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ORIGINAL ARTICLE

Early epigenetic downregulation of WNK2 kinase during pancreatic ductal adenocarcinoma development

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Pancreatic ductal adenocarcinoma (PDAC) is usually incurable. Contrary to genetic mechanisms involved in PDAC pathogenesis, epigenetic alterations are ill defined. Here, we determine the contribution of epigenetically silenced genes to the development of PDAC. We analyzed enriched, highly methylated DNAs from PDACs, chronic pancreatitis (CP) and normal tissues using CpG island microarrays and identified *WNK2* as a prominent candidate tumor suppressor gene being downregulated early in PDAC development. WNK2 was further investigated in tissue microarrays, methylation analysis of early pancreatic intraepithelial neoplasia (PanIN), mouse models for PDAC and pancreatitis, re-expression studies after demethylation, and cell growth assays using WNK2 overexpression. Demethylation assays confirmed the link between methylation and expression. *WNK2* hypermethylation was higher in tumor than in surrounding inflamed tissues and was observed in PanIN lesions as well as in a PDAC mouse model. *WNK2* mRNA and protein expressions were lower in PDAC and CP compared with normal tissues both in patients and mouse models. Overexpression of WNK2 led to reduced cell growth, and WNK2 expression in tissues correlated negatively with pERK1/2 expression, a downstream target of WNK2 responsible for cell proliferation. Downregulation of *WNK2* by promoter hypermethylation occurs early in PDAC pathogenesis and may support tumor cell growth via the ERK–MAPK pathway.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), representing about 80% of all malignant tumors of the exocrine pancreas, ranks among the most lethal human cancers. Its aggressive biology, the lack of clearly recognizable symptoms and the resistance to established therapeutic agents lead to the typical clinical presentation of an incurable disease at the time of diagnosis.[1] Hence, unraveling key molecular events and improving the understanding of pancreatic cancer biology are important requirements to develop more effective treatments.

Recent molecular characterization has identified a growing number of genetic[2,3] and epigenetic changes[4] in key processes and pathways of PDAC pathogenesis. Analyses using new high-throughput techniques have revealed that several hundreds of genes may present targets for aberrant methylation in pancreatic cancer.[5–7] It has been shown that the development of PDAC occurs through the progression of precursor lesions called pancreatic intraepithelial neoplasia (PanIN).[8] Downregulation of genes[9] and hypermethylation events[10–13] have already been observed in these lesions, but the pool of target genes silenced by hypermethylation throughout PDAC pathogenesis has not been comprehensively analyzed, especially in the context of chronic pancreatitis (CP). CP, an inflammatory pancreas disease, is known to be a risk factor for pancreatic cancer.[14] DNA methylation analyses performed in pancreatic juice or in circulating plasma DNA included samples from patients with CP[15–17] and showed both common but also differentially hypermethylated promoter regions between inflammatory disease and cancer. This suggests that some genes might be specifically silenced by hypermethylation during pancreatic cancer development and not by inflammation.

In order to discover new genes involved in PDAC development, we performed a genome-wide analysis of CpG methylation in DNAs from PDAC and CP patients as well as healthy donors. Methyl-CpG Immunoprecipitation (MCIp),[18] a technique not yet applied to pancreatic tissues, was used to enrich methylated DNA for subsequent hybridization on CpG island microarrays.

One of the key hypermethylated genes in our analysis was *WNK2*, encoding a cytoplasmic serine-threonine kinase structurally distinct from other members of its kinase superfamily, having the catalytic lysine in a noncanonical position. WNK2 inhibits cell proliferation by interfering with the activation of the MEK1/ERK1/2 MAP kinase pathway.[19,20] Silencing of *WNK2* expression via promoter hypermethylation was first described in gliomas,[21] and hypermethylation of the *WNK2* promoter was also observed

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in chronic lymphocytic leukemia, acute myeloid leukemia and meningioma.[22] We investigated both the methylation and expression patterns of this gene throughout PDAC pathogenesis, as well as the functional consequences of its silencing in PDAC development and progression.

RESULTS

Genome-wide profiling of CpG island methylation in PDAC reveals aberrantly methylated target genes

To identify hypermethylated CpG islands in PDAC, we hybridized MCIp-enriched methylated DNAs from seven PDAC, two CP and five normal pancreatic (NP) frozen tissues on human CpG island microarrays. About 3.8% of the ~27.800 CpG islands screened were identified as significantly hypermethylated in PDAC compared with NP and CP samples, after combination of three different selection strategies (see Materials and Methods). Hierarchical cluster analysis of the probes hypermethylated in PDAC according to the methylation threshold analysis separated the PDAC from the NP and CP samples, using the average linkage and Euclidian distance functions (Figure 1a).

Published data on promoter identity, gene expression in NP tissue, loss of heterozygosity and other genetic alterations, and known cancer relevance were used to further classify these islands and to select the most promising target genes for deeper analysis (Supplementary Materials and Methods, Supplementary Table 2). The Epityper assay was used to validate methylation of 13 candidate promoter CpG islands with the highest score. Eight islands (*CBS*, *FGF5*, *GABBR2*, *GRIN3A*, *ID4*, *PCDH17*, *PNLIPRP2* and *RORB*) showed hypermethylation in PDAC only, four (*DSCAML1*, *GRID1*, *TBX5* and *WNK2*) in PDAC and CP and one (*PCDH9*) did not show hypermethylation relative to the NP tissues (Figure 1b).

