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Activation of Wnt signalling in stroma from pancreatic cancer identified by gene expression profiling

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is characterized by an abundant desmoplastic stroma. Interactions between cancer and stromal cells play a critical role in tumour invasion, metastasis and chemoresistance. Therefore, we hypothesized that gene expression profile of the stromal components of pancreatic carcinoma is different from chronic pancreatitis and reflects the interaction with the tumour. We investigated the gene expression of eleven stromal tissues from PDAC, nine from chronic pancreatitis and cell lines of stromal origin using the Affymetrix U133 GeneChip set. The tissue samples were microdissected, the RNA was extracted, amplified and labelled using a repetitive *in vitro* transcription protocol. Differentially expressed genes were identified and validated using quantitative RT-PCR and immunohistochemistry. We found 255 genes to be overexpressed and 61 genes to be underexpressed within the stroma of pancreatic carcinoma compared to the stroma of chronic pancreatitis. Analysis of the involved signal transduction pathways revealed a number of genes associated with the Wnt pathway of which the differential expression of *SFRP1* and *WNT5a* was confirmed using immunohistochemistry. Moreover, we could demonstrate that *WNT5a* expression was induced in fibroblasts during cocultivation with a pancreatic carcinoma cell line. The identified differences in the expression profile of stroma cells derived from tumour compared to cells of inflammatory origin suggest a specific response of the tissue surrounding malignant cells. The overexpression of *WNT5a*, a gene involved in the non canonical Wnt signalling and chondrocyte development might contribute to the strong desmoplastic reaction seen in pancreatic cancer.

Keywords: pancreatic ductal adenocarcinoma • chronic pancreatitis • expression profiling • microarray • stroma • microdissection • WNT5a

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer-related death today. In the United States it ranks fourth, accounting for approximately 30,000 deaths per year [1]. Apart from surgery there is no effective therapy, and even most patients who had undergone tumour resection die within the first 3 years after surgery [2]. The main reason for this poor prognosis

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is local recurrence and/or the occurrence of distant metastases. Whereas the genetic and epigenetic changes of tumour epithelia in general have been investigated in great detail in the last decades [3], the contribution of tumour stroma during the natural history is still incomplete [4]. Stromal cells might revert a tumourigenic phenotype [5], but participation of stromal tissue during metastatic processes has also been described [6]. The stromal compartment of a tumour might also harbour genetic changes indicating a coevolutionary process of tumour epithelium and stroma compartment [7]. In PDAC, it has been demonstrated that radiation of stromal fibroblasts increase the invasiveness of PDAC [8, 9]. Several mediators of stromal epithelial interaction have been described [10], of which *TGF-β* takes part in the development of fibrotic changes within the stroma of pancreatic tissue [11]. Only recently the relevance of the Wnt signalling pathway in mediating stromal–epithelial interactions of tumours has been described [12]. However, in contrast to many other solid tumours the canonical Wnt signal transduction pathway is rarely activated in PDAC, since translocation of β-catenin to the nucleus is seldom observed [13, 14].

Recently, various research groups have applied DNA-microarray technology to identify differentially expressed genes in PDAC for new diagnostic and therapeutic approaches [15–20]. Most of these studies were performed on whole tissue samples which contain different cell types and focused on the gene expression differences of the epithelial compartment of the tumour. Those expression profiles cannot be attributed to one cell type and thus, microdissection is the method of choice to generate a more precise picture of gene expression changes in a specific cell type. We have established microdissection and microarray-based gene expression profiling in PDAC epithelia cells [21], and we have now compared now gene expression profiles from tumour-associated stromal tissue with expression profiles from benign stromal tissue using the Affymetrix U133 GeneChip set. Since there are only very few stromal cells adjacent to normal pancreatic ducts, we analysed stromal cells from the fibrotic tissue of chronic pancreatitis a benign tissue remodelling process of the pancreas [22]. We identified genes whose expression levels differed significantly between stroma from PDAC and from chronic pancreatitis. Within this set of genes, we detected an over-representation of genes encoding Wnt signalling molecules within the PDAC stroma and we were able to validate the overexpression of WNT5a suggesting that the non-canonical Wnt signalling cascade is activated.

Material and methods

Patients and tissues

Freshly frozen tissue samples of PDACs ($n = 11$) were obtained from surgical specimens from patients who underwent surgery at the Department of Visceral-, Thoracic- and Vascular-Surgery, University Hospital Carl Gustav Carus, Technical University of Dresden and the Department of General Surgery, University of Kiel, Germany, between 1996 and 2003. Chronic pancreatitis specimens ($n = 9$) were obtained from patients who underwent pancreatic resection. Prior to surgery, all patients had signed an informed consent form that had been approved by the local ethics committee. Immediately after surgical removal the specimens were sectioned and microscopically evaluated. Suitable samples of tumour tissue or normal tissue were snap frozen in liquid nitrogen and stored at −80°C until further processing.

Microdissection

Microdissection was performed in the laboratory of the Institute of Pathology, University Schleswig-Holstein Campus Kiel, Kiel, Germany as already described [21]. In brief, frozen tissue specimens were cut into 10 μm sections and slides were immediately fixed in 70% ethanol. The sections were briefly stained with haematoxylin and eosin and coverslipped. Suitable areas for microdissection without visible neoplastic cell contamination were marked on these slides and served as a template for microdissection. The estimated cellularity was approx. 10,000–11,000 cells per microdissected sample and the purity of the dissections was approx. 90%. For comparison, we included the gene expression data from our previous study of microdissected tumour epithelia [21] and seven additional epithelia samples (two normal, five PDAC).

