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TGF- β RII knock-down promotes tumor growth and chemoresistance to gemcitabine of pancreatic cancer cells via phosphorylation of STAT3

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Abstract

Pancreatic adenocarcinoma (PDAC) is one of the most deadly cancers in the western countries because of a lack of early diagnostic markers and efficient therapeutics. At the time of diagnosis, more than 80% of patients have metastasis or locally advanced cancer and are therefore not eligible for surgical resection. Pancreatic cancer cell also harbour a high resistance to chemotherapeutic drugs such as gemcitabine that is one of the main palliative treatment for PDAC.

TGF- β possesses both tumor-suppressive and oncogenic activities in pancreatic cancer. TGF- β signalling pathway plays complex role during carcinogenesis by initially inhibiting epithelial growth and later promoting the progression of advanced tumors and thus emerged as tumor suppressor pathway. TGF- β binds to its receptor TGF- β RII and activates different pathways: canonical pathway involving the Smad proteins and alternative pathways such as MAPKs. Smad4 is mutated in 50-80% of PDAC. Mutations of TGF- β RII also occurs (5-10%). In order to decipher the role of TGF- β in carcinogenesis and chemoresistance, we decided to characterize the knocking down of TGF- β RII that is the first actor of TGF- β signalling. We developed pancreatic cancer cell lines stably invalidated for TGF- β RII and studied the impact on biological properties of pancreatic cancer cells both in vitro and in vivo. We show that TGF- β RII silencing alters tumor growth and migration as well as resistance to. TGF- β RII silencing also leads to S727 STAT3 and S-63 c-Jun phosphorylation, decrease of MRP3 and increase of MRP4 ABC transporter expression and induction of a partial EMT phenotype.

In the future, the better understanding TGF- β signaling pathways and underlying cellular mechanisms in chemoresistance to gemcitabine may bring new therapeutic tools to clinicians.

Keywords: TGF- β RII receptor, STAT3, metastasis, gemcitabine, ABC transporters

Introduction

Pancreatic cancers (PC) are projected to become the second leading cause of cancer-related death by 2030 (Rahib et al., 2014). The survival curve is extremely short (6 months) and the survival rate at 5 years is very low (3%). This dramatic outcome is related to a lack of therapeutic tools and early diagnostic markers which makes pancreatic cancer the most deadly cancer. At the time of diagnosis, more than 80% of PC are already metastatic or locally advanced and only about 10 to 15% of patients are considered eligible for surgical resection (Vincent et al., 2011). Remaining patients that do not benefit of surgery will receive palliative chemotherapy and notably gemcitabine, a fluorinated analog of deoxycytidine that is a major chemotherapeutic drug used in firstline in advanced PC. Unfortunately, PC is characterized by an intrinsic and acquired chemoresistance that lead to relapse and death (Kleeff et al., 2016). Deciphering mechanisms responsible for PC cell resistance to gemcitabine is thus crucial to improve efficacy of the drug and propose more efficient therapies.

TGF- β signalling pathway plays a complex role during carcinogenesis. TGF- β initially inhibits epithelial growth whereas it appears to promote the progression of advanced tumors and thus emerged as tumor suppressor pathway in pancreatic cancer (Principe et al., 2014). TGF- β can act in an autocrine manner or as a paracrine factor secreted by the microenvironment (Derynck et al., 2001). After binding to its receptor TGF- β RII, TGF- β signals via activation of several pathways. The canonical pathway involves the Smad proteins, but activation of other pathways such as MAPKs, PI3K or small GTPases (Derynck et al., 2001) pathways may also mediate TGF- β effects. It is also interesting to note that Smad4/DPC4 (deleted in pancreatic cancer 4) is mutated in 50-80% of PDAC whereas mutations of TGF- β RII are less common (5-10%) (Kleeff et al., 2016; TCGA-Network., 2017). We previously showed that TGF- β can regulate gene expression via canonical or alternative signalling pathways (Jonckheere et al., 2004).

In order to design new therapeutic strategies, it is thus mandatory to better characterize the signaling pathways and complex gene networks that are altered during carcinogenesis progression. Therefore, to better understand the role and contribution of TGF- β RII in TGF- β signalling and biological properties of PC cells in vitro and in vivo, we developed PC cell lines stably invalidated for TGF- β RII. Our results show that TGF- β RII silencing alters tumor growth and migration and increases resistance to gemcitabine in vitro and in vivo. TGF- β RII silencing also leads to STAT3 and c-Jun phosphorylation, alteration of MRP3 and MRP4 ABC transporters expression and induction of a partial EMT phenotype.

This work underlies the importance of TGF- β signaling pathways and associated cellular mechanisms as inducers of chemoresistance to gemcitabine and proposes potential new therapeutic tools to clinicians, surgeons and anatomopathologists for this deadly disease.

Material and methods

Cell culture

CAPAN-1 and CAPAN-2 PC cell lines were cultured as previously described (Jonckheere et al., 2004). TGF- β RII-knocked down (KD) cells were obtained following stable transfection of CAPAN-1 and CAPAN-2 cells with four different pGeneClipTM puromycin vectors encoding TGF- β RII ShRNA (SA BiosciencesTM) as previously described (Jonckheere et al., 2012). The empty vector was used to raise control clones called Non Targeting (NT). Four selected clones of NT and each TGF- β RII-KD cells were pooled in order to avoid clonal variation and were designated TGF- β RIIKD6, TGF- β RIIKD7, TGF- β RIIKD8 and TGF- β RIIKD9. All cells were maintained in a 37°C incubator with 5% CO₂ and cultured as the parental cells.

qRT-PCR

Total RNA from PC cells was prepared using the NucleoSpin[®] RNA II kit (Macherey Nagel, Hoerd, Germany). cDNA was prepared as previously described (Van Seuning et al., 2000). Semi-quantitative PCR was performed as previously described (Mesquita et al., 2003). qPCR was performed using SsoFastTM Evagreen Supermix kit following the manufacturer's protocol using the CFX96 real time PCR system (Bio-Rad). Primer information is given in table 1. Each marker was assayed in triplicate in three independent experiments. Expression level of genes of interest was normalized to the mRNA level of GAPDH housekeeping gene.

