 1/250) was a gracious gift from Pr Dallas Swallow (University College London). Peroxydase-conjugated secondary antibodies (Pierce) were used and the chemo-luminescence was visualized using a LAS4000 apparatus (Fujifilm). The protein of interest/ β actin ratio was calculated after scanning protein bands with the Gel Analyst-gel Smart software (ClaraVision, Orsay, France).

2.5. Cell proliferation

1.5×10^5 cells were plated in 6-well plates and were counted daily during 6 days using a Malassez Chamber. Each experiment was carried out three times in triplicate.

2.6. Cell migration and invasion

2×10^5 cells were plated in 96-well plate. At confluence, a wound was created using the 96-pin WoundMaker device (Essen Bioscience). The plate was then placed in an incubator into an Incucyte™ chamber apparatus (Essen Bioscience) at 5% CO₂ and 37°C. Every 2 hours a picture of the wounds was taken and the wound closure percentages were calculated every 12h, from 0 to 36h. Cell invasion and migration was also studied using Boyden Chambers coated or not with Matrigel® (BD Bioscience), respectively. Cells that went through the matrix were counted 48h after the cells had been plated. The invasion index was calculated as recommended by the manufacturer (Invasion/Migration ratio). Each experiment was carried out three times in triplicate.

2.7. Subcutaneous xenografts

Subcutaneous xenografts of OE33 shMUC1 and Mock cells into SCID mice were performed with 150 μ l of RPMI 1640 medium (Invitrogen) containing 4×10^6 cells and 150 μ l of Matrigel® (BD 354262, BD Bioscience). The tumor progression was followed weekly and the tumor volumes (w/l^2) were calculated in cm^3 . For each cell clone, 10 mice were injected. All procedures were in accordance with the guideline of animal care committee (Comité Ethique Expérimentation Animal Nord Pas-de-Calais, #AF042008).

2.8. Immunohistochemistry

MUC1 (1/50), Akt (1/500), NF κ B p65 (1/500), TSG101 (1/100) and MCM6 (1/100) expression in human normal esophageal tissues, BE and EA lesions was studied by automatic (automated immunostainer ES, Ventana Medical System, Strasbourg, France) [18] and manual [21] immunohistochemistry (IHC), respectively. Antibodies against NF κ B p65 (sc-7151), TSG101 (M-19) and MCM6 (sc-9843) were purchased from Santa Cruz Biotechnology. Positive controls were included by staining tissues known to express the protein of interest and negative controls were run with 1X D-PBS instead of primary antibodies.

2.9. Statistics

The statistical analyses were carried out using both Excel and GraphPad 4.0 softwares (GraphPad softwares Inc., La Jolla, USA). The differences in the means of samples were analyzed using student t test or one way anova test with selected comparison using tukey post hoc test with differences less than 0.05 considered as statistically significant (*). ** indicates $p < 0.01$ and *** $p < 0.001$.

2. Results

3.1. *In vitro* biological properties of MUC1-deficient OE33 cells

To study the roles of MUC1 in the EA tumor progression, we established a stable OE33 shMUC1 cell line using a shRNA approach (Figure 1A). The complete loss of MUC1 protein expression in the shMUC1 cells was confirmed by western-blotting when compared to the control cells (Mock). The capacity of OE33 cells to proliferate in absence or presence of MUC1 was tested over a period of 6 days (Figure 1B). The results indicate that MUC1 deficient cells (shMUC1) significantly proliferate less than Mock cells as of day 4 ($p = 0.0158$) and this reduced proliferation is sustained until day 6 ($p = 0.0002$). The role of MUC1 in cell migration was studied using a wound-healing test (Figure 1C). We showed that in MUC1 deficient cells (shMUC1), the migration was impaired as of 12h ($p = 0.0179$) after the wound was made when compared to Mock cells. This was sustained until 36h ($p = 0.0025$). MUC1 role in OE33 capacity to invade a matrix was studied using Boyden Chamber coated with Matrigel® (Figure 1D). The results indicate that MUC1 deficient cells are significantly less invasive compared to Mock cells ($p = 0.0137$), with a reduction of the invasion index of 31% compared to the controls. This decreased invasion was correlated to a decreased expression of MMP13 in MUC1-deficient cells (Figure 1A). Moreover, OE33 cells transfected with MMP13 siRNA were less invasive (Figure 1E). This was not found for MMP3 (not shown). Knocking down expression of MMP3 or MMP13 had no impact on OE33 cell proliferation and migration properties (Figure S1A and B).

