

WNT5A – target of CUTL1 and potent modulator of tumor cell migration and invasion in pancreatic cancer

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ABSTRACT

Previously, we have identified the transcription factor CUTL1 as important mediator of tumor invasion and target of TGF-beta. Using high-throughput approaches, we identified several putative downstream effectors of CUTL1, among them WNT5A, a secreted member of the Wnt multi-gene family. The aim of this study was to investigate the role of WNT5A as a novel target of CUTL1 in pancreatic cancer. CUTL1 and WNT5A were stably overexpressed as well as transiently and stably knocked-down by RNA interference. Effects on proliferation, migration and invasiveness were investigated by thymidine incorporation, Boyden chamber experiments and time-lapse microscopy. Expression of WNT5A in pancreatic cancer tissues was analyzed by real-time PCR and immunohistochemistry. We found that CUTL1 transcriptionally upregulated WNT5A on RNA, protein and promoter level. WNT5A significantly enhanced migration, proliferation and invasiveness, mediating the pro-invasive effects of CUTL1 to a major extent. WNT5A effects were accompanied by a marked modulation of marker genes associated with epithelial-mesenchymal-transition (EMT). Using real-time PCR and immunohistochemistry, we found that WNT5A is up-regulated early during pancreatic cancerogenesis in pancreatic intraepithelial neoplasias (PanIN lesions) and in invasive pancreatic adenocarcinomas, as compared to normal pancreas tissues. These data identify WNT5A as important target of CUTL1 and as novel mediator of invasiveness and tumor progression in pancreatic cancer.

INTRODUCTION

The homeobox transcription factor CUTL1, also known as CDP (CCAAT displacement protein), is evolutionarily highly conserved and known to modulate cell growth and differentiation in *Drosophila* and mammals [1-6]. Knockout studies with the murine homologue, Cux-1, revealed reduced growth and numerous developmental defects including retarded differentiation of the lung epithelia, hair follicle defects, reduced male fertility and deficient T and B cell function [7-9]. In contrast, mice transgenic for Cux-1 showed organomegaly and multiorgan hyperplasia [10].

Recently, we have identified CUTL1 as an important mediator of tumor cell motility and invasiveness in a variety of tumor cell systems *in vitro* and *in vivo* [11]. In addition, we could show that CUTL1 is a transcriptional target of TGF-beta, thereby mediating pro-migratory effects of TGF-beta. The important role of CUTL1 in promoting cell motility and invasiveness is underlined by the fact that CUTL1 expression is strongly associated with a less differentiated phenotype and decreased patient survival in invasive breast cancer [11].

The Wnt family of proteins are secreted cysteine-rich glycoproteins with hydrophobic signal peptides. Wnt proteins lack transmembrane domains and are posttranslationally modified by N-linked glycosylation [12]. They associate with cell surfaces and the extracellular matrix, and many are known to bind to the Frizzled family of receptors [13]. Various Wnt genes are differentially expressed during embryogenesis and among various tissues, implicating distinct roles for each Wnt family member.

In addition to its pivotal role in embryogenesis and development, wnt signaling has been identified as major modulator of tumorigenesis. The abnormal expression of several Wnt family members including WNT1, WNT3A, WNT7A and, to a lesser extent, WNT2, WNT5B, and WNT7B, leads to transformation of various cell systems

[14;15]. In contrast, WNT4, WNT5A, and WNT6 fail to induce cell transformation in these assays [15]. Many Wnt members such as WNT1 can signal via Frizzled receptors to hyperphosphorylate and activate the disheveled gene (Dsh), resulting in the inhibition of GSK3beta-activity and the subsequent stabilization of its target beta-catenin [16]. Beta-catenin can then accumulate in the nucleus, altering chromatin structure, which results in differential gene expression [17]. Other Wnt members may also signal through alternative, “noncanonical” pathways such as the Wnt/calcium pathway involving activation of phospholipase C, increased turnover of membrane-bound phospholipids and subsequent release of intracellular calcium, which activates protein kinase C(PKC) and calmodulin-dependent protein kinase II (CamKII) [18;19].

The role of WNT5A in tumorigenesis remains ambiguous. In several cellular models including hematopoietic tissue, breast and uroepithelial cancers it has been shown to inhibit tumor cell proliferation [20-22]. Moreover, WNT5A has been identified as good prognostic marker in breast cancer patients [23].

However, there are several other lines of evidence indicating that increased WNT5A expression is indeed important for cancer progression. WNT5A has been demonstrated as potent enhancer of cell motility and invasiveness in melanomas [19] and is up-regulated in various malignancies including cancers of the lung, stomach, and prostate [24-27].

The aim of this study was to elucidate downstream targets of CUTL1 which are crucial for its effect on tumor invasion. Based on previous microarray results, we identify WNT5A as transcriptional target of CUTL1 and demonstrate its pivotal role as modulator of CUTL1-induced tumor cell migration and invasion. Furthermore, we show that WNT5A is progressively up-regulated during pancreatic tumorigenesis in pancreatic intraepithelial lesions (PanIN's) and is highly expressed in invasive pancreatic cancer.

MATERIALS AND METHODS

Materials and cell lines

Puromycin was obtained from Sigma-Aldrich (Saint Louis, MI) and used at a working concentration of 2,5µg/ml. Hygromycin B was purchased from Invitrogen (Karlsruhe, Germany) and used at a working concentration of 400µg/ml. Lithium chloride was obtained from Sigma and used at a working concentration of 10nM. MiaPaca2, PANC1 and HT1080 cell lines were obtained from ATCC. ImimPC1 cells were kindly provided by F.X. Real [28]. Cells were maintained in Dulbecco`s modified minimal essential medium (GIBCO, Invitrogen Corp., NY) supplemented with 10% fetal calf serum (GIBCO), 100µg/ml streptomycin and 100U/ml penicillin. All cell lines were grown at 37°C in 5% CO₂. Recombinant human WNT5A protein was purchased from Abcam (Cambridge, MA) and used at a concentration of 500ng/ml.

Plasmids and siRNA

Myc-tagged full-length human CUTL1 in pMX, as well as C-terminal CUTL1aa831-1336 in pXJ were kind gifts from A.Nepveu (McGill University, Canada). The hLef1 plasmid was a kind gift of D. Wedlich (University of Karlsruhe, Germany). The dominant negative hLef1 construct was a kind gift of D. Gradl (University of Karlsruhe, Germany). The shRNA pRetrosuper plasmid for human CUTL1 and the luciferase plasmid containing the WNT5A promoter were described previously [11]. The TOP/FOPflash TCF/LEF reporter constructs were purchased from Upstate Biotechnology (Schwalbach, Germany).

The open reading frame for human WNT5A was PCR-amplified from the human cDNA clone MGC:71588 IMAGE:30346200 and cloned into the tetracycline-inducible expression vector pBig2i [40], which was a kind gift of J.R. Hall, using the KpnI and

SpeI restriction sites. The pBig2i-Wnt5a and empty pBig2i vector were each transfected into PANC-1 cells using Transfast transfection reagent (Promega, Madison, WI, USA). Using this method, a transfection efficiency of 25-30% could be typically achieved in PANC1 cells, as determined by transfection of pEGFP-CUTL1aa831-1336 (Ripka et al., manuscript in preparation). For generation of stable clones, cells were cultured in the presence of 400µg/ml hygromycin B. Stable expression of WNT5A was confirmed after incubation with doxycyclin (6µg/ml for 24 hours) via immunoblot analysis.

For stable suppression of CUTL1 expression by shRNA in PANC1 cells, the puromycin-resistant pRetrosuper vector containing the silencing sequence for CUTL1 (pRS-shCUTL) was used as described previously [11]. Stable clones were selected by adding 2.5 ug/ml puromycin (Calbiochem) to the growth medium 24 hours after transfection with pRS-shCUTL or empty vector.

For transient transfection of hWnt5a siRNA, the following two sequences were used: hWNT5A_1 5'-GGUUGUAAUUGAAGCCAAUtt -3', hWNT5A_2 5'-GGACUUUCUCAAGGACAGAtt-3'. CUTL1 was transiently suppressed by using two different oligonucleotides (hCUTL1_1 and hCUTL1_2) as described previously [11]. The RNAi oligonucleotides and their Alexa-labeled versions were purchased from Ambion (Austin, Tx). PANC1 and HT1080 cells were transfected using Transmessenger transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To optimize transfection efficacy, cells were transfected with siRNA twice, with an interval of 24 hours. ImimPC1 cells were transfected with the siRNA transfection reagent X-tremeGene (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. As non-silencing control, the silencer negative control siRNA from Ambion was used.

Quantitative Real-Time PCR analysis

RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and first strand cDNA was synthesized using random hexamer primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Quantitative RT-PCR analysis was performed using an ABI PRISM 7700 Sequence Detector System and the SYBR Green PCR Master Mix kit (Applied Biosystems, Wellesley, MA) according to the manufacturer's instructions. The RT-PCR was performed with sequence-specific primer pairs determined with the PrimerExpress® program (Applied Biosystems, Wellesley, MA). Primers for cyclophilin A or the ribosomal protein RPLP0 (NM_001002) were used as internal standard. Primer sequences are available on request.

Immunoblotting

Cells were incubated in lysis buffer (50mM HEPES [pH 7,5], 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10 mM Na₄P₂O₇, 10% glycerol, 1% TritonX-100) supplemented with a cocktail of protease inhibitors (Complete, Roche Applied Science, Mannheim, Germany). Proteins were separated with 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Immunoblots were probed with primary antibodies against WNT5A (R&D Systems, MN), CUTL1 (antibody prepared as previously described) [11], E-Cadherin, phospho-GSK-3beta (serine 9), total GSK-3beta, beta-catenin, phospho-specific antibodies for PKC's including phospho-PKCalpha/beta (Thr638/641), phospho-PKC(pan)(betaII Ser660), phospho-PKCdelta (Thr505), phospho-PKCdelta (Ser643), phospho-PKD/PKC (Ser744/748), phospho-PKD/PKC (Ser916), phospho-PKCtheta (Thr538) and PhosphoPKCzeta/lambda (Thr410/403) (Cell Signaling Technologies, Danvers, MA), vimentin, lamin A/C and beta-actin (Sigma-Aldrich, Saint Louis, MI) and subsequently incubated with

peroxidase-conjugated secondary antibodies (Sigma). Blots were detected by ECL chemiluminescence (Amersham). CaM Kinase II activity was determined using an CaM Kinase II Assay Kit from Upstate according to the manufacturer's instructions. Nuclear and cytoplasmic extracts for beta-catenin blots were performed as described previously [29].