Protein extraction and western-blotting

Total cellular extracts were performed as previously described in Van Seuning et al. (Van Seuning et al., 1995) and Jonckheere et al. (Jonckheere et al., 2009). Western-blotting on nitrocellulose membrane (0.2 μ m, Whatman) was carried out as previously described (Piessen et al., 2007). Membranes were incubated with antibodies against STAT3 (79D7, Cell signalling), phospho S727 STAT3 (9134, signalling), c-Jun (60A8, Cell signalling), phospho S63 c-Jun (54B3, Cell signalling) and β -actin (AC-15, sigma). Antibodies were

diluted in 5% (w/v) non-fat dry milk in Tris-Buffered Saline Tween-20 (TBS-T). Peroxydase-conjugated secondary antibodies (Sigma-Aldrich) were used and immunoreactive bands were visualised using the West Pico chemoluminescent substrate (Thermo Scientific, Pierce, Brebières, France). Chemo-luminescence was visualised using LAS4000 apparatus (Fujifilm). Density of bands were integrated using Gel analyst software® (ClaraVision, Paris, France) and represented as histograms. Three independent experiments were performed.

Cell proliferation

Cells were seeded at 1×10^5 cells per well in 6-well plates. Cells were counted daily using a Malassez counting chamber using Trypan Blue exclusion dye (Life Technologies) during 96h. Experiments were performed three times in triplicate.

Wound healing test

1500 cells were seeded per wells in 96 well plates (Image Lock™ plates, Essen Bioscience) and cultured until confluence was reached. The wound was realized using IncuCyte wound maker (Essen BioScience). Cells were washed three times with PBS 1X and complete medium was added to the cells. Wound widths were analyzed using Incucyte platform (Live-Cell imaging System, Essen Bioscience) and pictures collected every 2h.

Cytotoxicity assay

Cells were seeded in growth medium into 96-well plates at a density of 10^4 cells per well. After 24h incubation, the medium was replaced by fresh medium containing gemcitabine at 35 nM and incubated for 72h at 37°C. The viability of cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma-Aldrich) as previously described (Skrypek et al., 2013). Percentage of viability = $[(A_{\text{treated}} - A_{\text{blank}})/(A_{\text{neg.}} - A_{\text{blank}})] \times 100$; where A_{treated} is the average of absorbance in wells containing cells treated with gemcitabine, $A_{\text{neg.}}$ is the average of wells containing cells without gemcitabine treatment, and A_{blank} is the average of wells containing medium without cells.

Subcutaneous xenografts

NT or TGF- β RII-KD CAPAN-1 (10^6 cells in 100 μ l Matrigel) and CAPAN-2 (2×10^6) cells were injected subcutaneously (SC) into the flank of seven-week-old male Severe Combined Immunodeficient (SCID) mice (CB17, Janvier, France). Six mice were used per group. Tumor size was evaluated weekly by measuring the length (l) and the width (L) and tumor volume was calculated with the formula ($l^2 \times L$). Once palpable tumors were developed (250 mm^3), gemcitabine (15 mg/kg) or PBS (200 μ l) were injected intra-peritoneously, twice a week. All procedures were in accordance with the guideline of animal care committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, #122012).

Immunohistochemistry

Xenografts were fixed in 10% (w/v) buffered formaldehyde, embedded in paraffin, cut at 4 μ m thickness and applied on SuperFrost® slides (Menzel-Glaser, Braunschweig, Germany). Manual IHC was carried out as previously described (van der Sluis et al., 2004). The antibodies were used as followed: anti-STAT3 (1:200, #483 Santa Cruz), anti-c-Jun (1:200, 60A8 Cell signaling), anti-E-Cadherin (1:200, 3195 Cell signalling) and anti-vimentin (1:200, sc5741, Santa Cruz). Intensity of staining was graded as weak (1), moderate (2) or strong (3). The percentage of ductal stained cells was graded as 1 (0–25%), 2 (25–50%), 3 (50–75%) and 4 (75–100%). Total score was calculated by multiplying the intensity score and percentage score.

Expression analysis in CCLE database

TGF- β RII, ABCB1/MDR1, ABCC1/2/3/4/5 and ABCG2 z-score expressions were extracted from databases available at cBioPortal for Cancer Genomics (Cerami et al., 2012; Gao et al., 2013). The queries were realized in CCLE (44 pancreatic samples, Broad Institute, Novartis Institutes for Biomedical Research) (Barretina et al., 2012).

Statistical analyses

Statistical analyses were performed using the Graphpad Prism 6.0 software (Graphpad softwares Inc., La Jolla, USA). Differences in data of two samples were analysed by the student's t test or ANOVA test with selected comparison using tukey post-hoc test and were considered significant for P-values <0.05 *, p<0.01 ** or p<0.001 ***.