3.2. *In vivo* biological properties of MUC1-deficient OE33 cells

Having shown that MUC1-deficient cells were less proliferative, less invasive and less migrating than Mock cells *in vitro*, we undertook to study the impact of MUC1 absence on tumor growth *in vivo* by performing subcutaneous xenografts of shMUC1 cells in SCID mice

(Figure 2). At 6 weeks post-injection, the shMUC1 tumors were much smaller (shMUC1-1: 0.57 ± 0.07 and shMUC1-2: 0.49 ± 0.16 cm³) than the Mock cell tumors (1.76 ± 0.07 cm³) with a 68 % and 72% reduction, respectively. This difference between the tumor volumes was highly significant between the Mock and the shMUC1-1 cells ($p < 0.01$) and extremely significant between the Mock and the shMUC1-2 cells ($p < 0.001$).

3.3. MUC1 deficiency impairs NF κ B p65, Akt and MAPK pathways

Having shown that MUC1 deficiency impairs proliferation, migration and invasion properties of OE33 cells, we undertook to study the intracellular mechanisms and signalling pathways that could mediate MUC1 effects. Our results indicate decreased activation of NF κ B p65 and Akt pathway in shMUC1 cells compared to Mock cells as well as decreased activation of the three major MAPK pathways (p44/42, JNK and p38) (Figure 3A). Involvement of the NF κ B p65 (Figure 3B) and PI3K/Akt (Figure 3C) pathways in cell proliferation was further confirmed using a siRNA approach with a significant decreased proliferation following siRNA treatment. No effect was found on cell migration (Figure S1B). Correlation between MUC1, NF κ B p65 and Akt expression was further confirmed in human esophageal tissues with immunohistochemical stainings indicating increased expression of MUC1, NF κ B p65 and Akt in BE and EA compared to normal squamous epithelium (Figure 4B).

Having previously found that the Tumor susceptibility gene 101 (TSG101) and the minichromosome maintenance complex-6 gene (MCM6) tumor-associated proteins were overexpressed in a MUC1-expressing rat model of esophageal cancer [10], we checked whether these two tumor markers were also altered in human OE33 MUC1-deficient cells and in human esophageal tissues. Accordingly, we found decreased expression of these two

proteins in shMUC1 cells (Figure 4A). Immunohistochemical studies in human esophageal tissues indicated expression of MCM6 in the nucleus of epithelial cells of normal squamous epithelium (Figure 4B). In BE and EA, MCM6 was expressed in the nuclei of epithelial cells constituting BE, with a higher number of labeled cells at the bottom of the glands compared to the surface villi (Figure 4B). In EA, MCM6 was also expressed in the nuclei of epithelial cells lining the pools of mucus. Whereas TSG101 was not expressed in normal esophagus, we observed TSG101 expression in the cytoplasm of basal cells in both BE and EA (Figure 4B). As expected, MUC1 mucin was expressed in epithelial cells of the superficial layers of the normal squamous epithelium and overexpressed in the early stage of BE and sustained in EA.

4. Discussion

In this report, we show that the MUC1 mucin plays a role in both *in vitro* (proliferation, migration, invasion) and *in vivo* (tumor growth) properties of esophageal adenocarcinomatous cells. Our results suggest that invasive properties of MUC1 may be mediated by the MMP13 metalloprotease. In support of this finding, previous data showed that MUC1 induces metastasis in esophageal squamous cell carcinoma (SCC) by upregulating MMP13 [22]. Other results showed that MMP13 has a key role in extracellular matrix degradation and tumor metastasis and that its overexpression was reported in esophageal SCC and was related to cancer aggressiveness [23]. MMP13 appears thus as a central element in invasive properties of esophageal cancer cells.