Cell culture

The cell lines CAPAN2, BxPC3, CAPAN1, ASPC1, COLO357, MiaPaCa2, Panc1, Panc89, PT45, PancTUI, Kif5, F13 [23] and immortalized primary stellate cells [24] were cultured in RPMI 1640 supplemented with 10% foetal bovine serum, 2 mM glutamine, non-essential amino acids (5 ml/l), penicillin (10,000 units/ml), and streptomycin (10 mg/ml) and passaged before they reached confluency. All cell culture materials were obtained from Invitrogen, Karlsruhe, Germany. 1.5×10^5 Panc89 and 1.5×10^5 Kif5 cells (human mesenchymal cells) were cocultured and separated by a 0.2 μm membrane (Becton Dickinson Labware, NJ, USA). After reaching confluence (48 hrs), the cells were trypsinized and transferred to new vessels to coculture cells for 60 additional hrs. Following trypsinization of the cells RNA isolation was performed using a Micro-to-Midi Total RNA Purification System (Invitrogen, Paisley, UK) according to the manufacturer's instruction.

RNA preparation and array hybridization

RNA preparation and array hybridization was performed as described earlier [21]. In brief, Poly A^+-RNA from the microdissected surgical specimens and cell cultures was prepared using a PolyATtract 1000 kit (Promega, Heidelberg, Germany). For each sample the cDNA synthesis and repetitive *in vitro* transcription were performed three times. First-strand cDNA synthesis was initiated using the Affymetrix T7-oligo-dT promotor-primer combination at 0.1 mM. After second-strand cDNA synthesis the *in vitro* transcription was performed using Ambion's Megascript kit (Ambion, Huntington, UK), as recommended by the manufacturer. From the generated aRNA a new first-strand synthesis was initiated using 0.025 mM of a

random hexamer as primer. After completion, the second-strand synthesis was performed using the Affymetrix T7-oligo-dT promotor-primer combination as primer at a concentration of 0.1 mM. A second *in vitro* transcription was performed and then the procedure was repeated one additional time. During the last *in vitro* transcription, biotin-labelled nucleotides were incorporated into the aRNA, as recommended by the Affymetrix protocol. Hybridization and detection of the labelled aRNA on the U133 A/B Affymetrix GeneChip set (Affymetrix, Santa Clara, CA, USA) were performed according to Affymetrix instructions.

Gene expression analysis

The U133 A/B Affymetrix GeneChip set utilized in this study consists of more than 44,000 probe sets. The Cel Files obtained from the Affymetrix MAS 5.0 software were loaded into dChip2006 (www.dchip.org), normalized and expression values were calculated using the PM/MM model [25]. To minimize the noise within the gene expression data set we used only the probe sets that displayed an intensity value of greater than 120 in more than 15% of the chips analysed and in which average intensity were below 4000. The cut-off of 120 for the intensity value was derived from the intensity values from the bacterial control probe sets for the Bacillus subtilis genes dapB, lys, pheA, thrC and trpE within the data set. Only 1% of those probe sets revealed intensity values above 120, thus limiting the probability of false positives due to random fluctuation of the background intensities. The expression values were exported and further explored using SAM (http://www-stat.stanford.edu/~tibs/SAM/) [26] and Excel (Microsoft, Seattle, WA, USA). We scored genes as differentially expressed if they met the following criteria: a fold change$>$2 and a q-value $\leq$5%. Identification of probe sets expression overexpressed only in pancreatic tumour stroma was done using dChip (cut-off: fold change $>$4 and *P*-value $<$ 0.05) by comparing the expression of tumour stroma samples (group 1) with chronic pancreatitis stroma, tumour epithelia and normal epithelia samples (group 2).

Quantitative RT-PCR

The aRNA from the second amplification cycle, as stated above, was reversely transcribed into cDNA. 1 ng of cDNA was used for a TaqMan assay (Applied Biosystems, Weiterstadt, Germany). The genes were amplified with the TaqMan Universal PCR Master Mix according to the manufacturer's instructions, with an ABI PRISM 5700 Sequence Detection System using gene specific primers and probes. Gene expression was quantified by the comparative cT-Method, normalizing cT-values to a housekeeping gene (*β-actin* or *G6PDH*) and calculating the relative expression values [27] using the following primers: G6PDH forward: 5′-acgtgatgcagaaccacctactg; G6PDH reverse: 5′- acgacggctgcaaaagtggcg-3′; SFRP1 forward: 5′-gatgcaggaggctcaggtgat-3′; SFRP1 reverse: 5′-tgtcctgtgtatctgctggcaac-3′; WNT5a forward: 5′-taggcacgaaagcacaggtc-3′; WNT5a reverse: 5′-cacggcatctctctttcacca-3′; CXCL14 forward: 5′-ctgtgatggcgagacaaatg-3′; CXCL14 reverse: 5′-gttgggaacctcacatgctt-3′; β-actin forward: 5′-aatgctatcacctcccctgtgt-3′ and β-actin reverse: 5′-aagccaccccacttctctctaa-3′.

For the cocultivation experiments total RNA was prepared and the reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers (Applied Biosystems, Foster City, CA, USA) according to the manufacture's suggestions. The subsequent quantitative PCR reaction was conducted using SYBR green Super Mix (Bio-Rad Laboratories GmbH, Munich, Germany), gene specific primers and the following PCR conditions: 95°C, 5 min. (single step); 95°C, 30 sec.; 60°C, 45 sec.; 72°C, 70 sec. (40 cycles). The fluorescence was determined by Bio-Rad's Opticon PCR-System.

Immunohistochemistry

For immunohistochemistry, 5 μm sections were routinely prepared using sialinized slides (superfrost slides from Menzel Gl‰ser, Braunschweig, Germany). Immunohistochemistry for WNT5a and SFRP1 was performed using the streptavidin-biotin-peroxidase method as described previously [28]. Antigen retrieval was carried out in a microwave oven (250 W for 30 min. in a citrate solution pH 6.0). The primary antibodies used were a mouse polyclonal antibody against the WNT5a protein (clone GT15034; 10 μg/ml; Neuromics, Northfield, MN and sc-23698, Santa Cruz, CA, USA), a polyclonal antiserum generated in rabbits with a synthesized peptide corresponding to amino acids 285-299 of the human SFRP1 protein (1:50) [29] and a goat antibody against the CXCL14 protein (AF866; 1:50; R&D Systems, Minneapolis, MN, USA). As negative control specimens were incubated without the primary antibody. Afterwards, the slides were briefly counterstained with haematoxylin and eosin. For the negative control, the primary antibody was omitted. The staining intensity was evaluated for each sample semi-quantitatively as absent, weak, moderate or strong by one pathologist (A.H. A.W. or G.K.) and by C.P. without knowledge of the histopathologic and molecular data. For the analysis of immunohistochemical staining of tumour and the adjacent normal tissue we employed the Wilcoxon Rank test.