Results

Generation and characterization of stable TGF- β RII-KD cellular clones

Expression of TGF- β RI, TGF- β RII, TGF- β RIII Smad2, Smad3, and Smad7 was confirmed by RT-PCR in CAPAN-1 and CAPAN-2 cells. Wild type Smad4, as it is mutated, is not detected in CAPAN-1 (Jonckheere et al., 2004; Schutte et al., 1996). Altogether this suggests that CAPAN-2 cells harbor a functional TGF- β signaling pathway whereas the canonical Smad pathway is not functional in CAPAN-1 cells. Moreover, strong TGF- β 1 and mild TGF- β 2 mRNA levels were observed in both cell lines suggesting TGF- β growth factor autocrine expression (Figure 1A).

We generated CAPAN-1 and CAPAN-2 stable cell lines in which TGF- β RII was knocked down (TGF- β RII-KD) by a shRNA approach. Four different shRNA sequences were used to establish four different cell lines designated as TGF- β RIIKD6, TGF- β RIIKD7, TGF- β RIIKD8 and TGF- β RIIKD9. Using qPCR, we confirmed that TGF- β RII mRNA levels are decreased in all CAPAN-1 and CAPAN-2 TGF- β RII-KD cells compared to NT control cells ($p < 0.005$, ***) (Figure 1B). We were not able to produce TGF- β RIIKD7 cell line in CAPAN-2.

In CAPAN-2 KD cells, the inhibition of TGF- β RII expression was correlated with a loss of activity of the Smad binding elements (SBE)-Luc synthetic promoter (Figure 1C). In CAPAN-2 NT cells, TGF- β treatment induces a 10-fold increase of SBE-Luc relative activity whereas this effect was lost in TGF- β RII-KD cells ($p < 0.001$). As expected, in CAPAN-1 cells mutated for Smad4, we did not observe any activity of SBE-Luc construct with or without TGF- β treatment (not shown). Interestingly, TGF- β RII knocking down led to decreased TGF- β 1 mRNA level in CAPAN-1 TGF- β RII-KD cells (44-87% decrease) (Figure 1D) whereas the effect was less pronounced (21-25%) in TGF- β RII-KD CAPAN-2 cell lines.

Involvement of TGF- β RII in PC cell biological properties

We investigated the effect of TGF- β RII silencing on CAPAN-1 and CAPAN-2 proliferation and migration properties. Cell migration was assessed by wound healing test. In CAPAN-2 NT cells, the wound was entirely closed at 60h. In CAPAN-2 TGF- β RII-KD cells, we observed a strong delay of wound closure that was statistically significant at 16-18h ($p < 0.001$, ***) (Figure 2A, left panel). Interestingly, we did not observe any statistically significant difference in wound closure in CAPAN-1 TGF- β RII-KD or NT cells suggesting the involvement of a functional Smad4 signaling pathway in wound closure (Figure 2A, right panel). TGF- β RII-KD CAPAN-1 or CAPAN-2 cells also showed a trend toward increased proliferation at 96h compared to the respective NT control cells but that remained not significant (not shown).

In order to determine the role of TGF- β RII on pancreatic carcinogenesis in vivo, CAPAN-1/-2 TGF- β RII-KD8 and NT SC xenograft studies were carried out. We selected the TGF- β RII-KD8 cell lines for in vivo studies as this cell line harboured the best KD in CAPAN-1 and CAPAN-2. The results indicate that the tumour volume was significantly higher in xenografted mice with CAPAN-1 TGF- β RII-KD8 compared to CAPAN-1 NT controls. The relative tumour volume was $2.26 \pm 0.1 \text{ cm}^3$ when compared to NT control tumour volume ($1.66 \pm 0.14 \text{ cm}^3$) at day 21. The increase was statistically significant (**, $p < 0.01$). Similar results were obtained with CAPAN-2 TGF- β RII-KD8 xenografts (0.423 ± 0.05 vs $0.828 \pm 0.08 \text{ cm}^3$) at day 42 (Figure 2B). Furthermore, we also evaluated the presence of micro-metastasis in the liver by detecting the presence of human GAPDH in the liver of the mouse by qPCR (Figure 2C). We detected micro-metastases in 5/7 (71%) CAPAN-2 controls whereas only 2/10 (20%) of CAPAN-1 TGF- β RII-KD8 xenografted mice harboured micro-metastases. Contingency analysis showed that difference was close to statistical significance ($p = 0.058$). We did not observe any difference in CAPAN-1 TGF- β RII-KD8 (4/6) compared to CAPAN-1 NT controls (4/6). No human GAPDH mRNA was detected in mice without xenografts. Our results suggest that TGF- β RII signalling is involved in tumor growth and migration of pancreatic cancer cells both in vitro and in vivo.

228

229 **Role of TGF- β RII on PC cells sensitivity to gemcitabine**

230 We investigated the effect of TGF- β RII silencing on CAPAN-1 and CAPAN-2 cell sensitivity
 231 to gemcitabine. We show that the lack of TGF- β RII induces a significant increase of
 232 resistance to gemcitabine treatment in both CAPAN-1 (87-152% increase of survival rate,
 233 Figure 3A) and CAPAN-2 (50-161% increase, Figure 3B) cell lines compared to NT control
 234 cells. All differences were statistically significant. We then carried out SC xenograft of NT or
 235 TGF- β RII-KD8 CAPAN-2 cells that were subsequently treated with gemcitabine for 46 days.
 236 Gemcitabine treatment decreased the normalized tumor volume in CAPAN-2 NT xenografts
 237 (2.7 ± 1.02 vs 1.4 ± 0.11 at D83) compared to initial tumor volume (D36). On the contrary, the
 238 tumor growth was exacerbated in TGF- β RII-KD xenografts following gemcitabine treatment
 239 (2.9 ± 1.7 vs 4.36 ± 1 at D83) (Figure 3C). Altogether, our results suggest that TGF- β RII alters
 240 sensitivity of PC cells to gemcitabine both in vitro and in vivo.