Furthermore, it has been shown that MUC1 is an oncoprotein involved in many epithelial cancers [11]. In EA, expression of MUC1 is progressively increased from metaplasia to adenocarcinoma, suggesting a potential role in degeneration of BE into adenocarcinoma [14]. MUC1 has been incriminated to be involved in (i) metastasis due to its anti-adhesive activity [24, 25] and to its binding properties to β -catenin and other family members [26], in (ii) cell interaction and (iii) transcriptional regulation [27]. Our results suggest that the increase of MMP13 expression may be thus an additional mechanism to promote MUC1 invasive properties.

To go further, we showed that absence of MUC1 in EA cells was correlated to the decreased expression of tumor-associated proteins TSG101 and MCM6, suggesting a link between MUC1 and these two proteins. This was also accompanied by an impaired proliferation capacity of EA cells. Since TSG101 is known to be involved in numerous cellular processes including apoptotic resistance [28] and G1/S Cell cycle checkpoint control [29] and MCM6 being involved in cell cycle control [30], these two proteins may be considered as partners of MUC1 to convey EA cell proliferative capacities.

We also showed that alteration of MUC1 expression in EA cells profoundly impaired NF κ B p65 and MAPK (p44/42, JNK and p38) pathways. Recently, it has been shown in colon cancer cells that MUC1 could inhibit apoptosis via activating JNK1 pathway in response to genotoxic anticancer agents such as cisplatin [31]. This suggests that MUC1, via the MAPK pathways, could perhaps be a target of choice to fight against chemoresistance in EA. In a recent study, Paterson *et al* demonstrated that the MAPK pathway was commonly activated in esophago-gastric cancer and that the constitutively active receptor tyrosine kinases (RTK) could be identified on a patient using RTK array analysis. This could be considered as a first step for a personalised treatment by targeting the signalling pathway in EA [32].

Alteration of NF κ B p65 pathway consecutively to MUC1 depletion altered EA cell proliferation properties. This is in agreement with increased NF κ B p65 expression observed in BE and EA. The NF κ B p65 increased expression in EA has been correlated to chemo-radiation resistance [33]. So MUC1 could potentially be considered as a factor of chemoresistance by the activation of the NF κ B p65 pathway.

Furthermore, previous studies have shown that tumor cells expressing MUC1 exhibit an increased capacity to resist the effect of chemotherapeutic drugs. Indeed, MUC1 blocks DNA damage-induced apoptosis by several mechanisms: (i) MUC1 C-terminal subunit is localized to the mitochondrial outer membrane to attenuate release of mitochondrial apoptogenic factors [34] and binds directly to Bax to block its function in activating the mitochondrial death pathway [35]; (ii) MUC1 sequesters c-Abl in the cytoplasm and inhibits apoptosis in response to genotoxic anticancer agents [36].

Whereas in esophageal SCC a high level of MUC1 expression was reported to be correlated with lymph node metastasis and associated with a poor prognosis [23], we previously did not show any correlation between the level of MUC1 expression in EA and histopathological variables, recurrence or survival in a series of 52 patients [15]. Even if

MUC1 may not serve as a potential biomarker in EA, our study suggests that MUC1 plays a role in EA cell proliferation and invasiveness and could be also considered as a potential therapeutic target for EA patients as a factor of chemoresistance.