Results

Gene expression analysis of stromal tissues

Using microdissection, stromal tissue adjacent to the tumour from 11 patients with PDAC and stromal tissue from nine patients with chronic pancreatitis were obtained and compared. The combination of fold change (cut-off: $>$2) and SAM q-value (cut-off: $\leq$5%) analysis yielded 331 differentially expressed probe sets from 316 genes. In total, 61 genes were under-expressed and 255 were overexpressed in the stromal tissue of PDAC (Table 1).

Hierarchical clustering of the stromal tissue samples using the 331 probe sets revealed two major clusters of stromal tissue of chronic pancreatitis and of PDAC (Fig. 1A).

Identifcation of genes overexpressed in tumour stroma

To identifiy genes highly expressed in pancreatic tumour stroma, we compared the expression of the 331 probe sets between pancreatic tumour stroma and other primary pancreatic tissues excluding pancreatic cell lines. 19 of the 331 probe sets representing 16 genes (including *CXCL14*, *COL10A1* and *COL11A1*) were up-regulated by a fold change of at least 4 (Fig. 1B).

Table 1 The 100 most differentially expressed genes in PDAC stromal tissue

Representative public ID	Affymetrix probeset ID	Fold change (TS/CP)	Gene symbol	Gene title
Up-regulated				
NM_005498	218261_at	22.67	*AP1M2*	Adaptor-related protein complex 1, mu 2 subunit
NM_004887	***218002_s_at***	**13.25**	**CXCL14**	***Chemokine (C-X-C motif) ligand 14***
AI739132	229479_at	8.97	*LOC646324*	Hypothetical LOC646324
NM_001854	204320_at	8.04	*COL11A1*	Collagen, type XI, α 1
NM_005940	203878_s_at	7.40	*MMP11*	Matrix metallopeptidase 11 (stromelysin 3)
NM_007036	208394_x_at	6.55	*ESM1*	Endothelial cell-specific molecule 1
BC006361	211050_x_at	6.21	*DKFZP434B2016*	Similar to hypothetical protein LOC284701
AF352728	221701_s_at	6.09	*STRA6*	Stimulated by retinoic acid gene 6 homologue (mouse)
AU156710	227123_at	5.68	*RAB3B*	RAB3B, member RAS oncogene family
AB033025	212942_s_at	5.25	*KIAA1199*	KIAA1199
AI822137	230135_at	5.17	*EST*	CDNA FLJ42405 fis, clone ASTRO3000474
BE327661	240649_at	4.89	*EST*	Transcribed locus
AA741296	238812_at	4.69	*ZFAND6*	Zinc finger, AN1-type domain 6
M86849	223278_at	4.64	*GJB2*	Gap junction protein, β 2, 26 kD (connexin 26)
AW972824	244765_at	4.63	*DUSP27*	Dual specificity phosphatase 27 (putative)
AK025453	228656_at	4.33	*PROX1*	Prospero-related homeobox 1
AW301806	217580_x_at	4.33	*ARL6IP2*	ADP-ribosylation factor-like 6 interacting protein 2
NM_006587	220356_at	4.18	*CORIN*	Corin, serine peptidase
AI376003	205941_s_at	4.08	*COL10A1*	Collagen, type X, α 1(Schmid metaphyseal chondrodysplasia)
AF288571	221558_s_at	3.90	*LEF1*	Lymphoid enhancer-binding factor 1
AA382425	232109_at	3.90	*UBXD3*	UBX domain containing 3
T10030	228210_at	3.87	*NXPH3*	Neurexophilin 3
NM_003248	204776_at	3.84	*THBS4*	Thrombospondin 4
M57707	204188_s_at	3.78	*RARG*	Retinoic acid receptor, γ
NM_003326	207426_s_at	3.76	*TNFSF4*	Tumour necrosis factor (ligand) superfamily, member 4
AB051466	232327_at	3.73	*THSD7B*	Thrombospondin, type I, domain containing 7B
AL136559	223536_at	3.61	*PSD2*	Pleckstrin and Sec7 domain containing 2
NM_012261	219463_at	3.60	*C20orf103*	Chromosome 20 open-reading frame 103
M31159	210095_s_at	3.50	*IGFBP3*	Insulin-like growth factor-binding protein 3
AW296153	235540_at	3.50	*EST*	—
AI206039	231187_at	3.41	*SLC28A1*	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 1
W74476	226997_at	3.41	*EST*	CDNA FLJ10196 fis, clone HEMBA1004776
AB032931	223229_at	3.34	*UBE2T*	Ubiquitin-conjugating enzyme E2T (putative)
AW593143	205134_s_at	3.32	*NUFIP1*	Nuclear fragile X mental retardation protein interacting protein 1
NM_016931	219773_at	3.30	*NOX4*	NADPH oxidase 4