241

242 **Identification of signalling pathways altered following TGF- β RII knocking down**

243 Impact of TGF- β RII knocking-down on intracellular signaling was studied using phospho
 244 array that detect relative site-specific phosphorylation of 43 proteins simultaneously (Figure
 245 4). Intensities of each spots for TGF- β RII were measured and normalized to the CAPAN-2
 246 NT proteins (Figure 4A). We observed an important increase of phosphorylation of S63 c-Jun
 247 (3.3-fold) and S727STAT3 (1.5-fold) in CAPAN-2 TGF- β RII-KD8 compared to NT cells. We
 248 also observed a mild decrease of phosphorylation of Y694 STAT5a (0.6-fold) and β -catenin
 249 (0.5-fold) (Figure 4B). Similar experiments were conducted for CAPAN-1 TGF- β RII-KD8 and
 250 NT cells. Only weak variations were observed (30%). By western blotting, we confirmed the
 251 increased of phospho-S727 STAT3 (4.49-fold) (Figure 5A) and phospho-S63 (7-fold)
 252 (supplemental figure 1A) in CAPAN-2 TGF- β RII-KD8 cells compared to CAPAN-2 NT cells.

Gemcitabine treatment also induced an increase of phospho-S727 STAT3 (1.95-fold) (Figure 5A) and phospho-S63 c-Jun (3.18-fold) (supplemental figure 1A) in NT cells (compared to untreated cells). This effect was not found in TGF- β RII-KD8 cells. We then performed immunohistochemistry for STAT3 and c-Jun in NT or TGF- β RII-KD8 CAPAN-2 SC xenografts (Figure 5B and supplemental figure 1B). Nuclear and cytoplasmic IHC staining were scored. We show that STAT3 nuclear H-score in TGF- β RII-KD8 tumors was significantly higher than in NT tumors (*, $p=0.0429$) (Figure 5C). We also observed that STAT3 nuclear staining was increased following gemcitabine treatment (*, $p=0.0286$). A mild increase of nuclear STAT3 was observed in TGF- β RII-KD8 tumors following gemcitabine treatment but was not statistically significant ($p=0.33$) (Figure 5C). No alteration of c-jun expression was observed in NT and TGF- β RII-KD8 untreated xenograft tumors (supplemental figure 1B). H score measurement indicates that gemcitabine treatment led to a significant decrease of cJun staining in TGF- β RII-KD8 tumors (Supplemental figure 1C). Altogether, our results indicate that TGF- β RII signalling implicates STAT3 and c-Jun phosphorylation in pancreatic cancer cells.

TGF- β RII silencing alters the expression of ABC transporters and EMT markers in PC cells

To go further and understand which molecular mechanisms could be responsible for the induced chemoresistance, we investigated the effect of TGF- β RII silencing on the expression of ATP-binding cassette (ABC) transporters that are commonly known to confer resistance to xenobiotics including chemotherapeutic drugs. Using qPCR, we investigated the expression of ABCB1/MDR1, ABCC1/MRP1, ABCC2/MRP2, ABCC3/MRP3, ABCC4/MRP4, ABCC5/MRP5 and ABCG2 in NT and TGF- β RII-KD CAPAN-1 and CAPAN-2 cells. MRP1 was not detected. We found that MDR1 (x4.2-fold, **), ABCG2 (x1.9-fold, ***) and MRP4 (x1.4-fold, *) mRNA levels were significantly increased in TGF- β RII-KD CAPAN-1 cells

compared to NT cells (Figure 6A). MRP3 mRNA level was decreased in TGF- β RII-KD CAPAN-1 cells (x0.42-fold, ***) and CAPAN-2 (x0.65-fold, p=0.13) (Figure 6A). TGF- β RII and ABC transporter expression was analyzed from 44 pancreatic cancer cell lines from CCLE. We showed that TGF- β RII mRNA relative level was correlated with expression of MRP3 (Pearson $r=0.3856$, $p=0.0097$) (Figure 6B) and inversely correlated with MRP4 (Pearson $r=-0.3691$, $p=0.037$) (Figure 6C).

Furthermore, TGF- β is commonly described as an inducer of epithelial-mesenchymal transition (EMT) that is associated with chemoresistance (Voulgari & Pintzas, 2009). We performed vimentin (mesenchymal marker) and E-cadherin (epithelial marker) immunohistochemical staining on FFPE sections of NT or TGF- β RII-KD CAPAN-2 xenografts treated with gemcitabine to check their status. Surprisingly, we observed a slight increase of vimentin in TGF- β RII-KD CAPAN-2 cells xenografts compared to NT tumors (supplemental figure 2). Moreover, we found that gemcitabine treatment induced a loss of E-cadherin staining and a gain of vimentin staining suggesting an EMT phenotype following gemcitabine treatment.

Altogether, these results suggest that TGF- β RII silencing alters MRP3 and MRP4 ABC transporters expression in pancreatic cancer cells and induces a partial EMT phenotype that could lead to chemoresistance to gemcitabine.