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Figure legends

Figure 1. *In vitro* biological properties of MUC1-deficient OE33 cells. (A) Expression of MUC1 and MMP13 in Mock and in shMUC1 OE33 cells by Western-Blotting. (B) Analysis of shMUC1 (grey line) and Mock (black line) cell proliferation *in vitro*. Countings were carried out with Malassez Chambers and were performed daily during 6 days. * corresponds to $p \leq 0.05$ and *** to $p \leq 0.001$ (student t test). (C) shMUC1 and Mock OE33 cell migration was studied by wound-healing test. The photographs of the wound closures were taken at 0h and 36h post-wounding. Representation of the wound closure in percentage from T0 to T36h. Comparison of the shMUC1 (grey line) to the Mock (black line) cells. * corresponds to $p \leq 0.05$ and * to $p \leq 0.01$ (student t test). (D) Representation of the invasion index of Mock (black) and shMUC1 (grey) cells using Boyden Chambers coated with Matrigel®. The invasion index is the ratio corresponding to the number of invasive cells reported to the number of migrating cells. * corresponds to $p \leq 0.05$ (student t test). (E) Representation of the invasion index of Mock cells (black), or cells transfected with non targeting (NT) siRNA (dark grey) or MMP13 siRNA using Boyden Chambers coated with Matrigel®.*** corresponding to $p \leq 0.001$ (student t test).

Figure 2. *In vivo* role of MUC1 on tumor growth. Means of tumor volumes in cm^3 obtained 6 weeks after SC injections of Mock or shMUC1 cells in SCID mice. ** corresponds to $p \leq 0.01$ and *** to $p \leq 0.001$ (one way anova with tukey post hoc).

Figure 3. Impact of MUC1 deficiency on NF κ B p65, Akt and MAPK pathways. (A) Expression of NF κ B p65, phospho-NF κ B p65, PI3K, Akt, phospho-Akt, MAPKs (p44/42, phospho-p44/42, JNK, phospho-JNK, p38, phospho-p38) and β actin in Mock and shMUC1

OE33 cells by Western-Blotting. (B) Expression of *NFκB p65* mRNA by RT-PCR and *in vitro* analysis of OE33 cell proliferation after siRNA treatment. (C) Expression of *PI3K* mRNA by RT-PCR and *in vitro* analysis of OE33 cell proliferation after siRNA treatment. Cell counting was carried out with Malassez Chambers and was performed daily during 6 days. * corresponds to $p \leq 0.05$ and ** to $p \leq 0.01$.

Figure 4. Expression of MUC1 and tumor-associated proteins in human MUC1-deficient cells and in human esophageal tissues. (A) Expression of TSG101, MCM6 and β actin in Mock and in shMUC1 OE33 cells by Western-Blotting. Protein intensity is reported to the β actin expression. * corresponds to $p \leq 0.05$ and *** to $p \leq 0.001$. (B) Immunohistochemical stainings of MUC1, Akt, *NFκB p65*, MCM6 and TSG101 in human esophageal tissues: normal esophagus x100, Barrett esophagus (BE) x100, esophageal adenocarcinoma (EA) x100. Inserts x 200.