Table 1 Continued

Representative public ID	Affymetrix probeset ID	Fold change (TS/CP)	Gene symbol	Gene title
AI081522	244486_at	3.29	*PINK1*	PTEN-induced putative kinase 1
AB015329	215138_s_at	3.27	*KIAA1026*	Kazrin
AI378647	230147_at	3.26	*F2RL2*	Coagulation factor II (thrombin) receptor-like 2
AW664964	230493_at	3.21	*TMEM46*	Transmembrane protein 46
AK024472	226530_at	3.15	*BMF*	Bcl2 modifying factor
NM_001845	211981_at	3.14	*COL4A1*	Collagen, type IV, α 1
BF476080	229779_at	3.13	*COL4A4*	Collagen, type IV, α 4
NM_021161	220727_at	3.10	*KCNK10*	Potassium channel, subfamily K, member 10
NM_002381	206091_at	3.09	*MATN3*	Matrilin 3
AI222435	230319_at	3.07	*C4orf31*	Chromosome 4 open-reading frame 31
U09716	203294_s_at	3.04	*LMAN1*	Lectin, mannose-binding, 1
U03115	217060_at	3.01	*EST*	T cell receptor b chain variable region (TCRB) mRNA, 5′ end
AI742057	226702_at	2.99	*LOC129607*	Hypothetical protein LOC129607
AA012950	239126_at	2.98	*C19orf23*	Chromosome 19 open reading frame 23
NM_003427	207494_s_at	2.97	*ZNF76*	Zinc finger protein 76 (expressed in testis)
Down-regulated				
NM_019598	220782_x_at	0.04	*KLK12*	Kallikrein-related peptidase 12
NM_000273	206696_at	0.05	*GPR143*	G protein-coupled receptor 143
NM_005867	207258_at	0.11	*DSCR4*	Down syndrome critical region gene 4
AI090768	224808_s_at	0.13	*C7orf20*	Chromosome 7 open reading frame 20
NM_006461	203145_at	0.14	*SPAG5*	Sperm-associated antigen 5
AF105378	228206_at	0.15	*HS3ST4*	Heparan sulphate (glucosamine) 3-O-sulfotransferase 4
N53051	229378_at	0.16	*STOX1*	Storkhead box 1
NM_017748	218655_s_at	0.17	*CCDC49*	Coiled-coil domain containing 49
BE273906	238980_x_at	0.18	*C17orf56*	Chromosome 17 open reading frame 56
NM_002580	205815_at	0.18	*REG3A*	Regenerating islet-derived 3 alpha
AA419275	224970_at	0.20	*NFIA*	nuclear factor I/A
U05598	209699_x_at	0.20	*AKR1C2*	Aldo-keto reductase family 1, member C2
M25915	208792_s_at	0.22	*CLU*	Clusterin
BF439728	238317_x_at	0.25	*RBMS1*	RNA binding motif, single-stranded interacting protein 1
NM_001458	207876_s_at	0.27	*FLNC*	Filamin C, γ (actin-binding protein 280)
NM_001353	204151_x_at	0.27	*AKR1C1*	Aldo-keto reductase family 1, member C1
M13452	212089_at	0.28	*LMNA*	Lamin A/C
NM_006179	231785_at	0.28	*NTF5*	Neurotrophin 5 (neurotrophin 4/5)
NM_003277	204482_at	0.30	*CLDN5*	Claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)
NM_001937	207977_s_at	0.31	*DPT*	Dermatopontin
AW157077	203197_s_at	0.31	*C1orf123*	Chromosome 1 open reading frame 123

Table 1 Continued

Representative public ID	Affymetrix probeset ID	Fold change (TS/CP)	Gene symbol	Gene title
AB056476	224339_s_at	0.32	*ANGPTL1*	Angiopoietin-like 1
BE646573	223217_s_at	0.33	*NFKBIZ*	Nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor
NM_002178	203851_at	0.35	*IGFBP6*	Insulin-like growth factor-binding protein 6
AA780381	215498_s_at	0.35	*MAP2K3*	Mitogen-activated protein kinase kinase 3
AA194312	227086_at	0.36	*HIRA*	HIR histone cell cycle regulation defective homologue A (S. cerevisiae)
AL353132	217021_at	0.37	*CYB5A*	Cytochrome b5 type A (microsomal)
NM_020190	218162_at	0.37	*OLFML3*	Olfactomedin-like 3
NM_001159	205083_at	0.39	*AOX1*	Aldehyde oxidase 1
NM_000504	205620_at	0.39	*F10*	Coagulation factor X
AW779917	230003_at	0.39	*EST*	Transcribed locus
AV757441	238675_x_at	0.40	*EST*	---
AK022266	233817_at	0.40	*NBPF10*	Neuroblastoma breakpoint family, member 10
AF109161	209357_at	0.40	*CITED2*	Cbp/p300-interacting transactivator
NM_014057	218730_s_at	0.40	*OGN*	Osteoglycin (osteoinductive factor, mimecan)
NM_006086	202154_x_at	0.41	*TUBB3*	Tubulin, β 3
NM_003012	***202037_s_at***	***0.42***	***SFRP1***	***secreted frizzled-related protein 1***
AW157094	209291_at	0.42	*ID4*	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
AB028976	212845_at	0.42	*SAMD4A*	Sterile a motif domain containing 4A
AI830227	222065_s_at	0.42	*FLII*	Flightless I homologue (Drosophila)
M24317	209612_s_at	0.43	*ADH1B*	Alcohol dehydrogenase IB (class I), β polypeptide
AF043290	209447_at	0.43	*SYNE1*	Spectrin repeat containing, nuclear envelope 1
AI989530	227197_at	0.45	*SGEF*	Src homology 3 domain-containing guanine nucleotide exchange factor
NM_001449	201540_at	0.45	*FHL1*	Four and a half LIM domains 1
AK023795	222162_s_at	0.45	*ADAMTS1*	ADAM metallopeptidase with thrombospondin type 1 motif, 1
AB011538	203812_at	0.46	*EST*	CDNA clone IMAGE:5922621
BE967532	203636_at	0.46	*MID1*	Midline 1 (Opitz/BBB syndrome)
NM_001801	204154_at	0.46	*CDO1*	Cysteine dioxygenase, type I
U36764	208756_at	0.47	*EIF3S2*	Eukaryotic translation initiation factor 3, subunit 2 β, 36 kD
AL136694	223938_at	0.47	*C1orf49*	Chromosome 1 open-reading frame 49

Gene selected for further validation are marked in bold and italic. TS, tumour stroma; CP, stroma of chronic pancreatitis. The complete table is available as supplemental data.