Discussion

In the present manuscript, we characterized pancreatic cancer cell lines stably invalidated for TGF- β RII and investigated the consequences on both biological properties and response to chemotherapy in vitro and in vivo. We show an increase of tumor growth and a reduction of cell migration. We also show for the first time an increased resistance to gemcitabine that could be mediated by S727 STAT3 phosphorylation and via deregulation of MRP3 and MRP4 ABC transporter expression. TGF- β signalling pathway has been described as a double edge sword during carcinogenesis (Akhurst & Derynck, 2001); acting as a tumor suppressor in the early stages but promoting metastasis in the advanced carcinoma (Principe et al., 2014). Moses's laboratory generated TGF- β RII knock out mice crossed with Ptf1a-Cre; LSL-KrasG12D and showed that compound mice developed well differentiated PDAC (Ijichi et al., 2006) mostly highlighting the role as a tumor suppressor. TGF β R2 targeting by a monoclonal antibody is also effective at reducing metastasis (Ostapoff et al., 2014). In our cellular models, we confirmed that TGF- β RII inhibition led to an increased tumor growth in vivo and TGF- β RIIKD CAPAN-2 tumors led to less metastasis in the liver.

STAT3 targeting has been proposed as a therapeutic target in pancreatic adenocarcinoma (Sahu et al., 2017). Combined treatments of gemcitabine and a JAK inhibitor (AZD1480) led to stroma remodeling, increased density of microvessel, enhanced drug delivery and improved survival of in Ptf1a-Cre; LSL-KrasG12D; TGF- β RII^{KO} vivo models suggesting an effect of the treatment via the stroma (Nagathihalli et al., 2015). S727 phosphorylation was previously studied in prostate carcinogenesis and was shown to promote cell survival and cell invasion (Qin et al., 2008). In the present work, we also showed that TGF- β RII inhibition led to STAT3 S727 phosphorylation and increased gemcitabine resistance of the tumor cells suggesting the crucial role of STAT3 in both tumor and stromal cells. STAT3 knockdown was shown to be associated with increased response to gemcitabine in pancreatic cancer cells

(Venkatasubbarao et al., 2013). It is interesting to note that Erlotinib treatment that inhibits epidermal growth factor receptor (EGFR) tyrosine kinase also inhibited phosphorylation of STAT3 (Miyabayashi et al., 2013). Among the targeted therapy for PDAC, erlotinib associated with gemcitabine is the only drug showing statistically significantly improved survival (Moore et al., 2007).

TGF- β signaling is mediated through canonical SMAD and non-canonical non-SMAD pathways (Principe et al., 2014). Accordingly, we only observed increase of c-Jun and STAT3 phosphorylation in CAPAN-2 TGF- β RIIKD cells but not in the CAPAN-1 model that is SMAD4 mutated. It was previously shown that STAT3-induced senescence requires functional TGF β R signaling and notably a functional SMAD3/SMAD4 pathway. STAT3 promotes SMAD3 nuclear localization (Bryson et al., 2017). We hypothesize that the TGF- β RIIKD-induced gemcitabine resistance, shown in the present manuscript, is mediated by STAT3 which similarly requires SMAD3/SMAD4 dependent pathway.

In Smad4 mutated CAPAN-1 cells we observed an increased expression of MRP4, ABCG2 and MDR1. This increased expression could be responsible for the gemcitabine resistance of CAPAN-1 TGF- β RIIKD cells. The link between ABC transporters and TGF- β pathway is scarcely described. TGF- β 1 has been shown to upregulate ABCG2 expression in MiaPACA2 pancreatic cells which is contradictory with our findings (Kali et al., 2017). In breast cancer cells, silencing of TGF- β RII leads to overexpression of multidrug resistance protein ABCG2 and tamoxifen resistance (Busch et al., 2015).

TGF- β is usually considered as a bona fide inducer of EMT (Wendt et al., 2009). However, we were surprised to observe that TGF- β RII inhibition led to a partial EMT with an increase of vimentin. STAT3 signaling is linked to cancer cell plasticity and is able to promote EMT and CSC expansion (Junk et al., 2017). Previous work also showed that IL6, secreted by pancreatic stellate cells, triggers STAT3 activation in pancreatic cells which subsequently

350 induces EMT via Nrf2 (Wu et al., 2017). Therefore, we hypothesize that the paradoxal EMT
351 observed in TGF- β RII cells is a consequence of the STAT3 phosphorylation on S727.

352

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References

- Akhurst, R.J. & Derynck, R. TGF-beta signaling in cancer--a double-edged sword *Trends Cell Biol* 2001. 11: S44-51.
- Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A.A., Kim, S., et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity *Nature* 2012. 483: 603-7.
- Bryson, B.L., Junk, D.J., Cipriano, R. & Jackson, M.W. STAT3-mediated SMAD3 activation underlies Oncostatin M-induced Senescence *Cell Cycle* 2017. 16: 319-334.
- Busch, S., Sims, A.H., Stal, O., Ferno, M. & Landberg, G. Loss of TGFbeta Receptor Type 2 Expression Impairs Estrogen Response and Confers Tamoxifen Resistance *Cancer Res* 2015. 75: 1457-69.
- Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data *Cancer Discov* 2012. 2: 401-4.
- Derynck, R., Akhurst, R.J. & Balmain, A. TGF-beta signaling in tumor suppression and cancer progression *Nat Genet* 2001. 29: 117-29.
- Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal *Sci Signal* 2013. 6: p11.
- Ijichi, H., Chytil, A., Gorska, A.E., Aakre, M.E., Fujitani, Y., Fujitani, S., et al. Aggressive pancreatic ductal adenocarcinoma in mice caused by pancreas-specific blockade of transforming growth factor-beta signaling in cooperation with active Kras expression *Genes Dev* 2006. 20: 3147-60.
- Jonckheere, N., Fauquette, V., Stechly, L., Saint-Laurent, N., Aubert, S., Susini, C., et al. Tumour growth and resistance to gemcitabine of pancreatic cancer cells are decreased by AP-2alpha overexpression *Br J Cancer* 2009. 101: 637-44.
- Jonckheere, N., Perrais, M., Mariette, C., Batra, S.K., Aubert, J.P., Pigny, P., et al. A role for human MUC4 mucin gene, the ErbB2 ligand, as a target of TGF-beta in pancreatic carcinogenesis *Oncogene* 2004. 23: 5729-38.