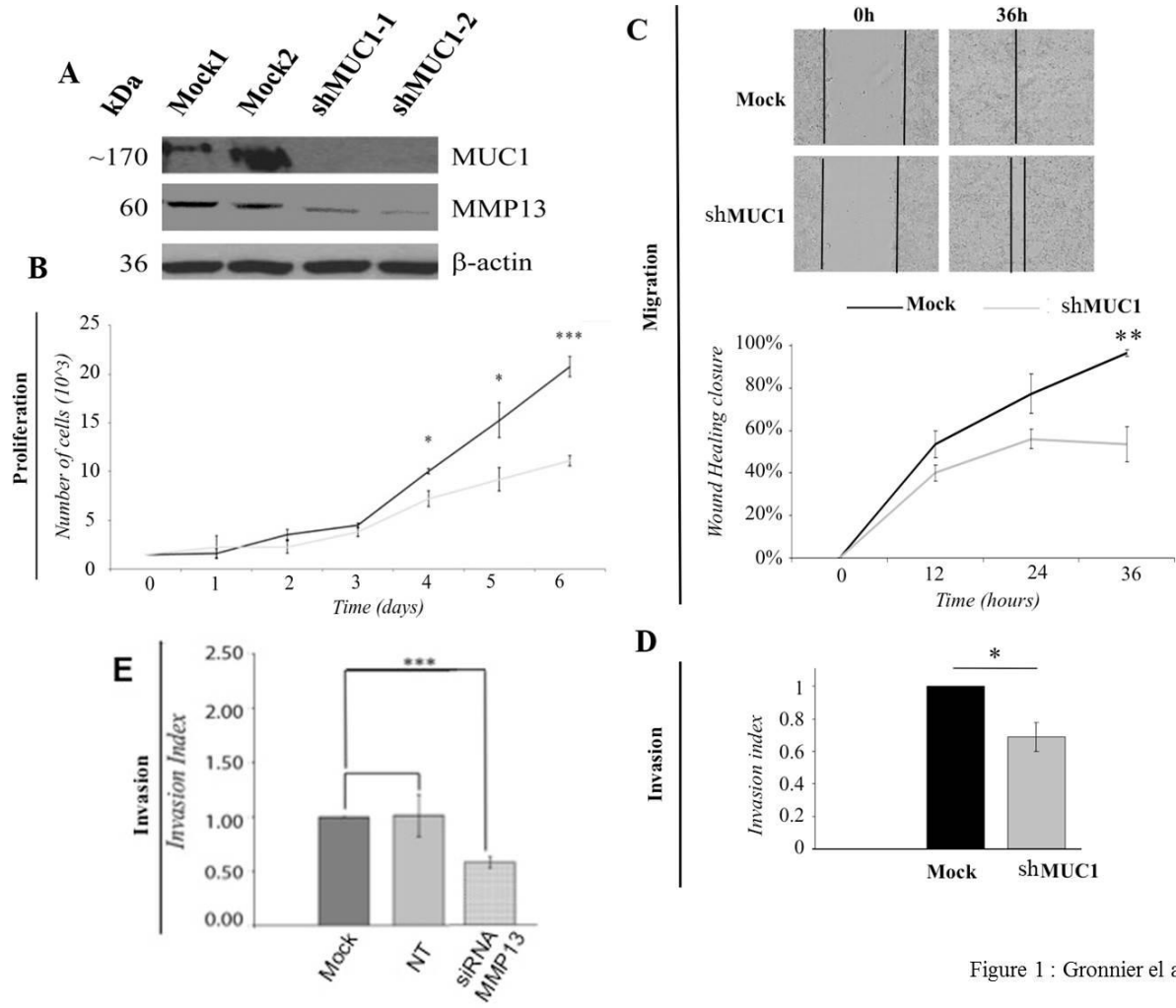


Figure 1 : Gronnier et al.