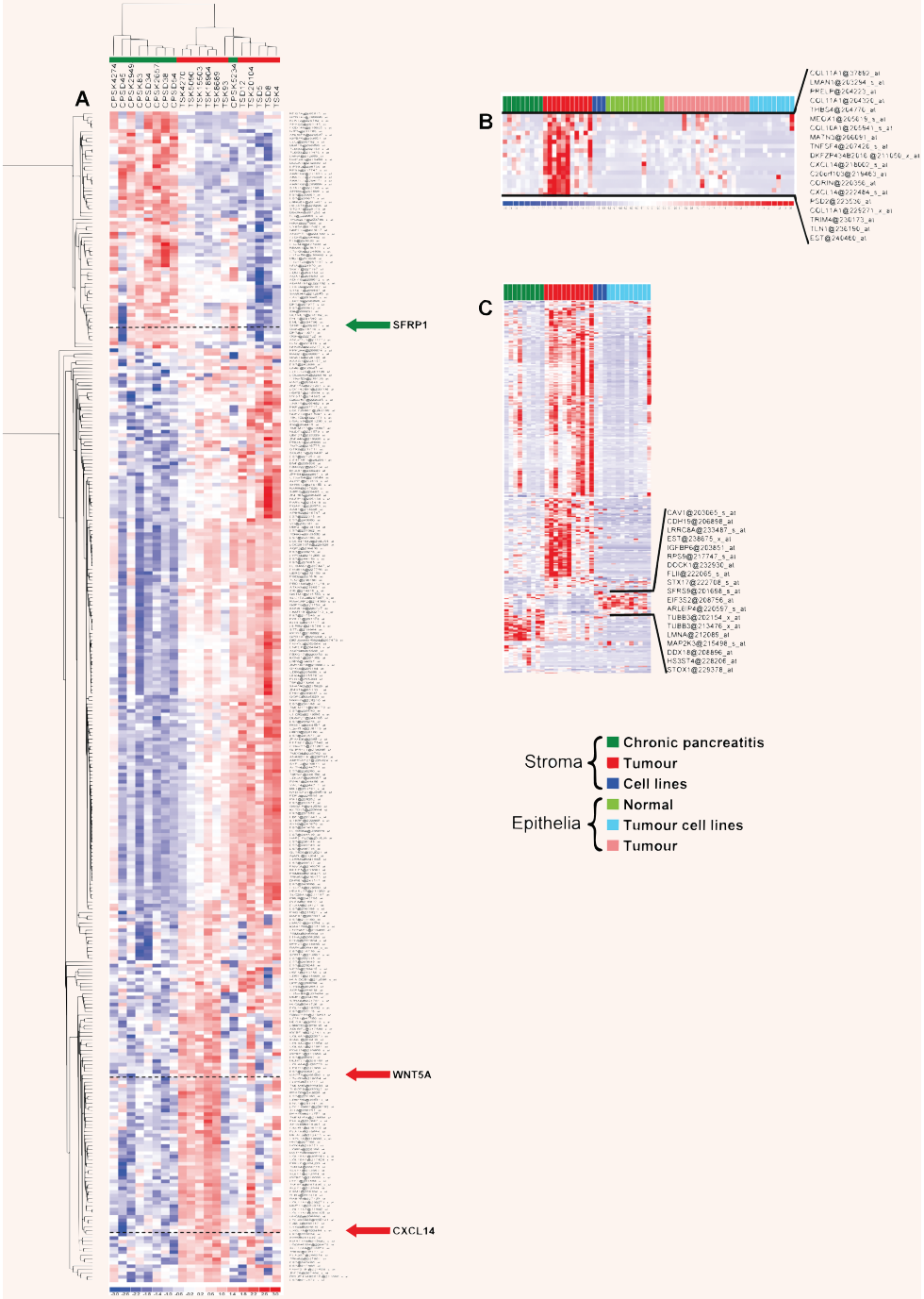
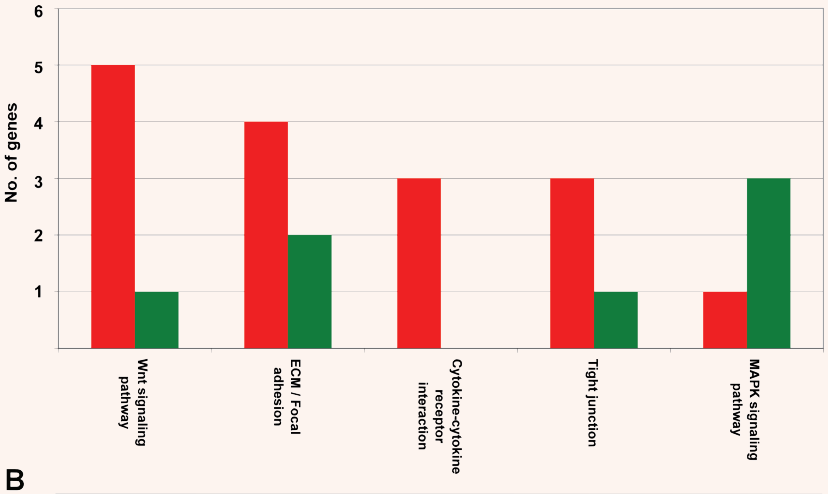


Fig. 1 (**A**) Hierarchical clustering of the stromal tissue samples using the identified differentially expressed genes. (**B**) Hierarchical clustering of pancreatic tissues and cell lines using the 205 probe sets identified as differentially expressed between tumour stroma and all other primary tissues from the 331 probe set. Probe set within the black lines are highly expressed in tumour stroma and the genes of this cluster are depicted on the right. (**C**) Heatmap of the expression of the 331 probe sets in the data set. Probe sets within the black line are highly expressed in cell lines and are depicted on the right. The cell line PT45 is the last column to the right in Fig. 1**B** and **C**.