398 Jonckheere, N., Skrypek, N., Merlin, J., Dessein, A.F., Dumont, P., Leteurtre, E., et al. The mucin
399 MUC4 and its membrane partner ErbB2 regulate biological properties of human CAPAN-2
400 pancreatic cancer cells via different signalling pathways *PLoS One* 2012. 7: e32232.

401 Junk, D.J., Bryson, B.L., Smigiel, J.M., Parameswaran, N., Bartel, C.A. & Jackson, M.W. Oncostatin M
402 promotes cancer cell plasticity through cooperative STAT3-SMAD3 signaling *Oncogene* 2017.
403 36: 4001-4013.

404 Kali, A., Ostapchuk, Y.O. & Belyaev, N.N. TNFalpha and TGFbeta-1 synergistically increase the
405 cancer stem cell properties of MiaPaCa-2 cells *Oncol Lett* 2017. 14: 4647-4658.

406 Kleeff, J., Korc, M., Apte, M., La Vecchia, C., Johnson, C.D., Biankin, A.V., et al. Pancreatic cancer
407 *Nat Rev Dis Primers* 2016. 2: 16022.

408 Mesquita, P., Jonckheere, N., Almeida, R., Ducourouble, M.P., Serpa, J., Silva, E., et al. Human
409 MUC2 mucin gene is transcriptionally regulated by Cdx homeodomain proteins in
410 gastrointestinal carcinoma cell lines *J Biol Chem* 2003. 278: 51549-56.

411 Miyabayashi, K., Ijichi, H., Mohri, D., Tada, M., Yamamoto, K., Asaoka, Y., et al. Erlotinib prolongs
412 survival in pancreatic cancer by blocking gemcitabine-induced MAPK signals *Cancer Res*
413 2013. 73: 2221-34.

414 Moore, M.J., Goldstein, D., Hamm, J., Figer, A., Hecht, J.R., Gallinger, S., et al. Erlotinib plus
415 gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a
416 phase III trial of the National Cancer Institute of Canada Clinical Trials Group *J Clin Oncol*
417 2007. 25: 1960-6.

418 Nagathihalli, N.S., Castellanos, J.A., Shi, C., Beesetty, Y., Reyzer, M.L., Caprioli, R., et al. Signal
419 Transducer and Activator of Transcription 3, Mediated Remodeling of the Tumor
420 Microenvironment Results in Enhanced Tumor Drug Delivery in a Mouse Model of Pancreatic
421 Cancer *Gastroenterology* 2015. 149: 1932-1943 e9.

422 Ostapoff, K.T., Cenik, B.K., Wang, M., Ye, R., Xu, X., Nugent, D., et al. Neutralizing murine
423 TGFbetaR2 promotes a differentiated tumor cell phenotype and inhibits pancreatic cancer
424 metastasis *Cancer Res* 2014. 74: 4996-5007.

425 Piessen, G., Jonckheere, N., Vincent, A., Hemon, B., Ducourouble, M.P., Copin, M.C., et al.
426 Regulation of the human mucin MUC4 by taurodeoxycholic and taurochenodeoxycholic bile

acids in oesophageal cancer cells is mediated by hepatocyte nuclear factor 1alpha *Biochem J* 2007. 402: 81-91.

Principe, D.R., Doll, J.A., Bauer, J., Jung, B., Munshi, H.G., Bartholin, L., et al. TGF-beta: duality of function between tumor prevention and carcinogenesis *J Natl Cancer Inst* 2014. 106: djt369.

Qin, H.R., Kim, H.J., Kim, J.Y., Hurt, E.M., Klarmann, G.J., Kawasaki, B.T., et al. Activation of signal transducer and activator of transcription 3 through a phosphomimetic serine 727 promotes prostate tumorigenesis independent of tyrosine 705 phosphorylation *Cancer Res* 2008. 68: 7736-41.

Rahib, L., Smith, B.D., Aizenberg, R., Rosenzweig, A.B., Fleshman, J.M. & Matrisian, L.M. Projecting Cancer Incidence and Deaths to 2030: The Unexpected Burden of Thyroid, Liver, and Pancreas Cancers in the United States *Cancer Res* 2014.

Sahu, N., Chan, E., Chu, F., Pham, T., Koeppen, H., Forrest, W., et al. Cotargeting of MEK and PDGFR/STAT3 Pathways to Treat Pancreatic Ductal Adenocarcinoma *Mol Cancer Ther* 2017. 16: 1729-1738.

Schutte, M., Hruban, R.H., Hedrick, L., Cho, K.R., Nadasdy, G.M., Weinstein, C.L., et al. DPC4 gene in various tumor types *Cancer Res* 1996. 56: 2527-30.

Skrypek, N., Duchene, B., Hebbar, M., Leteurtre, E., van Seuning, I. & Jonckheere, N. The MUC4 mucin mediates gemcitabine resistance of human pancreatic cancer cells via the Concentrative Nucleoside Transporter family *Oncogene* 2013. 32: 1714-23.

TCGA-Network. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma *Cancer Cell* 2017. 32: 185-203 e13.

van der Sluis, M., Melis, M.H., Jonckheere, N., Ducourouble, M.P., Buller, H.A., Renes, I., et al. The murine Muc2 mucin gene is transcriptionally regulated by the zinc-finger GATA-4 transcription factor in intestinal cells *Biochem Biophys Res Commun* 2004. 325: 952-60.