Luciferase reporter assays

PANC1 cells were transfected with the luciferase plasmids using Transfast transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions, and harvested 24 hours after transfection. Luciferase assays were performed with a DLReady™ Luminometer (Berthold Technologies) and the Dual-Luciferase®-Reporter Assay System (Promega, Madison, Wis, USA). Because the internal control plasmid is itself often repressed by CUTL1 and its targets, as a control for transfection efficiency, the purified beta-galactosidase protein (Sigma) was included in the transfection mix, as previously described [4], [30], [11]. Beta-Galactosidase activity was detected using 2-Nitrophenyl beta-D-galactopyranoside (ONPG, Sigma) in substrate buffer (1 mM MgCl₂, 1 mg/ml ONPG, 75 mM sodium phosphate buffer [pH 7,4], 60 mM β-mercaptoethanol). After 30 minutes incubation at 37°C, activity was measured at 420nm.

[³H]Thymidin Incorporation

Transiently transfected cells were seeded at a density of 5x10⁴ cells/well in 24-well plates. 24 hours later, 5 μCi/well of [³H]thymidine were added at 37°C for 4 hours. Subsequently, cells were treated with 5% ice-cold TCA for 30 min, washed twice with ice-cold water, and solubilized in 1N sodium hydroxide. The cell-associated radioactivity was determined by liquid scintillation counting.

Two-chamber migration and invasion assays

Migration and invasion assays were performed as described previously [11]. In brief, cells transfected with siRNA were trypsinated, counted and used for modified two-chamber migration assay (8 μm pore size, BD Biosciences, Bedford, MA) or invasion assay (BD BioCoat Matrigel invasion Chamber, 8 μm , BD Biosciences) as described [11].

The number of migrated/invaded cells was measured by counting of the fixed and stained cells (crystal violet 0.2% / methanol 20%) at the bottom of the membrane or by the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega, Madison, WI, USA). This assay determines the number of viable cells which have migrated through the membrane based on quantitation of ATP that indicates the presence of metabolically active cells. The number of migrated cells was normalized to the total number of proliferating cells in a neighbor well.

In migration assays with cells transiently transfected with expression plasmids, cells which migrated through the pores were counted under the microscope. To selectively assess the migration of transiently transfected cells, co-transfections with LacZ plasmid were performed. After 4 hours, cells which had migrated through the pores were fixed and stained by X-Gal stain (Sigma). Migration was calculated by counting blue-stained cells. The number of migrated cells was normalized to the total number of transfected cells in a adjacent well.

Time-lapse microscopy

For time-lapse microscopy, 1×10^5 cells were seeded on a coverslip and incubated for 12 hours at 37°C and 5% CO₂. Subsequently, imaging was performed over 20 hours

in serum-containing medium at 37°C. Images were obtained with an Olympus IX-71 microscope every 15 minutes. Migration of each cell was analyzed by measuring the distance traveled by a cell nucleus over the 20-h time period using the ImageJ software. The average migration speed was calculated by analyzing at least 30 cells/group.

Tissues and immunohistochemistry

For immunohistochemistry, eight human pancreatic adenocarcinoma tissues and adjacent normal tissues were provided by the Department of Surgery at the University of Ulm. All tissues were obtained after approval by the local ethics committee. In addition, eight pancreatic adenocarcinoma tissues and matched adjacent normal tissues were used from a Petagen **AccuMax A207(III)** multiple tissue array (Biomol, Hamburg, Germany). Immunohistochemical analysis was performed as previously described [31]. In short, paraffin sections were stained with rabbit anti-WNT5A (1:200). Antibody binding was visualized using a biotinylated secondary antibody, avidine conjugated peroxidase (ABC method; Vector Laboratories) and 3,3'-diaminobenzidine tetrachloride (DAB) as a substrate and hematoxylin as counterstain.

Microdissection and linear amplification of pancreatic tissues

Microdissected tissues were prepared as described previously [32]. All tissues were obtained after approval by the local ethics committees and informed consent was obtained from all patients undergoing surgery. In brief, normal pancreatic ducts, pancreatic intraepithelial neoplasias (PanIN1-3) and pancreatic adenocarcinomas, derived from 5 patients each, were microdissected from frozen pancreatic tissues. Subsequently, 5–50 ng of purified total RNA was linearly amplified in the presence of

UTP and 5-(3-aminoallyl)-UTP (each 3.75mM) using the MessageAMPt aRNA Kit (Ambion), according to the manufacturer's instructions [32]. The quality and quantity of the total and amplified RNA samples was determined with a 2100 Agilent Bioanalyser (Agilent Technologies, Palo Alto, CA, USA), as described previously [32].

Statistical analysis

Statistical analyses were performed using the double-sided unpaired students t-test after Bonferroni correction for multiple testing, where appropriate.

RESULTS

CUTL1 induces WNT5A expression

In our search for transcriptional downstream targets of CUTL1 which are crucial for its effect on tumor invasion, we have previously performed microarray analyses using the highly motile NIH3T3 cell line with stable knock-down of CUTL1 by RNA interference. We found that, among others, the mRNA coding for WNT5A was significantly induced by CUTL1 [11].

Based on these high-throughput results, we examined whether WNT5A is indeed a transcriptional target of CUTL1. For most of our experiments, we knocked-down CUTL1 and/or WNT5A by siRNA to avoid potential biases introduced by over-expression techniques. To minimize off-target effects, we confirmed all results by two different siRNA oligonucleotides targeting different regions of the CUTL1 or WNT5A mRNA's. All of these silencing sequences were able to decrease CUTL1 or WNT5A

protein expression by at least 70%. By using Alexa-labelled siRNA oligos, we confirmed that more than 80% of the cells were typically transfected by siRNA (data not shown).

Using PANC1 and ImimPC1 pancreatic cancer cells and the fibrosarcoma cell line HT1080, we show that transient knock-down of CUTL1 by siRNA results in a significant decrease of WNT5A mRNA, as measured by real-time PCR (Figure 1A). These results could be confirmed on protein level by immunoblotting: Knock-down of CUTL1 leads to a marked decrease in WNT5A protein levels in all tested pancreatic cancer cell lines and HT1080 cells (Figure 1B). In addition, we transiently overexpressed the C-terminal fragment of CUTL1 containing amino acids 831-1336 which has been shown to be more transcriptionally active than full-length CUTL1 [33]. Overexpression of CUTL1 resulted in elevated WNT5A protein levels in PANC1 and HT1080 cells (Figure 1C). These results suggest that WNT5A is induced by CUTL1 on mRNA and protein levels. To further confirm the transcriptional regulation of WNT5A by CUTL1, we performed luciferase assays using a plasmid containing the putative WNT5A promoter including 2kB upstream of the transcriptional start site of WNT5A mRNA [11]. Using these assays we could show that CUTL1 leads to a significant activation of the WNT5A promoter (Figure 1D), which corroborates the transcriptional activation of WNT5A induced by CUTL1. Since CUTL1 has been described to be proteolytically cleaved into a transcriptionally more active 110 kD fragment [33], we confirmed the presence of both full-length CUTL1 and the transcriptionally more active 110 kD C-terminal endogenous cleavage product (Figure 1D, lower panel).

WNT5A enhances migration and invasion of pancreatic cancer cells

Recently, we described CUTL1 as important mediator of TGFbeta-induced cell migration and invasiveness [11]. To investigate whether WNT5A mediates the effect of CUTL1, we performed migration assays in PANC1, ImimPC1, MiaPaca2 and HT1080 cells. First, we could demonstrate that with transient knock-down of WNT5A by siRNA, we could achieve a marked reduction of WNT5A protein levels (Figure 2A). Knock-down of WNT5A results in significantly reduced migration in all examined cell lines, as measured by Boyden chamber assays (Figure 2A). The proportion of migrating cells in the Boyden chambers was normalized to the total number of proliferating cells in a neighbor well to avoid a potential bias due to differences in proliferation. In addition, we performed time-lapse microscopy experiments with single-cell tracking using the highly motile mesenchymal cell line HT1080. By using these assays, we also observed significantly reduced motility when endogenous WNT5A was suppressed (Figure 2B).

To examine whether WNT5A also affects invasiveness involving movement through a three-dimensional matrix, we performed invasion assays with PANC1, ImimPC1 and MiaPaca2 pancreatic cancer cells and HT1080 fibrosarcoma cells. Invasion through a membrane with 8µm pores coated with a 30µm layer of Matrigel consisting of extracellular matrix proteins was markedly reduced up to 70% in PANC1, ImimPC1 and HT1080 cell lines, reaching significance for PANC1 and HT1080 cells, when endogenous WNT5A was transiently suppressed by siRNA transfection (Figure 2C). Interestingly, MiaPaca2 cells, which show a significant decrease in migration, respond only minimally to WNT5A knock-down in invasion assays (Figure 2C).

In addition to experiments with transient knock-down of WNT5A, we generated PANC1 cells stably transfected with a tetracyclin-inducible WNT5A expression

plasmid (WNT5A-pBIG2i). After induction of WNT5A expression by addition of doxycyclin, we observed a significant increase in both migration and invasion, as measured by modified Boyden assays (Figure 2D).

Since WNT5A is known to act as a secreted ligand, we performed further experiments with exogenously added recombinant human WNT5A protein. Stimulation with WNT5A protein resulted in a marked increase in migration, which reached significance in ImimPC1, MiaPaca and HT1080 cells (Figure 2E).

These data indicate that WNT5A enhances the migration and invasion of most examined cell lines to a significant extent.

WNT5A enhances proliferation of several pancreatic cancer cells

In addition to its effects on cell motility, CUTL1 is known to induce proliferation in a variety of cell lines [3]. We therefore examined whether its target WNT5A also mediates the effects of CUTL1 on proliferation of pancreatic cancer cells. Thymidin incorporation assays revealed that transient knock-down of WNT5A reduces cell proliferation in several cell lines such as MiaPaca2 and PANC1 pancreatic cancer cell lines and HT1080 cell lines to various degrees (Figure 3). In contrast, WNT5A has no significant effect on proliferation in ImimPC1 cells (Figure 3), despite of sufficient reduction in WNT5A protein levels (not shown) and a significant effect on migration (Figure 2A).

WNT5A is an important mediator of CUTL1-induced migration and invasion

To test the biological significance of WNT5A in CUTL1-induced migration and invasion, the effect of CUTL1 knock-down on migration and invasion was compared to the effect of WNT5A knock-down in the presence of CUTL1. Transient knock-down of WNT5A decreased both migration (Figure 4A) and invasion (Figure 4B) of PANC1

cells to a similar extent as the knock-down of CUTL1. Further knock-down of WNT5A in CUTL1 knock-down cells led to an additional decrease in migration, which was, however, less pronounced than the effect of knocked-down WNT5A in control cells with endogenous CUTL1 levels. Furthermore, transient over-expression of WNT5A in cells with stable CUTL1 knock-down was able to rescue the CUTL1 effect on migration (Figure 4C). These data indicate that WNT5A is a major downstream effector of the pro-migratory effects of CUTL1 although it can not be excluded that both CUTL1 and WNT5A interact with other additional signalling pathways and effector molecules.