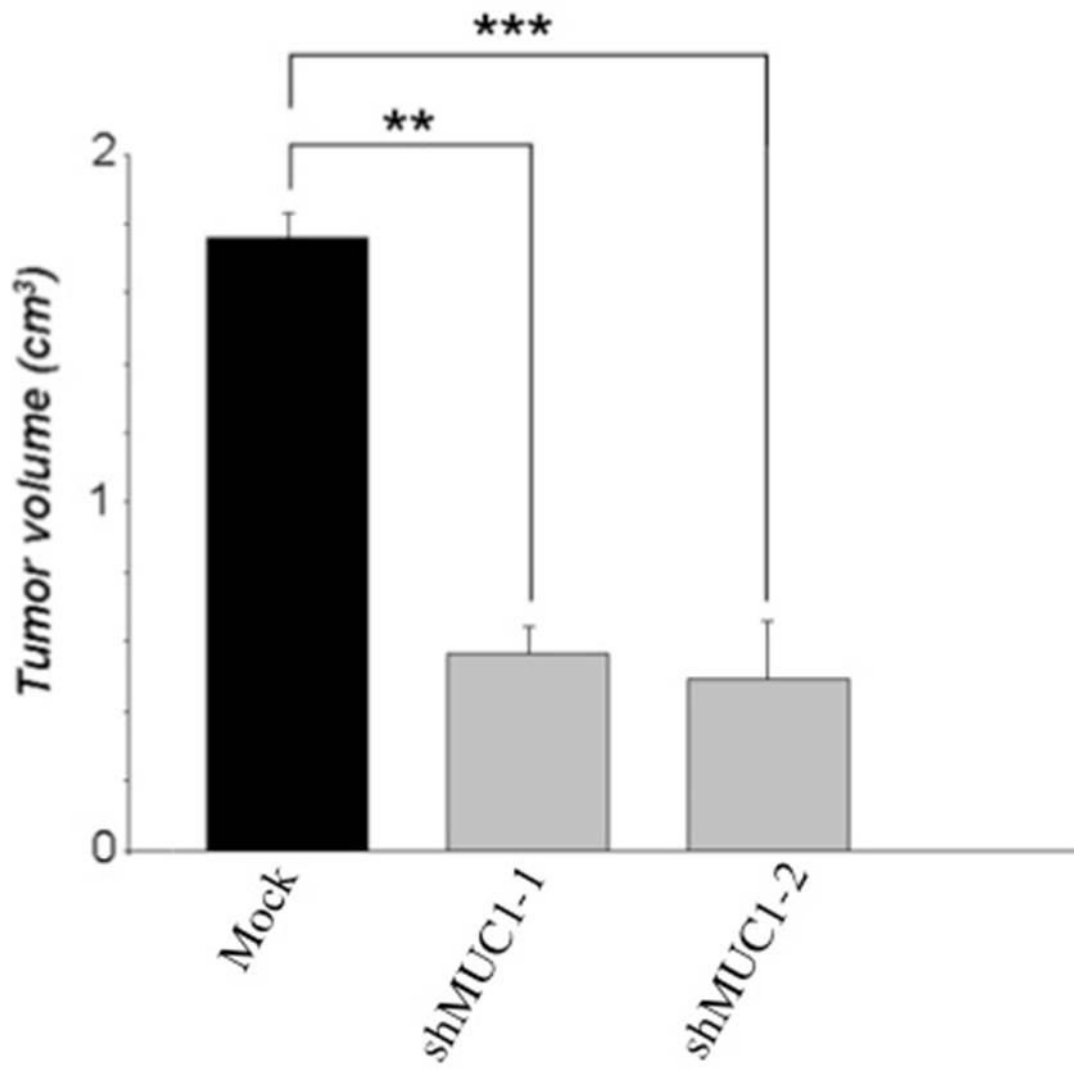


Figure 2 : Gronnier et al.

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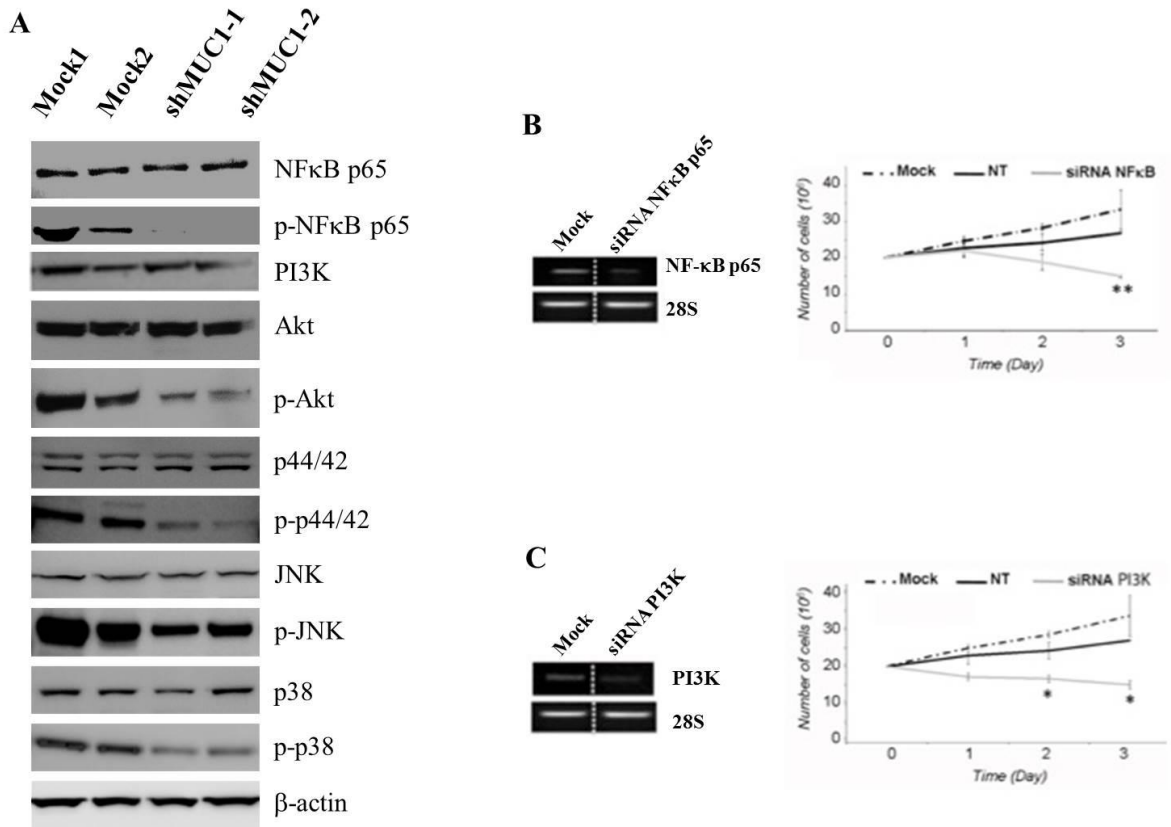