A



B

KEGG pathways	Genes in TS	Genes in CP
Wnt signaling pathway	WNT5a; WNT2; MMP7; LRP6; LEF1;	SFRP1
ECM / Focal adhesion	THBS4; COL4A2; COL4A1; COL11A1	FLNC; CAV1
Cytokine-cytokine receptor interaction	TNFSF4; CXCL14; PRLR	
Tight junction	PPP2R4; RAB3B; F2RL2	CLDN5
MAPK signaling pathway	RASGRP2	MAP2K3; NTF5; FLNC

Fig. 2 (**A**) Association of the differentially expressed genes with KEGG signal transduction pathways. Green: genes underexpressed; red: genes overexpressed in pancreatic ductal adenocarcinoma (PDAC) stroma compared to stroma from chronic pancreatitis. (**B**) Genes identified in the KEGG pathways, TS, tumour stroma; CP, stroma of chronic pancreatitis.

Signal transduction pathway analysis of differentially expressed genes

Using the FatiGO$^+$ tool in conjunction with the KEGG database from the Babelomics web server, we were able to assign 45 out of the 316 differentially expressed genes to a KEGG pathway (Fig. 2) [30]. Interestingly, five overexpressed and one underexpressed genes were associated with the general Wnt signal transduction cascade indicating an activation of the pathway in PDAC stroma. Moreover, the KEGG pathway analysis showed that three genes of the Mitogen-activated protein kinases (MAPK) signalling pathway were underexpressed and one was overexpressed in PDAC stroma (Fig. 2A and B).

Validation of differential expression of *WNT5a*, *CXCL14* and *SFRP1*

We choose to validate the differential expression of *CXCL14*, *SFRP1* and *WNT5a* using quantitative RT-PCR and immunohistochemistry. The selection of those three genes was based on their assignment to important signal transduction pathways and on their differential expression. We could show that all genes, the underexpressed *SFRP1*, the only lightly overexpressed *WNT5a* and the highly overexpressed *CXCL14* are differentially expressed in the stroma of PDACs compared to the stromal tissue of chronic pancreatitis. Quantitative RT-PCR showed that *SFRP1* is down-regulated by a fold change of 5.27, whereas *WNT5a* is up-regulated by a fold change of 2.06 and *CXCL14* is up-regulated by a fold change of 13.25 in the stroma of PDACs (Fig. 3). Using immunohistochemistry, we analysed stroma tissue from PDAC, peritumoural chronic pancreatitis or peritumoural benign stromal tissue for the expression of *WNT5a, CXCL14* and *SFRP1*. For WNT5a, we detected an up-regulation in 92% of the cases (47/51, $P < 0.01$, Fig. 4A and B). Analysis of the stromal tissue surrounding normal ducts revealed only rarely positive cells for *WNT5a*. Recently, it has been described that *WNT5a* is regulated by *CUTL1* in PDAC [31]. Analysis of the *CUTL1* expression in our gene expression data revealed mean arbitrary intensity units for *CUTL1* in tumour stroma of 251 and 252 in chronic pancreatitis stroma indicating that *CUTL1* is expressed in pancreatic stroma tissue, but not overexpressed. For CXCL14, an overexpression could be observed in 54% of the cases of PDAC-associated stroma compared to peritumoural chronic pancreatitis tissue (7/13 Fig. 4C and D) and for SFRP1, we observed a loss of protein in stromal PDAC tissue in 65% of the cases analysed (24/37, Fig. 4E and F).

Induction of *WNT5a* by cocultivation

We also analysed three cell lines from stromal origin for their expression of the identified 331 probe sets. The stromal cell lines F13, Kif5 and Stellate, as well as the cell lines derived from PDAC tissues except PT45 expressed most of the genes in only low amounts under standard cultivation conditions (Fig. 1C). However, cocultivation of the human foreskin fibroblast cell line Kif5 with the pancreas cancer cell line Panc89 resulted in a median 2.82-fold induction of *WNT5a* RNA in the fibroblasts. This indicates that WNT5a expression might be induced by soluble factors derived from pancreatic tumours, since the two cell lines were separated by a membrane with 0.2 μm pore size (Fig. 3G).