WNT5A is up-regulated by TGFbeta via CUTL1, is associated with features of epithelial-mesenchymal transition (EMT) and involves beta-catenin-dependent signaling pathways

Previously, we identified CUTL1 as target of TGFbeta. Based on our observation that WNT5A is an important mediator of cell migration and invasion, we examined whether TGFbeta up-regulates WNT5A and whether CUTL1 is required for TGFbeta-induced up-regulation of WNT5A. Indeed, we found that WNT5A protein levels are increased upon TGFbeta treatment (Figure 5A). After transient knock-down of CUTL1, this effect was absent (Figure 5A), indicating that TGFbeta-induced WNT5A up-regulation functions via CUTL1. TGFbeta is known to enhance epithelial-mesenchymal transition (EMT) which is a characteristic feature of tumor progression and associated with enhanced cell migration and tumor invasiveness. Therefore, we were interested whether WNT5A as effector downstream of TGFbeta and CUTL1 leads to alterations in markers for EMT, such as E-cadherin and vimentin. As cell model, we used PANC1 cells which have previously been described to undergo EMT [34]. Interestingly, knock-down of WNT5A markedly increases expression of E-

cadherin, a marker for the epithelial phenotype. Moreover, knock-down of WNT5A decreases expression of vimentin as marker for mesenchymal differentiation (Figure 5B). These data suggest that the pro-migratory effects of WNT5A are associated with features of epithelial-mesenchymal transition in pancreatic cancer cells such as PANC1.

WNT5A has been described to signal via non-canonical calcium-dependent pathways as well as via the canonical, TCF-dependent pathway, depending on the receptor context [35]. Using PANC1 cells, we could show that addition of recombinant WNT5A caused a significant increase in TCF/LEF-dependent reporter activity (Figure 5C). Similar results were obtained in ImimPC1, MiaPaca and HT1080 cells (data not shown). Furthermore, induction of WNT5A expression with doxycyclin in pBig2i-WNT5A transfected cells led to a similar increase in TCF/LEF-dependent reporter activity, which was blocked upon cotransfection of dominant negative Lef1 (Figure 5D). The increase in TCF/LEF-dependent reporter activity was always significant, but did not reach the same extent as seen after cotransfection with Lef1 which was used as positive control (Figure 5D). Using the pancreatic cancer cell lines PANC1, ImimPC1 and MiaPaca, we could show that knock-down of WNT5A significantly inhibits TCF/LEF-dependent reporter activity (Figure 5E). In addition, knock-down of WNT5A led to a marked decrease in nuclear beta-catenin in all examined pancreatic cancer cells (Figure 5F). Moreover, addition of exogenous recombinant WNT5A protein resulted in increased nuclear beta-catenin in PANC1 cells, although did not significantly alter the cytoplasmic beta-catenin levels (Figure 5G). In addition, we performed experiments using lithium chloride as known activator of the canonical pathway and inhibitor of GSK-3beta. Interestingly, knock-down of WNT5A was able to decrease the phosphorylation of GSK-3beta at serine 9 which was induced by lithium chloride (Figure 5H). Although it is being discussed

controversely in the literature, several reports suggest that GSK-3beta is inhibited in canonical wnt signalling by phosphorylation at serine 9 [42],[43]. Therefore, inhibited phosphorylation of GSK-3beta by knock-down of WNT5A may also indicate involvement of the canonical pathway.

These data suggest that mediators of the canonical signalling pathway are involved in the signalling cascades induced by WNT5A in pancreatic cancer cells. In contrast, we did not detect any significant alterations in the phosphorylation of various PKC's using a panel of phospho-specific antibodies, among them antibodies for phospho-PKCalpha/beta (Thr638/641) and phospho-PKC(pan)(betaII Ser660) (Figure 5I). All other tested phospho-specific antibodies including phospho-PKCDelta (Thr505), phospho-PKCDelta (Ser643), phospho-PKD/PKC (Ser744/748), phospho-PKD/PKC (Ser916), phospho-PKCtheta (Thr538) and PhosphoPKCzeta/lambda (Thr410/403) did not reveal any significant phosphorylations in PANC1 cells (not shown). Calmodulin-dependent protein kinase II activity was slightly decreased upon WNT5A knock-down (Figure 5J), which, however, did never reach significance. This indicates that non-canonical, PKC or CamKII-dependent pathways do not play a major role in mediating WNT5A-induced migration and invasion in pancreatic cancer cells.

WNT5A expression increases during pancreatic carcinogenesis and is highly expressed in pancreatic cancer tissues

As reduction in WNT5A expression leads to reduced proliferation, motility and invasiveness, and WNT5A up-regulation is associated with increased motility and invasion, we sought to elucidate the physiological relevance of WNT5A in tumor progression and metastasis by studying WNT5A expression in pancreatic cancer samples and pancreatic cancer precursor lesions. Similar to colon cancer, a tumor

progression model has been described for pancreatic cancer. Precursor lesions preceding invasive pancreatic cancer have been defined histologically as Pancreatic Intraepithelial Neoplasia 1-3 (PanIN1-3). These lesions are characterized by progressive nuclear abnormalities and disturbed epithelial architecture [36].

We microdissected normal ducts, PanIN1-3 lesions and tumor cells from invasive ductal adenocarcinomas as described previously [32]. Interestingly, real-time PCR of RNA from 25 microdissected tissues including normal ducts, PanIN1-3 lesions and invasive adenocarcinomas revealed that WNT5A expression is significantly increased in advanced PanIN lesions 2 and 3, reaching levels comparable to invasive pancreatic adenocarcinomas (PDAC) (Figure 6A). This indicates that WNT5A is already up-regulated in advanced, but still pre-invasive precursor lesions, thereby possibly enhancing its transition in malignant, invasive carcinomas.

To confirm our data for carcinomas on protein level, we performed immunohistochemistry on an independent series of 16 pancreatic cancer tissues and adjacent normal tissue. 13 / 16 pancreatic cancer tissues showed a markedly stronger WNT5A protein staining in tumor cells compared to normal pancreatic tissue which showed only faint staining, mainly of ductal cells and islet cells (Figure 6B). These data on RNA and protein levels strongly indicate that elevated levels of WNT5A expression may play an important role in enhancing invasiveness and tumor progression in pancreatic cancer.

DISCUSSION

Previously, we identified the homeodomain transcription factor CUTL1 as target of TGFbeta and important mediator of its effects on cell migration and

invasiveness [11]. Based on previous microarray results, we show in this study that WNT5A, a member of the Wnt family of secreted glycoproteins, is up-regulated by CUTL1 on mRNA and protein level. Increased activity of a luciferase construct containing the putative WNT5A promoter upon CUTL1 over-expression suggests that CUTL1 transcriptionally activates WNT5A. However, further studies are warranted to define whether CUTL1 binds directly to the WNT5A promoter. In addition, we demonstrate that WNT5A is a crucial downstream mediator of tumor cell migration, invasion and proliferation induced by CUTL1. In order to substantiate these findings on a broad panel of cell lines with different biological behaviour, we used a panel of four cancer cell lines, three of them being pancreatic cancer cells of epithelial origin, and one being a highly motile fibrosarcoma cell line of non-epithelial origin. All used cell lines are known to differ in their proliferative, migratory and invasive potential which has been shown by our group and others [11], [34], and express different baseline levels of both CUTL1 and WNT5A. This might explain why the degree of migratory, invasive and proliferative effects induced by WNT5A varied from cell line to cell line. Enhanced migration induced by WNT5A appears to be accompanied by certain features of epithelial-mesenchymal transition (EMT), a cardinal hallmark of TGFbeta-induced tumor progression. Interestingly, WNT5A appears to mediate its pro-invasive effects in the pancreatic cancer cells to a significant extent through canonical, TCF-dependent pathways rather than non-canonical pathways. The important role of WNT5A in pancreatic tumorigenesis is further supported by the fact that WNT5A is progressively up-regulated in advanced stages of pre-invasive pancreatic cancer precursor lesions (PanIN's) and by its high expression in invasive pancreatic carcinomas.

To date, the pathophysiological role of WNT5A in tumorigenesis and tumor progression remains controversial. On the one hand, it has been identified as inhibitor

of tumor progression in tumors such as breast and renal cell carcinomas and hematopoietic malignancies. WNT5A may even be able to revert the transforming effects of other WNT members. Mouse C57MG mammary cell transformation by an antisense WNT5A mimics WNT1-mediated transformation [20] and in vivo, WNT5A may serve as good prognostic marker in patients with breast cancer [23]. In renal carcinoma cells, ectopic expression of WNT5A reverts tumorigenesis [21]. WNT5A hemizygous mice develop myeloid leukemias and B cell lymphomas that display loss of WNT5A function in tumor tissues [22].

On the other hand, accumulating data from other tumor entities are contradictory to these reports which describe WNT5A as putative tumor suppressor. There is increasing evidence that WNT5A is also able to enhance cancer progression. In addition to its role as stimulator of cell motility and invasiveness in melanomas [19], WNT5A is highly expressed in various other solid tumors including lung cancers and adenocarcinomas of the stomach and the prostate [24-26]. Being highly expressed in aggressive non-small cell lung cancers (NSCLC), WNT5A is associated with a poor prognosis for these patients [27]. Our data clearly demonstrate that WNT5A enhances invasiveness in pancreatic cancer. Although previous microarray data reports suggested that WNT5A is down-regulated in pancreatic cancer [37], our results on RNA and protein level indicate that WNT5A is markedly up-regulated in the majority of invasive pancreatic cancers. Moreover, the fact that WNT5A is already up-regulated in pre-invasive precursor lesions of pancreatic cancer (PanIN's) suggests that WNT5A plays an important role during tumorigenesis. Previous reports describing WNT5A as crucial player in embryogenesis and development demonstrate that WNT5A is required for the proliferation of progenitor cells within the progress zone and the paraxial mesoderm [38]. Since the modulation of proliferation and migration in embryogenesis is

frequently recapitulated in tumors, it can be speculated that WNT5A acts as oncofetal protein by enhancing both early steps in tumorigenesis and tumour progression in advanced invasive carcinomas.