Van Seuning, I., Ostrowski, J., Bustelo, X.R., Sleath, P.R. & Bomsztyk, K. The K protein domain that recruits the interleukin 1-responsive K protein kinase lies adjacent to a cluster of c-Src and Vav SH3-binding sites. Implications that K protein acts as a docking platform *J Biol Chem* 1995. 270: 26976-85.

- 455 Van Seuning, I., Perrais, M., Pigny, P., Porchet, N. & Aubert, J.P. Sequence of the 5'-flanking region
456 and promoter activity of the human mucin gene MUC5B in different phenotypes of colon
457 cancer cells *Biochem J* 2000. 348 Pt 3: 675-86.
- 458 Venkatasubbarao, K., Peterson, L., Zhao, S., Hill, P., Cao, L., Zhou, Q., et al. Inhibiting signal
459 transducer and activator of transcription-3 increases response to gemcitabine and delays
460 progression of pancreatic cancer *Mol Cancer* 2013. 12: 104.
- 461 Vincent, A., Herman, J., Schulick, R., Hruban, R.H. & Goggins, M. Pancreatic cancer *Lancet* 2011.
462 378: 607-20.
- 463 Voulgari, A. & Pintzas, A. Epithelial-mesenchymal transition in cancer metastasis: mechanisms,
464 markers and strategies to overcome drug resistance in the clinic *Biochim Biophys Acta* 2009.
465 1796: 75-90.
- 466 Wendt, M.K., Allington, T.M. & Schiemann, W.P. Mechanisms of the epithelial-mesenchymal transition
467 by TGF-beta *Future Oncol* 2009. 5: 1145-68.
- 468 Wu, Y.S., Chung, I., Wong, W.F., Masamune, A., Sim, M.S. & Looi, C.Y. Paracrine IL-6 signaling
469 mediates the effects of pancreatic stellate cells on epithelial-mesenchymal transition via
470 Stat3/Nrf2 pathway in pancreatic cancer cells *Biochim Biophys Acta* 2017. 1861: 296-306.

Figure legends

Figure 1: Establishment of TGF- β RII-KD CAPAN-1 and CAPAN-2 cell lines. (A) Analysis of mRNA expression of TGF- β 1, TGF- β 2, TGF- β RI, TGF- β RII, TGF- β RIII, Smad2, Smad3, Smad4, Smad ATP7A, ATP7B and 28S in CAPAN-1, CAPAN-2 cells by RT-PCR. (B) Analysis of mRNA relative expression of TGF- β RII in NT and TGF- β RII-KD CAPAN-1 and CAPAN-2 cell lines. Expression in NT cells was arbitrarily set to 1. (C) Smad-Binding-Elements (SBE) relative luciferase activity in untreated and TGF- β treated NT and TGF- β RII-KD CAPAN-2 cells. Relative luciferase activity was expressed as a ratio of SBE-Luc normalized with pGL3 basic activity. (D) Analysis of mRNA relative expression of TGF- β 1 in NT and TGF- β RII-KD CAPAN-1 and CAPAN-2 cell lines.

Figure 2: TGF- β RII alters tumor growth and migration in pancreatic cancer cells. (A) Wound healing closure of NT and TGF- β RII-KD CAPAN-1 and CAPAN-2 cell lines using the IncuCyte™ chamber apparatus. (B) Subcutaneous xenografts of NT/TGF- β RII-KD8 CAPAN-1 and CAPAN-2 cells in scid mice. Tumour growth (mm³) was evaluated until sacrifice. **p < 0.01 and ***p < 0.001 indicate statistical significance of TGF- β RII-KD1 compared with the NT control. Ns: not significant. (C) Evaluation of the presence of micro-metastasis in the liver by detecting the presence of human GAPDH in the liver of NT and TGF- β RII-KD CAPAN-1 and CAPAN-2 xenografted mice by qPCR.

Figure 3: TGF- β RII alters sensitivity to gemcitabine in pancreatic cancer cells in vitro and in vivo. Survival rates in different TGF- β RII-KD CAPAN-1 (A) and CAPAN-2 (B) cell lines or their NT control cells were measured following treatment with gemcitabine using the MTT assay. Results are expressed as % of cell survival (/untreated cells). Three independent experiments were performed. (C) Subcutaneous xenografts of NT and TGF- β RII-KD8 CAPAN-2 cells in scid mice. Gemcitabine (15 mg/kg) or PBS (200 μ l) were injected intra-peritoneously, twice a week once palpable tumors were developed. Normalized tumor growth

is expressed as the ratio of tumor progression relative to tumor volume on the first day of gemcitabine treatment. Right graph represents tumor growth over time. Left graph represents final tumor volume at day 83 (normalized as initial tumor volume at D36 equal to 1).

Figure 4: Impact of TGF- β RII knocking-down on signaling pathways. (A) Impact of TGF- β RII knocking-down on intracellular signaling was studied using phospho array that detect relative site-specific phosphorylation of 43 proteins. Boxes highlight spots for S63 c-Jun and S727 STAT3. (B) Heatmap representing the intensities of each spots (TGF- β RII vs NT) that were measured and normalized to the reference spots for CAPAN-1 and CAPAN-2 cells.