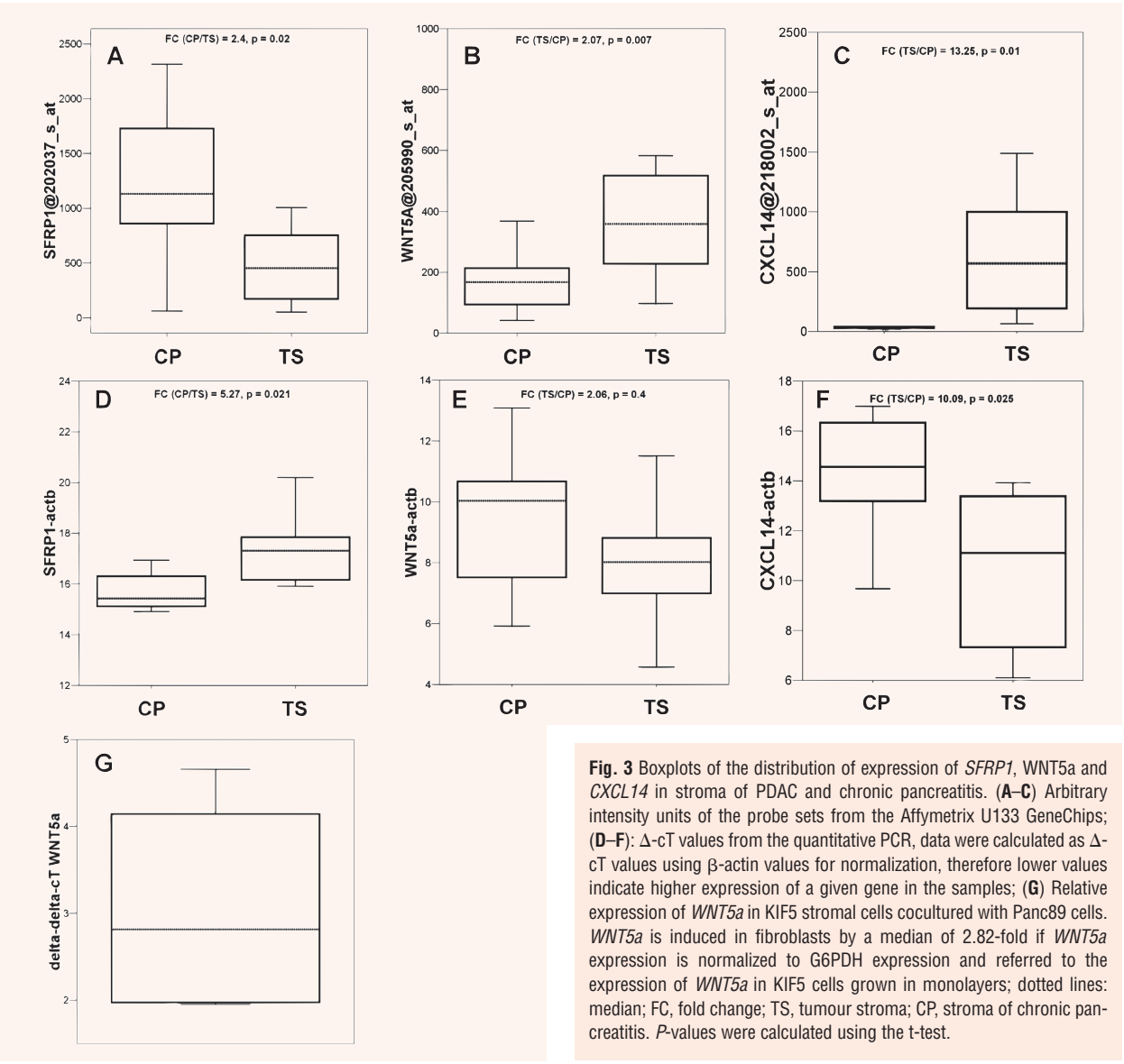


Fig. 3 Boxplots of the distribution of expression of *SFRP1*, WNT5a and *CXCL14* in stroma of PDAC and chronic pancreatitis. (**A**–**C**) Arbitrary intensity units of the probe sets from the Affymetrix U133 GeneChips; (**D**–**F**): Δ-cT values from the quantitative PCR, data were calculated as Δ-cT values using β-actin values for normalization, therefore lower values indicate higher expression of a given gene in the samples; (**G**) Relative expression of *WNT5a* in KIF5 stromal cells cocultured with Panc89 cells. *WNT5a* is induced in fibroblasts by a median of 2.82-fold if *WNT5a* expression is normalized to G6PDH expression and referred to the expression of *WNT5a* in KIF5 cells grown in monolayers; dotted lines: median; FC, fold change; TS, tumour stroma; CP, stroma of chronic pancreatitis. *P*-values were calculated using the t-test.

Discussion

A strong desmoplastic reaction is typical for PDACs, but may also be found in other carcinomas, such as colon or breast carcinoma [32–34]. Since tumour desmoplasia seems to have an association with tumour progression, analysis of stromal gene expression may increase our understanding of this process. Using the Affymetrix U133 GeneChip set we performed the first whole genome gene expression analysis of microdissected cells from PDAC stroma and chronic pancreatitis stroma. Thus the tumour-specific stromal reaction can be discriminated from the severe, but benign reaction represented by chronic pancreatitis. We identified 316 differentially expressed genes represented by 331 Affymetrix probe sets, of which 255 were up-regulated and 61 genes down-regulated in the stromal tissue of PDAC compared to chronic pancreatitis stroma. Within the set of genes up-regulated in PDAC stromal tissue, we identified *MMP11* and *CXCL14* which has been shown to be activated in stromal tissue other carcinomas [35–37]. Despite the importance of the stromal tissue for tumour development and maintenance, few gene expression studies on stromal tissue have been performed. This might be due to the fact that microdissection is a prerequisite for profiling stromal