The molecular basis for the diverse effects of WNT5A seen in different tumor types remains to be elucidated. Moreover, the downstream signalling mechanisms induced by WNT5A are controversially discussed [35], [41]. In addition to activating non-canonical, calcium-dependent signalling pathways, WNT5A has been described to inhibit canonical beta-catenin/TCF signalling in several cell systems. However, recent reports indicate that WNT5A may also activate canonical beta-catenin/TCF signalling depending on the receptor context, in particular depending on the presence of the Frizzled 4 and LRP5 receptors [35]. Our data support these reports and suggest that WNT5A exerts its tumor-promoting effects in pancreatic cancers cells via beta-catenin/TCF-dependent pathways, in analogy to the beta-catenin/TCF-dependent tumor-promoting effects of other WNT members seen in colon carcinomas. Interestingly, knock-down of WNT5A was able to decrease the phosphorylation of GSK-3beta at serine 9 which was induced by lithium chloride. LiCl is known as activator of the canonical pathway and inhibitor of GSK-3beta. Although it is being discussed controversially in the literature, several reports suggest that in canonical wnt signalling, GSK-3beta is inhibited by phosphorylation at serine 9, the same residue phosphorylated by Akt/PKB [42], [43]. Based on these data, inhibited phosphorylation of GSK-3beta by knock-down of WNT5A might serve as another indicator of involvement of the canonical pathway. However, further studies are warranted to elucidate the signalling events downstream WNT5A in greater detail.

In this study, we showed that WNT5A is an important downstream target of CUTL1. Previously, we had identified CUTL1 as target of TGFbeta [11]. We therefore propose that the up-regulation of WNT5A by CUTL1 might represent a significant

new pathway through which TGFbeta exerts its pro-migratory and pro-invasive effects. TGFbeta, however, is known to play a dual role in tumorigenesis and tumor progression and may inhibit or promote cell proliferation and tumor progression depending on the cellular context. While the growth-suppressing effects of TGFbeta are mainly seen in normal epithelial cells or early stages of differentiated carcinomas, in advanced stages of the disease, anti-proliferative actions of TGFbeta are lost and its tumor-promoting and pro-invasive effects become predominant. The mechanisms leading to the loss of TGFbeta-induced growth suppression remain to be fully elucidated [39]. WNT5A and its up-regulation by CUTL1 may turn out to be a key pathway in this context: Activation of the pro-proliferative and pro-invasive transcription factor CUTL1 by TGFbeta leading to subsequent activation of WNT5A may represent one mechanism of overcoming TGFbeta-induced growth suppression and enhancing tumor progression. Further studies are warranted to determine the activation status of the pathway TGFbeta-CUTL1-WNT5A dependent on cell type, grade of differentiation and stage of the tumor.

Our data indicate that the effect of WNT5A on migration and invasion is paralleled by changes in marker proteins for epithelial-mesenchymal transition (EMT). EMT has been described as important prerequisite for invasiveness in various epithelial tumor types including pancreatic cancer [34]. Previously, we could not detect significant effects of the TGFbeta-target CUTL1 on the expression of EMT markers by using mammary epithelial Eph4 cells as cell model [11]. In the present study, however, a clear effect of the CUTL1-downstream target WNT5A on EMT markers such as E-cadherin and vimentin was seen in the pancreatic cancer cell line PANC1, a cell line which has been shown to undergo EMT in various previous reports [34]. Reasons for this discrepancy between the effect of CUTL1 and its downstream target WNT5A on EMT might be due to differences in the genetic

background of the two tumor entities. Alternatively, it is conceivable that the effect of CUTL1 as upstream transcription factor targeting numerous downstream genes is not as pronounced as the effect of individual downstream effectors such as WNT5A. However, the demonstrated changes in the marker proteins E-cadherin and vimentin are not sufficient to justify the conclusion that WNT5A mediates EMT. Further detailed analyses are necessary to confirm that WNT5A induces the morphological, biochemical and transcriptional changes typical for this complex process.

The data presented here demonstrate that WNT5A is transcriptionally up-regulated by the TGFbeta-target CUTL1 and plays a critical role in mediating its effects on cancer cell motility and invasive behavior. Previously, we showed that CUTL1 is highly expressed in invasive breast and pancreatic cancers [11]. The present study demonstrates a high expression of its target WNT5A in pancreatic cancers and its precursor lesions. Based on our data, we propose the pathway TGFbeta - CUTL1 – WNT5A as a novel signaling cascade mediating migration, invasion and tumor progression in pancreatic cancer.

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REFERENCES

1. Nepveu,A. (2001) Role of the multifunctional CDP/Cut/Cux homeodomain transcription factor in regulating differentiation, cell growth and development. *Gene*, **270**, 1-15.
2. Van Wijnen,A.J., van Gulp,M.F., de Ridder,M.C., Tufarelli,C., Last,T.J., Birnbaum,M., Vaughan,P.S., Giordano,A., Krek,W., Neufeld,E.J., Stein,J.L., and Stein,G.S. (1996) CDP/cut is the DNA-binding subunit of histone gene transcription factor HiNF-D: a mechanism for gene regulation at the G1/S phase cell cycle transition point independent of transcription factor E2F. *Proc.Natl.Acad.Sci.U.S.A*, **93**, 11516-11521.
3. Sansregret,L., Goulet,B., Harada,R., Wilson,B., Leduy,L., Bertoglio,J, Nepveu,A. (2006) The p110 isoform of the CDP/Cux transcription factor accelerates entry into S phase. *Mol. Cell. Biol.*, **26**, 2441-55.
4. Truscott,M., Raynal,L., Premdas,P., Goulet,B., Leduy,L., Berube,G., and Nepveu,A. (2003) CDP/Cux stimulates transcription from the DNA polymerase alpha gene promoter. *Mol.Cell Biol.*, **23**, 3013-3028.
5. van Gulp,M.F., Pratap,J., Luong,M., Javed,A., Hoffmann,H., Giordano,A., Stein,J.L., Neufeld,E.J., Lian,J.B., Stein,G.S., and Van Wijnen,A.J. (1999) The CCAAT displacement protein/cut homeodomain protein represses osteocalcin gene transcription and forms complexes with the retinoblastoma protein-related protein p107 and cyclin A. *Cancer Res.*, **59**, 5980-5988.
6. Skalnik,D.G., Strauss,E.C., and Orkin,S.H. (1991) CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91-phox gene promoter. *J.Biol.Chem.*, **266**, 16736-16744.
7. Ellis,T., Gambardella,L., Horcher,M., Tschanz,S., Capol,J., Bertram,P., Jochum,W., Barrandon,Y., and Busslinger,M. (2001) The transcriptional repressor CDP (Cutl1) is essential for epithelial cell differentiation of the lung and the hair follicle. *Genes Dev.*, **15**, 2307-2319.
8. Luong,M.X., van der Meijden,C.M., Xing,D., Hesselton,R., Monuki,E.S., Jones,S.N., Lian,J.B., Stein,J.L., Stein,G.S., Neufeld,E.J., and Van Wijnen,A.J. (2002) Genetic ablation of the CDP/Cux protein C terminus results in hair cycle defects and reduced male fertility. *Mol.Cell Biol.*, **22**, 1424-1437.
9. Sinclair,A.M., Lee,J.A., Goldstein,A., Xing,D., Liu,S., Ju,R., Tucker,P.W., Neufeld,E.J., and Scheuermann,R.H. (2001) Lymphoid apoptosis and myeloid hyperplasia in CCAAT displacement protein mutant mice. *Blood*, **98**, 3658-3667.
10. Ledford,A.W., Brantley,J.G., Kemeny,G., Foreman,T.L., Quaggin,S.E., Igarashi,P., Oberhaus,S.M., Rodova,M., Calvet,J.P., and Vanden Heuvel,G.B. (2002) Deregulated expression of the homeobox gene Cux-1 in transgenic mice results in downregulation of p27(kip1) expression during nephrogenesis, glomerular abnormalities, and multiorgan hyperplasia. *Dev.Biol.*, **245**, 157-171.
11. Michl,P., Ramjaun,A.R., Pardo,O.E., Warne,P.H., Wagner,M., Poulsom,R., D'Arrigo,C., Ryder,K., Menke,A., Gress,T., and Downward,J. (2005) CUTL1 is a target of TGFbeta signaling that enhances cancer cell motility and invasiveness. *Cancer Cell*, **7**, 521-532.

12. Tanaka,K., Kitagawa,Y., and Kadowaki,T. (2002) Drosophila segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum. *J.Biol.Chem.*, **277**, 12816-12823.
13. Yang-Snyder,J., Miller,J.R., Brown,J.D., Lai,C.J., and Moon,R.T. (1996) A frizzled homolog functions in a vertebrate Wnt signaling pathway. *Curr.Biol.*, **6**, 1302-1306.
14. Jue,S.F., Bradley,R.S., Rudnicki,J.A., Varmus,H.E., and Brown,A.M. (1992) The mouse Wnt-1 gene can act via a paracrine mechanism in transformation of mammary epithelial cells. *Mol.Cell Biol.* , **12**, 321-328.
15. Wong,G.T., Gavin,B.J., and McMahon,A.P. (1994) Differential transformation of mammary epithelial cells by Wnt genes. *Mol.Cell Biol.*, **14**, 6278-6286.
16. Miller,J.R., Hocking,A.M., Brown,J.D., and Moon,R.T. (1999) Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca²⁺ pathways. *Oncogene.*, **18**, 7860-7872.
17. Sharpe,C., Lawrence,N., and Martinez,A.A. (2001) Wnt signalling: a theme with nuclear variations. *Bioessays.*, **23**, 311-318.
18. Kuhl,M., Sheldahl,L.C., Malbon,C.C., and Moon,R.T. (2000) Ca²⁺/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. *J.Biol.Chem.*, **275**, 12701-12711.
19. Weeraratna,A.T., Jiang,Y., Hostetter,G., Rosenblatt,K., Duray,P., Bittner,M., and Trent,J.M. (2002) Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer Cell*, **1**, 279-288.
20. Olson,D.J. and Gibo,D.M. (1998) Antisense wnt-5a mimics wnt-1-mediated C57MG mammary epithelial cell transformation. *Exp.Cell Res.*, **241**, 134-141.
21. Olson,D.J., Gibo,D.M., Saggars,G., Debinski,W., and Kumar,R. (1997) Reversion of uroepithelial cell tumorigenesis by the ectopic expression of human wnt-5a. *Cell Growth Differ.*, **8**, 417-423.
22. Liang,H., Chen,Q., Coles,A.H., Anderson,S.J., Pihan,G., Bradley,A., Gerstein,R., Jurecic,R., and Jones,S.N. (2003) Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. *Cancer Cell*, **4**, 349-360.
23. Dejmek,J., Leandersson,K., Manjer,J., Bjartell,A., Emdin,S.O., Vogel,W.F., Landberg,G., and Andersson,T. (2005) Expression and signaling activity of Wnt-5a/discoidin domain receptor-1 and Syk plays distinct but decisive roles in breast cancer patient survival. *Clin.Cancer Res.*, **11**, 520-528.
24. Iozzo,R.V., Eichstetter,I., and Danielson,K.G. (1995) Aberrant expression of the growth factor Wnt-5A in human malignancy. *Cancer Res.*, **55**, 3495-3499.
25. Lejeune,S., Huguette,E.L., Hamby,A., Poulosom,R., and Harris,A.L. (1995) Wnt5a cloning, expression, and up-regulation in human primary breast cancers. *Clin.Cancer Res.*, **1**, 215-222.
26. Saitoh,T., Mine,T., and Kato,M. (2002) Frequent up-regulation of WNT5A mRNA in primary gastric cancer. *Int.J.Mol.Med.*, **9**, 515-519.
27. Huang,C.L., Liu,D., Nakano,J., Ishikawa,S., Kontani,K., Yokomise,H., and Ueno,M. (2005) Wnt5a expression is associated with the tumor proliferation and the stromal