Figure 5: TGF- β RII knockdown promotes STAT3 phosphorylation and nuclear localisation in CAPAN-2 cells. (A) STAT3, phospho-S727 STAT3 and β -actin expression was analysed by western blotting. Bands intensities were quantified by densitometry and ratios (KD vs NT or treated/untreated) are indicated in the graphs. Expression in NT (for TGF- β RIIKD) or untreated (for gemcitabine/TGF- β) cells was arbitrarily set to 1. (B) IHC analysis of STAT3 on extracted xenografted NT and TGF- β RIIKD tumors. (C) Nuclear and cytoplasmic IHC staining were scored in NT and TGF- β RIIKD xenografted tumors that were treated with gemcitabine or PBS. * $p < 0.05$ indicates statistical significance of TGF- β RII-KD1 compared with the NT control.

Figure 6: TGF- β RII silencing alters ABC transporters expression. (A) mRNA expression of *TGF- β RII*, *c-Jun*, *STAT3*, *MRP1*, *MRP2*, *MRP3*, *MRP4*, *MRP5*, *ABCG2* and *MDR1* was analyzed in NT and TGF- β RII-KD CAPAN-1 and CAPAN-2 cells by qRT-PCR. The histogram represents the ratio of their expression in TGF- β RII-KD compared with NT cells. Three independent experiments were performed. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistical significance of TGF- β RII-KD1 compared with the NT control. TGF- β RII, MRP3 (B) and MRP4 (C) mRNA expression was extracted from pancreatic cell lines from

529 Cancer Cell Line Encyclopedia (CCLE). Statistical analyses of MRP3/TGF β RII and
 530 MRP3/TGF- β RII correlations were analysed Pearson's correlation coefficient.

531

532 **Table 1 : Primers used for RT-PCR and qPCR experiments**
 533

Supplemental material

Supplemental Figure 1: TGF- β RII knockdown promotes c-Jun S-63 phosphorylation in CAPAN-2 cells. (A) c-Jun, phospho-S63 c-Jun and β -actin expression was analysed by western blotting. Bands intensities were quantified by densitometry and ratios (KD vs NT or treated/untreated) are indicated in the graphs. Expression in NT (for TGF- β RIIKD) or untreated (for gemcitabine/TGF- β) cells was arbitrarily set to 1. (B) IHC analysis of c-Jun on extracted xenografted NT and TGF- β RIIKD tumors. (C) IHC staining was scored in NT and TGF- β RIIKD xenografted tumors that were treated with gemcitabine or PBS. * $p < 0.05$ indicate statistical significance of TGF- β RII-KD compared with the NT control.

Supplemental Figure 2: TGF- β RII knockdown promotes partial EMT-like phenotype. IHC analysis of E-cadherin and vimentin on extracted xenografted NT and TGF- β RIIKD tumors. IHC staining was scored in NT and TGF- β RIIKD xenografted tumors that were treated with gemcitabine or PBS. * $p < 0.05$, ** $p < 0.01$ indicate statistical significance of TGF- β RII-KD compared with the NT control.

Figure 1

	Metastasis (%)	p, Fisher test
CAPAN-1 NT	4/6 (66%)	1.0
CAPAN-1 TGF- β R2KD8	4/6 (66%)	
CAPAN-2 NT	5/7 (50%)	0.058
CAPAN-2 TGF- β R2KD8	2/10 (20%)	
No xenograft	0/3 (0%)	

Figure 2

A

B

C

Figure 4

Figure 5

Figure 6

Table 1 : Primers used for RT-PCR and qPCR experiments

Gene	Orientation	Sequences of primers (5'-3')	T ^o m (°C)	Expected size
RT-PCR				
<i>28S</i>	Forward Reverse	GCAGGGCGAAGCAGAAGGAAACT TGAGATCGTTTCGGCCCCAA	59	231
<i>TGF-β1</i>	Forward Reverse	GAGGTGACCTGGCCACCATTCAT CCAGCCGAGGTCCCTTGCGGA	60	194
<i>TGF-β2</i>	Forward Reverse	GCTTTTCTGATCCTGCATCTG CAATACCTGCAAATCTTGCTTC	56	823
<i>TGF-βRI</i>	Forward Reverse	CTCTCCTTTTTTCTTCAGATCTGC AATCCAACCTCCTTTGCCCTT	55	328
<i>TGF-βRII</i>	Forward Reverse	GCCAACAACATCAACCACAACACA TAGTGTTTAGGGAGCCGTCTTCAG	61	1003
<i>TGF-βRIII</i>	Forward Reverse	TGCCTTACTTCTCTTGCCTTAA GCAAAGTGGCATCATATTATT	56	100
<i>Smad2</i>	Forward Reverse	GTCCATCTTGCCATTCACG TGGTGATGGCTTTCTCAAGC	55	192
<i>Smad3</i>	Forward Reverse	GGGCTCCCTCATGTCATCTA GGCTCGCAGTAGGTAAGTGG	60	443
<i>Smad4</i>	Forward Reverse	CTCCTGAGTATTGGTGTTC CTAAAGGTTGTGGGTCTGC	56	796
<i>Smad7</i>	Forward Reverse	GGCTCGCAGTAGGTAAGTGG TTGTTGTCCGAATTGAGCTG	55	448
qPCR				
<i>TGF-β1</i>	Forward Reverse	CACTCTCAAACCTTTACGAGACC CGTTGCTAGGGGCGAAGATG	58	131
<i>TGF-βRII</i>	Forward Reverse	AGGAGTATGCCTCTTGGAAGAC AGCCAGTATTGTTTCCCCAAC	58	123
<i>Human GADPH</i>	Forward Reverse	CCACATCGCTCAGACACCAT CCAGGCGCCCAATACG	58	70
<i>Mouse GADPH</i>	Forward Reverse	AGGTCGGTGTGAACGGATTTG TGTAGACCATGTAGTTGAGGTCA	58	129