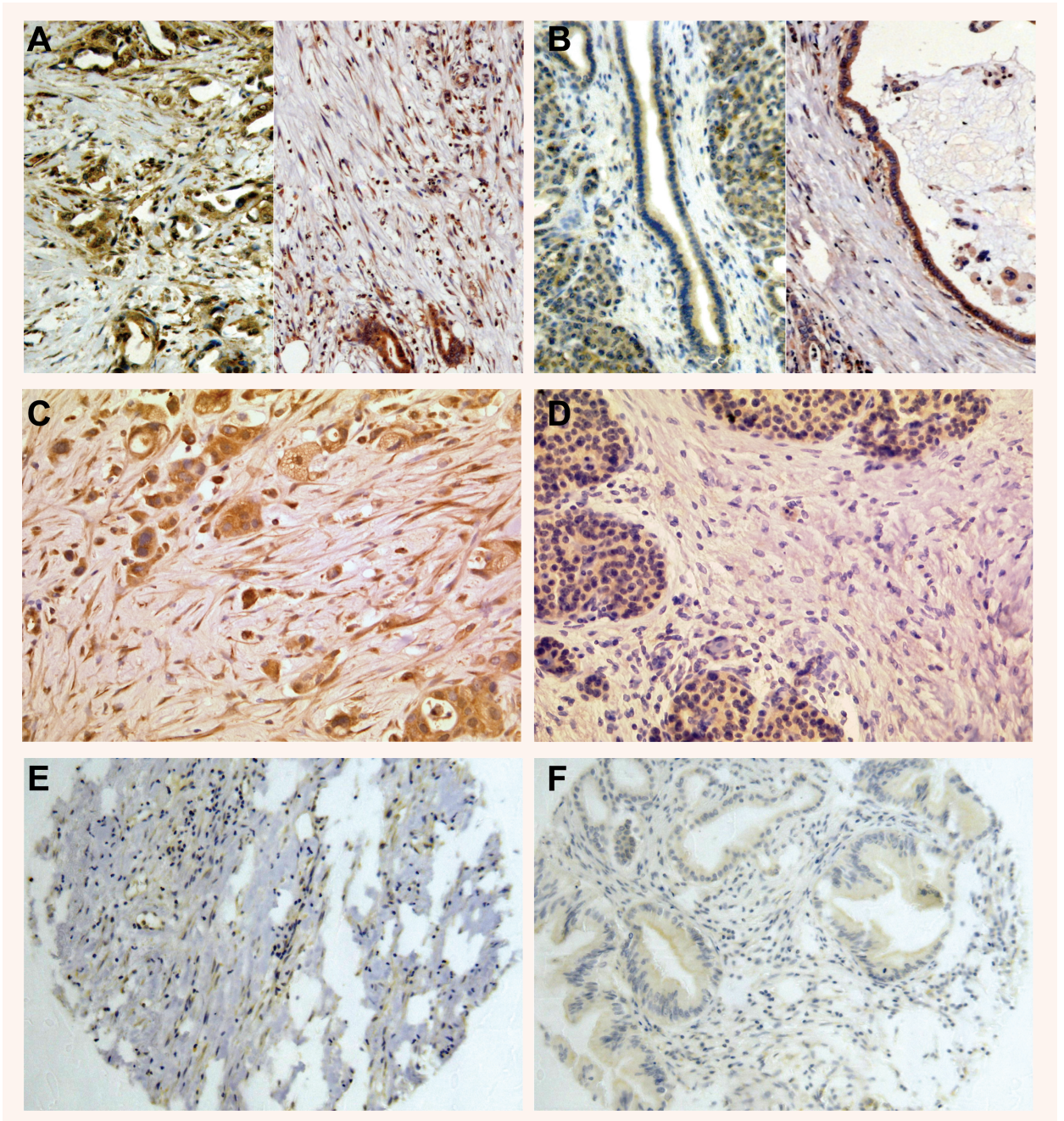


Fig. 4 Immunohistochemistry of Wnt pathway associated genes. (**A**) Strong expression of *WNT5a* in PDAC stroma cell and (**B**) lack of expression of *WNT5a* in stromal tissue adjacent to normal ducts in two different samples. The surrounding acinar cells of the pancreas show staining for *WNT5a*, whereas the normal duct epithelia show only a faint stain and staining is absent within the stromal cells; (**C**) *CXCL14* expression in PDAC epithelia and adjacent stromal cells. (**D**) Lack of expression of *CXCL14* in a stroma of chronic pancreatitis. (**E**) Expression of *SFRP1* in the stroma of chronic pancreatitis and blood vessels; (**F**) Loss of *SFRP1* expression in the stroma of a well-differentiated PDAC. (**A**–**F**) 200×.

tissue. We compared our data with the data set published by Binkley *et al.* and found an overlap of six genes (*COL10A1*, *COL4A2*, *IFI30*, *IFI6*, *POSTN* and *UCP2*) [38]. The reason for this small overlap might be found in the different types of analysis. Binkley *et al.* used a bioinformatic approach to identify stromal gene expression by subtracting epithelial gene expression data from their data of pancreatic cancer gene expression; whereas, we analysed the gene expression of tumours stroma directly. The different GeneChip formats might also be another cause for the small overlap, we observed as Binkley *et al.* analysed only 6800 human genes, whereas we analysed of the whole human transcriptome (~30,000 genes). Of the 61 genes underexpressed in PDAC stroma, some are highly expressed in normal epithelia. This might be owed to spurious epithelial cells within the stroma of chronic pancreatitis which cannot be eliminated by manual microdissection. However, the immunohistochemical validation of *WNT5a*, *SFRP1* and *CXCL14* clearly demonstrates that the genes identified by our approach are differentially expressed between PDAC and chronic pancreatitis stroma.

We classified the genes according to their involvement in signal transduction pathways and found a high proportion of Wnt pathway members as dysregulated suggesting that this pathway plays a role in stroma–tumour interaction in PDACs. Interestingly, we found a down-regulation of *SFRP1* in PDAC stroma. As it has been shown that *SFRP1* expression is lost by hypermethylation in the majority of cancers and PDACs [39, 40], this is the first report of the down-regulation of SFRP1 in primary stromal tissue. We may speculate that *SFRP1* down-regulation in PDAC stroma is linked to hypermethylation of the *SFRP1* gene in PDAC stroma as observed in cancer epithelia. *SFRP1* is an inhibitor of canonical Wnt action and it is also capable of transducing signals without the participation of Wnt molecules *via* Frizzled receptors [41]. As *WNT5a* is known to be able to inactivate the canonical pathway, our observed overexpression of *WNT5a* in the stromal and the epithelial compartment of PDAC might be a reason for the dormancy of the canonical Wnt pathway [42]. *WNT5a* has been shown to activate *MMP7* and to enhance the invasiveness of breast cancer cells *via* the non-canonical JNK pathway which might also occur in PDAC stroma, since we observed a *MMP7* overexpression in those cells [43]. Interestingly, in PDAC cells *WNT5a* might also promote growth and invasion [31] indicating that the stromal compartment might contribute to these processes. However, despite the overexpression of *WNT5a* in PDAC stromal tissue we did not observe an overexpression of *CUTL1* suggesting that also other signalling pathways might contribute to the transcriptional activation of *WNT5a* and may act *via* soluble factors as the induction of *WNT5a* expression in fibroblasts demonstrated. These factors are most likely proteins of the hedgehog family, which are overexpressed in PDAC and known to regulate *WNT5a* expression [44, 45]. *WNT5a* has also been characterized as a modulator of chondrocyte development and associated with chondrocyte hypertrophy [46]. Chondrocyte hypertrophy is also associated with the expression of COL10A1 [47] which we and others identified to be overexpressed in PDAC stroma [38]. Therefore, *WNT5a* overexpression in tumour stroma might result in overexpression of *COL10A1*. *COL10A1* belongs to the type of network-forming collagens; whereas, *COL11A1* which we also observed as overexpressed in PDAC stroma is a fibrillar collagen [48]. The overexpression of these two types of collagen might have profound effects on the density of the extracellular matrix surrounding the PDAC cells and might contribute to the callous form of tumours seen in PDAC. Gene expression profiling of microdissected tissues has been shown feasible and generates a complete picture of changes not only in the tumour epithelia [49], but also as shown here in the stromal tissue surrounding the tumour. The differential expression we have observed in our data might therefore lead to a better understanding of the generation of desmoplasia in PDAC.

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