- vascular endothelial growth factor--an expression in non-small-cell lung cancer. *J.Clin.Oncol.*, **23**, 8765-8773.
28. Vila,M.R., Lloreta,J., Schussler,M.H., Berrozpe,G., Welt,S., and Real,F.X. (1995) New pancreas cancers cell lines that represent distinct stages of ductal differentiation. *Lab Invest.*, **72**, 395-404.
 29. Schreiber,E., Matthias,P., Muller,M.M., and Schaffner,W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.*, **17**, 6419.
 30. Howcroft,T.K., Kirshner,S.L., and Singer,D.S. (1997) Measure of transient transfection efficiency using beta-galactosidase protein. *Anal.Biochem.*, **244**, 22-27.
 31. Wagner,M., Kunsch,S., Duerschmied,D., Beil,M., Adler,G., Mueller,F., and Gress,T.M. (2003) Transgenic overexpression of the oncofetal RNA binding protein KOC leads to remodeling of the exocrine pancreas. *Gastroenterology*, **124**, 1901-1914.
 32. Buchholz,M., Braun,M., Heidenblut,A., Kestler,H.A., Kloppel,G., Schmiegel,W., Hahn,S.A., Luttges,J., and Gress,T.M. (2005) Transcriptome analysis of microdissected pancreatic intraepithelial neoplastic lesions. *Oncogene.*, **24**, 6626-6636.
 33. Goulet,B., Baruch,A., Moon,N.S., Poirier,M., Sansregret,L.L., Erickson,A., Bogyo,M., and Nepveu,A. (2004) A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor. *Mol.Cell*, **14**, 207-219.
 34. Ellenrieder,V., Hendler,S.F., Boeck,W., Seufferlein,T., Menke,A., Ruhland,C., Adler,G., and Gress,T.M. (2001) Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. *Cancer Res.*, **61**, 4222-4228.
 35. Mikels,A.J. and Nusse,R. (2006) Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS.Biol.*, **4**, e115.
 36. Hruban,R.H., Takaori,K., Klimstra,D.S., Adsay,N.V., Albores-Saavedra,J., Biankin,A.V., Biankin,S.A., Compton,C., Fukushima,N., Furukawa,T., Goggins,M., Kato,Y., Kloppel,G., Longnecker,D.S., Luttges,J., Maitra,A., Offerhaus,G.J., Shimizu,M., and Yonezawa,S. (2004) An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. *Am.J.Surg.Pathol.*, **28**, 977-987.
 37. Crnogorac-Jurcevic,T., Efthimiou,E., Capelli,P., Blaveri,E., Baron,A., Terris,B., Jones,M., Tyson,K., Bassi,C., Scarpa,A., and Lemoine,N.R. (2001) Gene expression profiles of pancreatic cancer and stromal desmoplasia. *Oncogene*, **20**, 7437-7446.
 38. Yamaguchi,T.P., Bradley,A., McMahon,A.P., and Jones,S. (1999) A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development.*, **126**, 1211-1223.
 39. Siegel,P.M. and Massague,J. (2003) Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat.Rev.Cancer*, **3**, 807-821.
 40. Strathdee, C.A., McLeod, M.R., Hall, J.R. (1999) Efficient control of tetracycline-responsive gene expression from an autoregulated bi-directional expression vector. *Gene*, **229**, 21-9.

41. Pukrop, T., Klemm, F., Hagemann, Th., Gradl, D., Schulz, M., Siemes S., Trümper, L., Binder, C. (2006) Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines. *Proc. Natl. Acad. Sci. U. S. A.*, **103**, 5454-9.
42. Yuan, H., Mao, J., Li, L., Wu, D. (1999) Suppression of glycogen synthase kinase activity is not sufficient for leukemia enhancer factor-1 activation. *J. Biol. Chem.*, **274**, 30419-23.
43. Fukumoto, S., Hsieh, C.M., Maemura, K., Layne, M.D., Yet, S.F., Lee, K.H., Matsui, T., Rosenzweig, A., Taylor, W.G., Rubin, J.S., Perrella, M.A., Lee, M.E. (2001) Akt participation in the Wnt signaling pathway through Dishevelled. *J. Biol. Chem.*, **276**, 17479-83.

LEGENDS TO FIGURES

Figure 1. CUTL1 induces WNT5A expression

- A. Knock-down of CUTL1 reduces WNT5A mRNA levels. PANC1, ImimPC1 and HT0180 cells were transiently transfected with hCUTL1_1 siRNA (siCUTL1) or control siRNA (siControl) and WNT5A mRNA was quantified by real-time PCR 24 hours after transfection. WNT5A mRNA levels were normalized to RPLP0 expression as housekeeping gene. Results are representative for 3 independent experiments. Assays were performed in triplicates and are shown as mean \pm SD. * indicates $p < 0.05$ as compared to control cells. Similar results were obtained with CUTL1_2 siRNA.
- B. Knock-down of CUTL1 reduces WNT5A protein levels. PANC1, ImimPC1, MiaPaca and HT0180 cells were transiently transfected with hCUTL1_1 siRNA or control siRNA and WNT5A protein levels were detected by immunoblotting with the anti-WNT5A antibody 48 hours after transfection. Similar results were obtained with hCUTL1_2 siRNA. To control for equal loading the blots were reprobed with beta-actin antibody.

- C. Overexpression of CUTL1 increases WNT5A protein levels. PANC1 and HT1080 cells were transiently transfected with the transcriptionally active C-terminal CUTL1aa831-1336 plasmid or an empty control vector. 24 hours after transfection, WNT5A protein was detected by immunoblotting with anti-WNT5A. The blot was reprobed with the beta-actin antibody to control for equal loading.
- D. Overexpression of CUTL1 increases WNT5A promoter activity. PANC1 cells were transiently transfected with the CUTL1 full-length plasmid, resulting in expression of full-length CUTL1 protein and the transcriptionally more active p110 cleavage product (demonstrated by immunoblot in the lower panel), or an empty vector (Control), the WNT5A promoter luciferase plasmid and beta-galactosidase protein. 24 hours after transfection, WNT5A promoter activity was measured by luciferase assays. Results are normalized to beta-galactosidase levels, are representative for three independent experiments which were performed in triplicates and are shown as mean \pm -SD. * indicates $p < 0.05$ as compared to control.

Figure 2. WNT5A enhances migration and invasion of pancreatic cancer cells

- A. Knock-down of WNT5A reduces migration. PANC1, ImimPC1, MiaPaca and HT0180 cells were transiently transfected with hWNT5A_1 siRNA or control siRNA and migration over a time period of 4 hours (HT1080 cells) or 8 hours (all other cells) was quantified with a modified two-chamber migration assay. The number of migrated cells was measured by the CellTiter-Glo[®] Luminescent Cell Viability Assay and normalized to the total number of proliferating cells in a neighbor well. Knock-down of WNT5A in each cell line was confirmed by Western blot (lower panel). Data are representative for three

independent experiments which were performed in triplicates and are shown as mean \pm SD. * indicates $P < 0.05$ as compared to control cells. Similar results were obtained using hWNT5A_2 siRNA.

- B. Knock-down of WNT5A reduces migration in time-lapse microscopy. HT0180 cells were transiently transfected with hWNT5A_1 siRNA or control siRNA and imaging was performed over 20 hours in serum-containing medium at 37°C. Velocity of the cells was measured by single-cell tracking. Data are representative for three independent experiments. 25 cells per group were tracked and are shown as mean \pm SEM. * : $P < 0.05$ as compared to control cells.
- C. Knock-down of WNT5A reduces invasion. PANC1, ImimPC1, MiaPaca and HT0180 cells were transiently transfected with hWNT5A_1 siRNA or control siRNA and invasion over a time period of 6 hours (HT1080, PANC1, MiaPaca cells) or 12 hours (ImimPC1) was quantified with a modified two-chamber invasion assay through a Matrigel-coated 8 μ m-membrane. The number of invading cells was measured by the CellTiter-Glo[®] Luminescent Cell Viability Assay and normalized to the total number of proliferating cells in a neighbor well. Data are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SD. * : $P < 0.05$ as compared to control cells. Similar results were obtained using hWNT5A_2 siRNA.
- D. Overexpression of WNT5A enhances migration and invasion. PANC1 cells stably transfected with WNT5A-pBIG2i were stimulated with doxycyclin or DMSO for 24 hours. Increased expression of WNT5A protein was confirmed by Western blot (lower panel). Subsequently, migration and invasion over a time period of 6 hours was quantified with two-chamber migration or invasion assays. The number of invading cells was measured by the CellTiter-Glo[®]

Luminescent Cell Viability Assay and normalized to the total number of proliferating cells in a neighboring well. Data are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SD. * : $P < 0.05$ as compared to unstimulated control cells.

- E. Exogenously added WNT5A protein enhances migration. PANC1, HT1080, ImimPC1 and MiaPaca cells were stimulated with 500ng WNT5a protein/ml for 24 hours. Subsequently, migration was quantified with a modified two-chamber migration assay over a time period of 6h (HT1080) or 12h (all other cells). The number of migrated cells was measured by the CellTiter-Glo[®] Luminescent Cell Viability Assay and normalized to untreated control cells. Data are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SD. * : $P < 0.05$ as compared to unstimulated control cells.

Figure 3. Knock-down of WNT5A reduces proliferation. PANC1, ImimPC1, MiaPaca and HT0180 cells were transiently transfected with hWNT5A_1 siRNA or control siRNA and proliferation was quantified by [³H]thymidine incorporation assays. Data are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SD. * : $P < 0.05$ as compared to control cells. Similar results were obtained using hWNT5A_2 siRNA.

Figure 4. WNT5A is an important mediator of CUTL1-induced migration and invasion.

- A. PANC1 cells with stably reduced CUTL1 expression by shRNA (shCUTL) or empty-vector transfected control cells (shControl) were transiently transfected

with hWNT5A_1 siRNA or control siRNA. Migration was assayed by modified Boyden chamber assays. The number of migrating cells was measured by the cell counting of the fixed and stained cells at the bottom of the membrane. Data are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SD. * indicates $P<0.05$ as compared to shControl cells.