Figure 3 : Gronnier et al.

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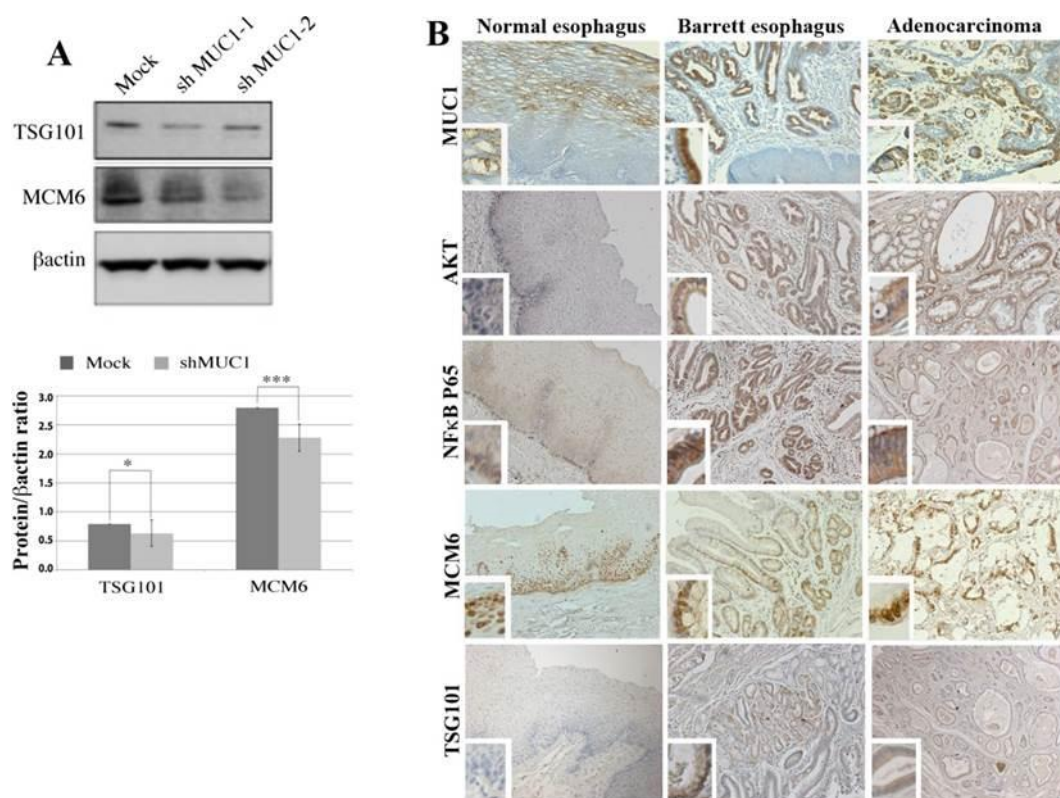


Figure 4 : Gronnier et al.

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