B. PANC1 cells with stably reduced CUTL1 expression by shRNA (shCUTL) or empty-vector transfected control cells (shControl) were transiently transfected with hWNT5A_1 siRNA or control siRNA. Invasion was assayed by modified Boyden chamber assays. The number of invading cells was measured by the cell counting of the fixed and stained cells at the bottom of the membrane. Data are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SD. * indicates $P<0.05$ as compared to control cells.

C. PANC1 cells with stably reduced CUTL1 expression by shRNA (shCUTL) or empty-vector transfected control cells (shControl) were transiently transfected with pWNT5A expression plasmid or empty vector. To selectively assess the migration of transiently transfected cells, co-transfections with LacZ plasmid were performed. Migration was assayed by modified Boyden chamber assays and was calculated by counting the fixed X-Gal stained cells. The number of migrated cells was normalized to the total number of transfected cells in a adjacent well. Data are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SD. * indicates $P<0.05$ as compared to shControl cells without WNT5A.

Figure 5. WNT5A is up-regulated by TGFbeta via CUTL1, is associated with features of epithelial-mesenchymal transition (EMT) and signals via beta-catenin/TCF-dependent pathways

- A. PANC1 cells were transiently transfected with CUTL1 siRNA or control siRNA and stimulated with TGFbeta (10ng/ml) for 18 hours. WNT5A levels were determined by immunoblotting using the anti-WNT5A antibody. The blot was reprobed with the beta-actin antibody to control for equal loading.
- B. WNT5A is associated with regulation of EMT marker proteins. PANC1 cells were transiently transfected with WNT5A siRNA and expression of the EMT marker genes E-cadherin and vimentin was determined by immunoblotting using specific antibodies. The blot was reprobed with the beta-actin antibody to control for equal loading.
- C. Addition of recombinant human WNT5A increases TCF/LEF-dependent promoter activity. PANC1 cells were transiently transfected with TOPflash or FOPflash and beta-galactosidase protein. Recombinant human WNT5A protein was added for 12 hours at a concentration of 500 ng/ml. 24 hours after transfection, the TCF/LEF-dependent promoter activity was measured by luciferase assays. Results are normalized to beta-galactosidase and shown as relative to TOPflash transfected controls, are representative for three independent experiments which were performed in triplicates and are shown as mean+/-SEM. * indicates $p < 0.05$ as compared to TOPflash control.
- D. Tetracyclin-inducible WNT5A expression increases TCF/LEF-dependent promoter activity which is inhibited by dominant negative hLef1. PANC1 cells stably transfected with pBig2i-WNT5A were transiently transfected with TOPflash, FOPflash and beta-galactosidase protein as well as hLef1 or dominant negative hLef1, as indicated. WNT5A expression was induced for 24

hours with doxycyclin at a concentration of 6 $\mu\text{g/ml}$. 24 hours after transfection, the TCF/LEF-dependent promoter activity was measured by luciferase assays. hLef1 cotransfection served as positive control. Results are normalized to beta-galactosidase and shown as relative to TOPflash transfected controls, are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SEM. FOPflash activity was not significantly regulated among all groups (not shown). * indicates $p < 0.05$ as compared to TOPflash control.

- E. Knock-down of WNT5A decreases TCF/LEF-dependent promoter activity. PANC1, ImimPC1 and MiaPaca cells were transiently transfected with WNT5A siRNA or control siRNA, the TOPflash or FOPflash luciferase plasmid and beta-galactosidase protein. 24 hours after transfection with TOPflash, the TCF/LEF-dependent promoter activity was measured by luciferase assays. Results are normalized to beta-galactosidase levels, are representative for three independent experiments which were performed in triplicates and are shown as mean \pm SD. FOPflash activity was not significantly regulated among all groups (not shown). * indicates $p < 0.05$ as compared to control.
- F. Knock-down of WNT5A decreases nuclear beta-catenin levels. PANC1, ImimPC1 and MiaPaca cells were transiently transfected with WNT5A siRNA or control siRNA. Expression of beta-catenin was determined from nuclear extracts by immunoblotting using a specific antibody. As positive control for canonical wnt signalling, nuclear beta-catenin was analysed after addition of LiCl (10nM) in PANC1 cells. The blots were reprobated with the lamin A/C antibody to control for equal loading.
- G. Recombinant WNT5a protein increases nuclear levels of beta-catenin in PANC1 cells. PANC1 cells were stimulated for 24 hours in serum-free media

with 500ng WNT5A protein/ml. Cytosolic beta-catenin and nuclear levels of beta-catenin were detected after 12 hours by immunoblotting with a specific beta-catenin antibody after preparation of cytosolic and nuclear extracts. The blot was reprobated with the lamin A/C antibody to control for equal loading of the nuclear extracts.

- H. WNT5A knock-down inhibits lithium chloride-induced GSK-3beta phosphorylation. PANC1 cells were transiently transfected with hWNT5A_1 siRNA or control siRNA. The canonical wnt pathway was activated using lithium chloride at 10nM and 30 nM. LiCl-induced phosphorylation of GSK-3beta at serine 9 was determined by a phospho-specific antibody. Total GSK-3beta and beta-actin were used as loading control.
- I. WNT5A does not alter the phosphorylation of various PKC's. PANC1 cells were transiently transfected with hWNT5A_1 siRNA or control siRNA. Knock-down of WNT5A was confirmed by Western blot. Phosphorylation of various PKC's was detected by specific antibodies for phospho-PKCalpha/beta (Thr638/641) and phospho-PKC(pan)(betaII Ser660). The blot was reprobated with the beta-actin antibody to control for equal loading.
- J. WNT5A does not significantly alter the activity of Calmodulin-dependent kinase II. PANC1 cells were transiently transfected with hWNT5A_1 siRNA or control siRNA. CamK II activity assays were performed according to the manufacturer's instruction and radioactivity was measured by scintillation counting. Results are representative for four independent experiments which were performed in triplicates and are shown as mean+/-SD.

Figure 6. WNT5A expression increases during pancreatic carcinogenesis and is highly expressed in pancreatic cancer tissues

- A. WNT5A mRNA expression is up-regulated in pancreatic intraepithelial neoplasia (PanIN) lesions. WNT5A mRNA expression in amplified RNA from microdissected PanIN1-3 lesions and pancreatic ductal adenocarcinomas (PDAC) derived from frozen surgical specimens was quantified by real-time PCR and normalized to RPLP0 expression. Data are expressed as mean +/- SD from 5 individual samples each. * indicates $p < 0.05$ compared to normal duct cells.
- B. WNT5A protein is highly expressed in invasive pancreatic adenocarcinomas (PDAC). WNT5A was detected by immunohistochemistry using the anti-WNT5A antibody. The pictures show three non-malignant pancreatic tissues (upper row) and three pancreatic adenocarcinomas (lower row) and are representative for 13 / 16 examined pancreatic cancer tissues and 16 / 16 adjacent pancreatic tissue. Bars represent 200 μm .

Fig.1A

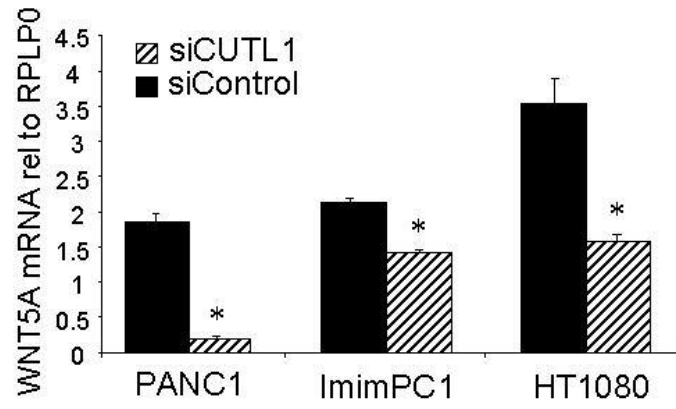


Fig.1B

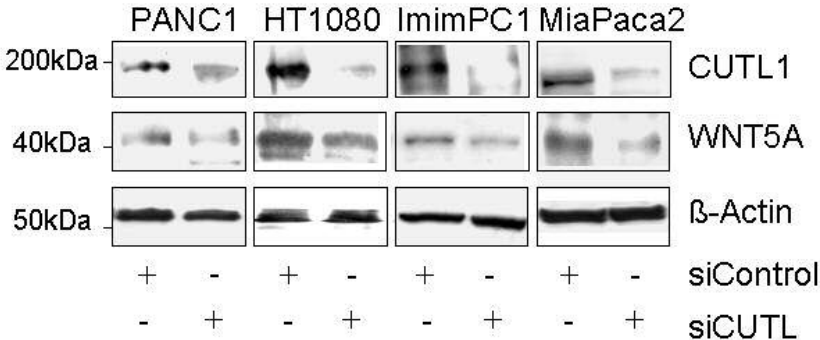


Fig. 1C

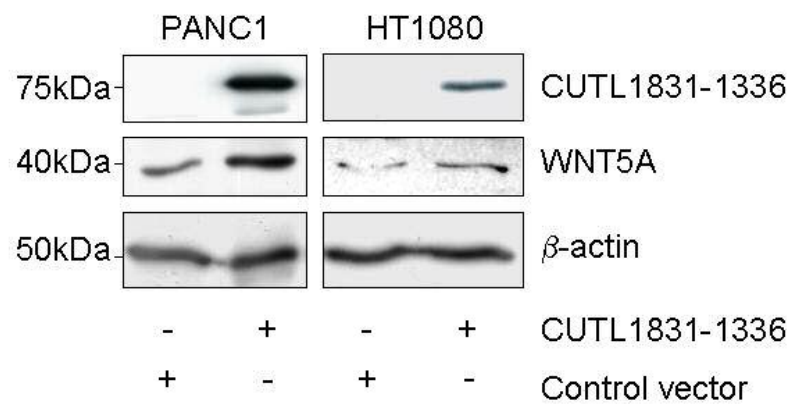


Fig.1D

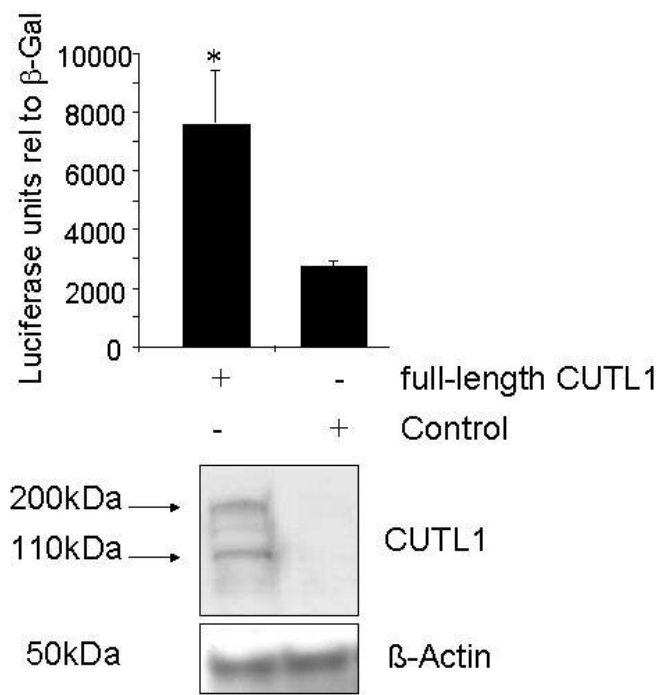


Fig.2A

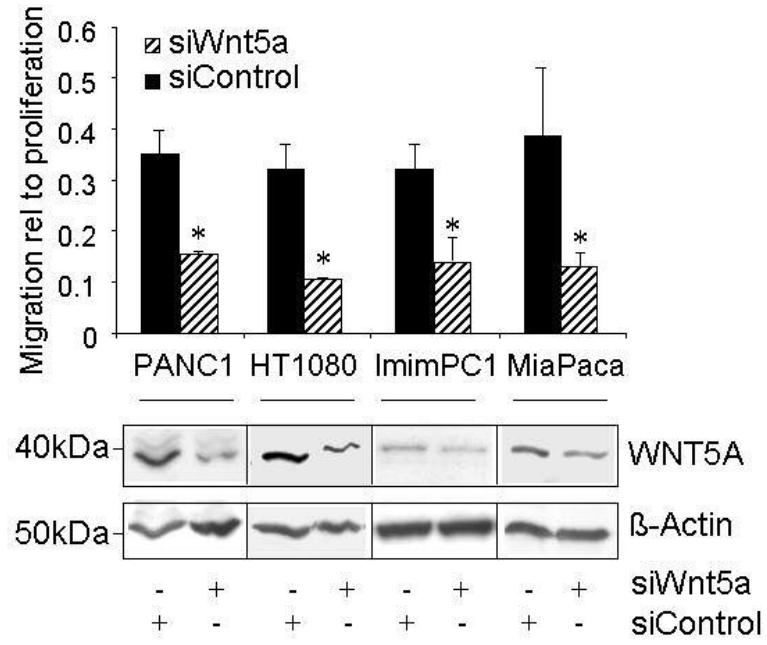


Fig.2B

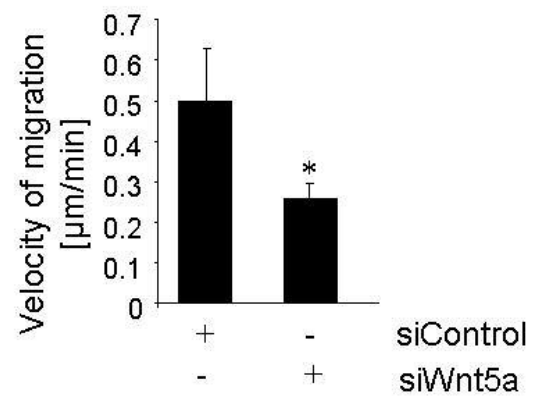


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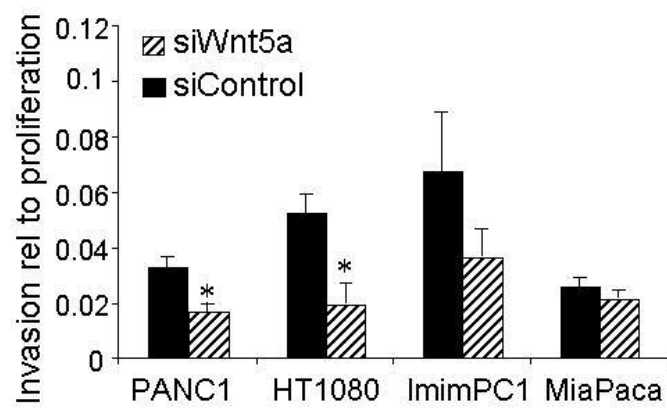


Fig. 2D

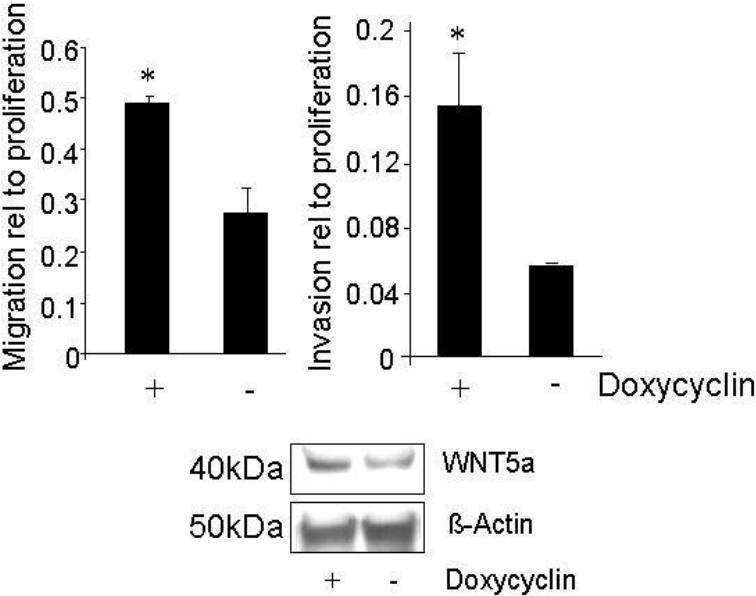


Fig. 2E

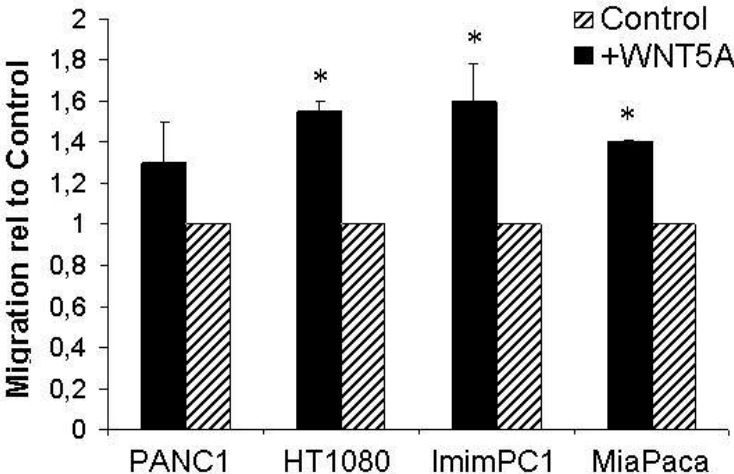


Fig.3

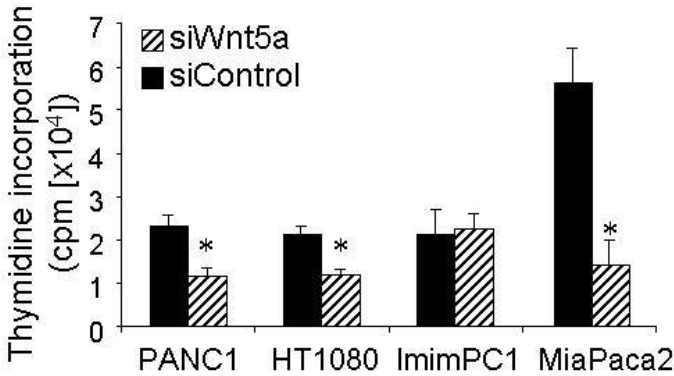


Fig.4A

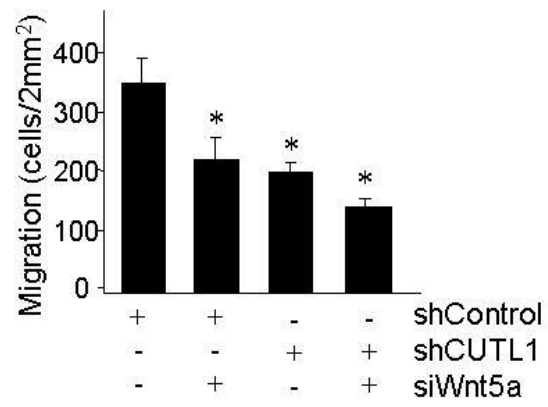


Fig.4B

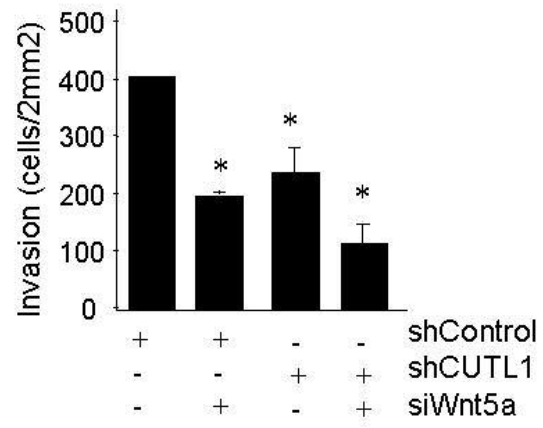


Fig.4C

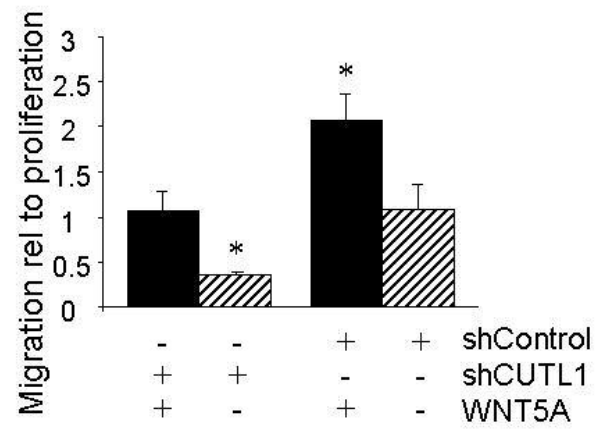


Fig.5A

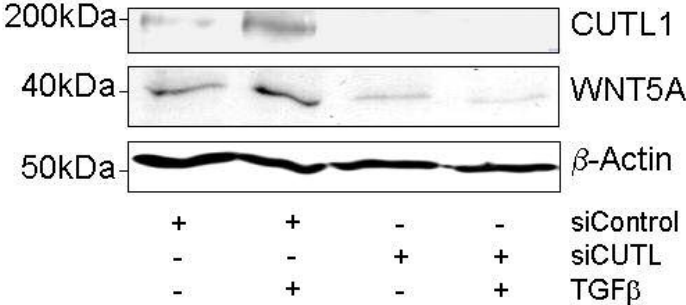


Fig.5B

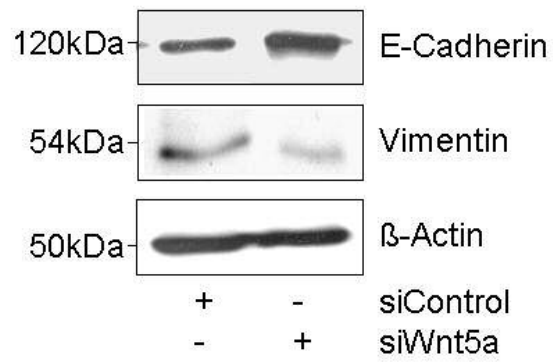


Fig.5C

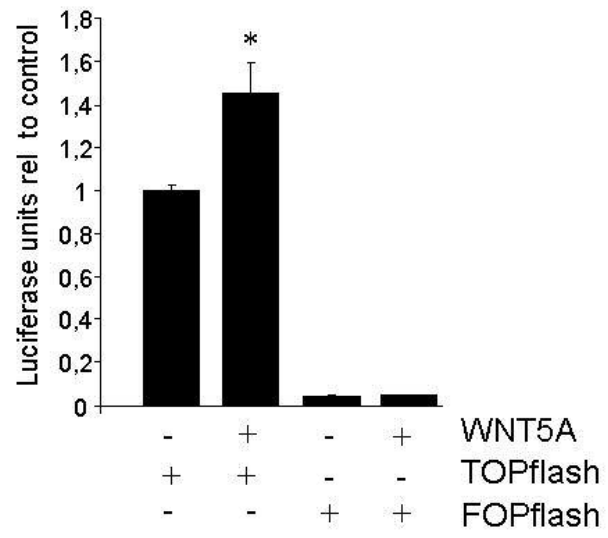


Fig.5D

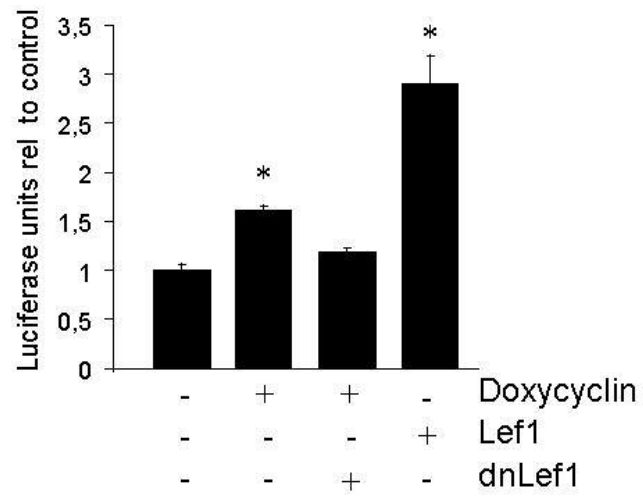


Fig.5E

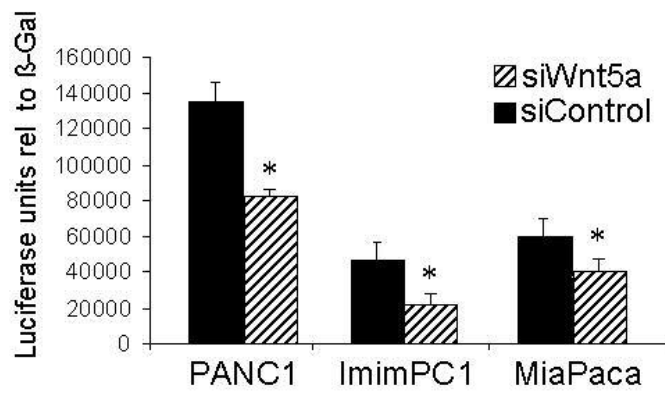


Fig.5F

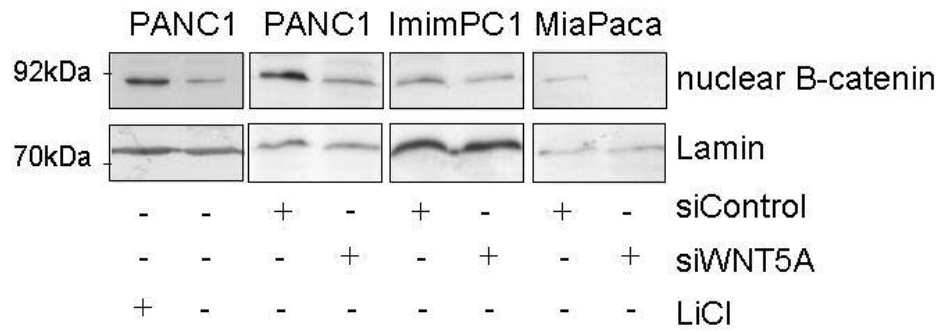


Fig.5G

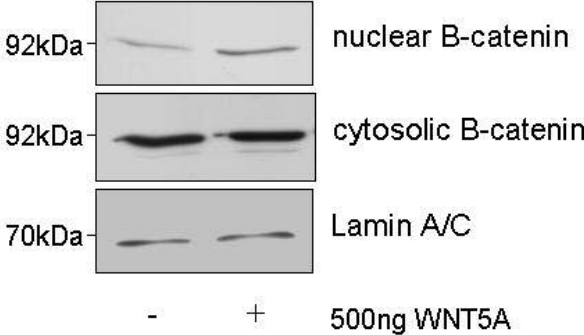


Fig.5H

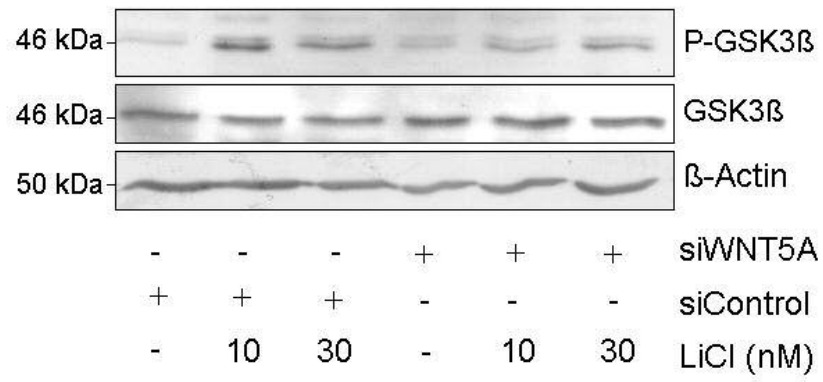


Fig.5I

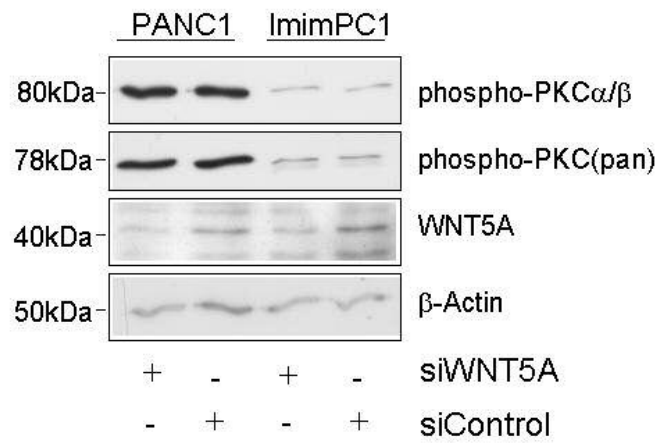


Fig.5J

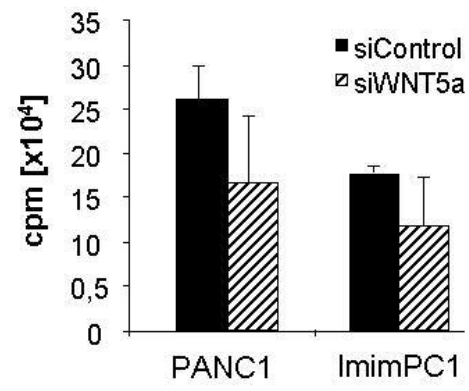


Fig.6A

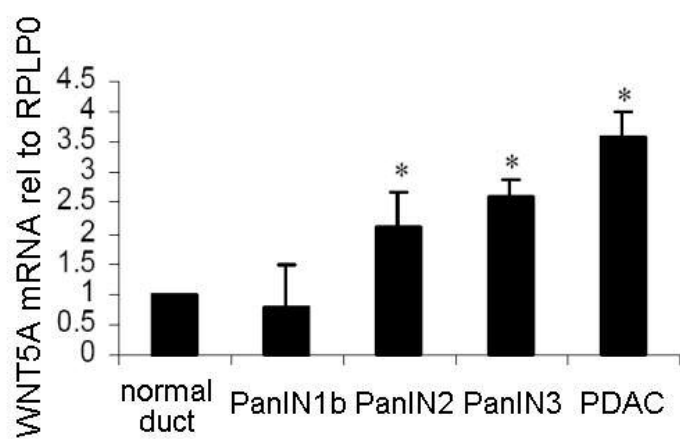
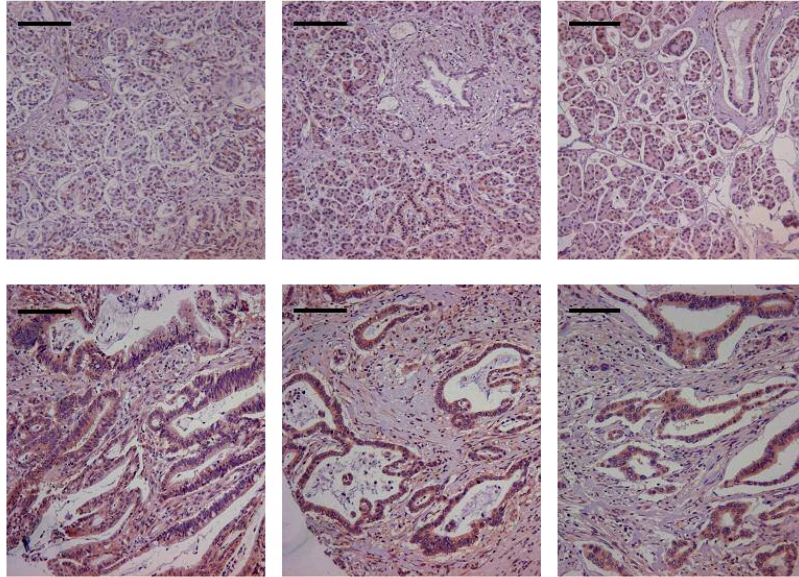


Fig.6B

Non-neoplastic pancreas



PDAC