WNK2 is hypermethylated and downregulated in PDAC

Expression levels of the 12 confirmed hypermethylated genes were assessed by qRT–PCR in a subset of PDAC, CP and NP tissues including those samples used for the CpG island microarray analysis (Figure 1c). Four genes were downregulated in PDAC relative to NP tissues: *cystathionine beta-synthase* (*CBS*), *inhibitor of DNA-Binding 4* (*ID4*), *pancreatic lipase-related protein 2* (*PNLIPRP2*) and *lysine-deficient protein kinase 2* (*WNK2*). The expression of *GRIN3A*, *RORB* and *TBX5* was too low to be measured. The unique CpG island of *PNLIPRP2* was far from the promoter region and thus not further analyzed. The *WNK2* promoter showed the most pronounced hypermethylation and was therefore investigated for *WNK2* regulation and its role in pancreatic carcinogenesis.

WNK2 showed significant promoter CpG island hypermethylation in PDAC compared with NP and downregulation in both PDAC and CP (Figures 2a and b). WNK2 protein expression was analyzed by immunohistochemistry using tissue microarrays (TMAs) comprising 247 human pancreatic tissue samples (Figure 3). Micrographs of immunostained sections were prepared to illustrate the WNK2 expression pattern in NP, CP and PDAC specimens (Figure 3a). In NP tissue sections, acinar cells did not show WNK2 expression, whereas ductal cells displayed cytoplasmic positivity. This was confirmed by additional analyses using double immunofluorescence staining for the WNK2 and the centroacinar marker cytokeratin 19 (Supplementary Figure 5). Further, in CP and within inflammatory altered lobules, acinar cells were transformed into WNK2-positive ductal cells, whereas in PDAC the duct-like atypical epithelial tumor cells showed frequently negative immunoreaction for WNK2. Most of the NP showed high WNK2 expression, whereas only about 40 and 50% of the PDAC and CP cells, respectively, had high WNK2 protein levels (Figure 3b).

DNA methylation contributes to the regulation of WNK2 expression

To validate the association between promoter hypermethylation and loss of gene expression, *WNK2* methylation and mRNA expression were compared before and after treatment of normal and tumor cell lines (Supplementary Materials and Methods) with 5-aza-dC, an inhibitor of DNA methyltransferases. *WNK2* was highly methylated (Figure 2c) and expressed at very low levels (Supplementary Figure 2 and Supplementary Table 8) in the tested cell lines. *WNK2* methylation was 15% lower in the normal cell line HPDE6-E6E7 as compared with the tumor cell lines COLO357, MIA PaCa-2 and PANC-1, which showed about 100% methylation. Global demethylation of the repetitive element *LINE1* confirmed success of the 5-aza-dC treatment in these cells (data not shown). 5-aza-dC treatment resulted in *WNK2* promoter demethylation of up to 40% in all cell lines. This was associated with an increase in *WNK2* mRNA expression in Mia PaCa2 (34%), PANC-1 (60%) and COLO357 cells (65%), although the expression level in the normal HPDE6-E6E7 cell line did not change much (Figure 2d). These results suggest a functional link between *WNK2* expression and methylation of its promoter-associated CpG island.

The 3′ end of the WNK2 promoter-associated CpG island shows specific hypermethylation in PDAC tumor cells

Our CpG island microarray experiments identified only one region of the *WNK2* promoter-associated CpG island (amplicon A1 shown in Figure 4a) as hypermethylated in PDAC (Supplementary Figure 3). In order to analyze the whole CpG island, four additional PCR amplicons (Figure 4a, A2–A5) were designed, and methylation was quantified in a subset of DNAs from PDAC, CP and NP tissues from the replication sample set, including those samples used for the CpG island microarray analysis (Figure 4b). Hypermethylation in PDAC versus NP tissues could be confirmed in amplicon A1 and was also observed at lower levels in amplicons A2 and A3. Amplicons A4 and A5, located at the 5′ end of the CpG island, upstream of *WNK2* transcription start site, did not show any difference in methylation between NP, CP and PDAC tissues.

Sixty-three additional tissue samples were analyzed and classified according to their tumor cell content (Figure 4c). Significant hypermethylation could be observed in PDAC versus NP tissues from healthy donors and in PDAC versus CP tissues ($P<0.001$ for both comparisons). Remarkably, high methylation levels ($>62\%$) were only measured in PDAC tumor cells. Further, a significant increase in *WNK2* methylation from NP to CP to PanIN and PDAC tissues was observed ($P<0.001$; Jonkheere–Terpstra trend test). This suggests that *WNK2* hypermethylation is linked to PDAC development. DNA methylation was also measured in four matched pairs of tumor and adjacent normal tissue. All DNAs from tumor tissue showed higher methylation than their matched normal samples, with methylation differences ranging from 18 to 70% (Supplementary Figure 4).

WNK2 hypermethylation and downregulation are early events in PDAC carcinogenesis

In order to investigate *WNK2* methylation in early neoplastic lesions, we isolated DNA from PanINs and assessed amplicon A1 methylation using an Epityper assay. PanINs were significantly hypermethylated compared with NP tissues ($P<0.01$; Figure 4c). The average methylation differences ranged from 44 to 92%, similar to the values obtained for PDAC samples with $\geqslant 50\%$ tumor cell content (42–89%).

To investigate the hypothesis that WNK2 has an early role in pancreatic carcinogenesis, we analyzed *WNK2* methylation in pancreatitis and PDAC mouse models (Figures 5a,b,d,e). Amplicon A1, which shows hypermethylation in human PanIN and PDAC tissues, is highly conserved between human and mouse genomes

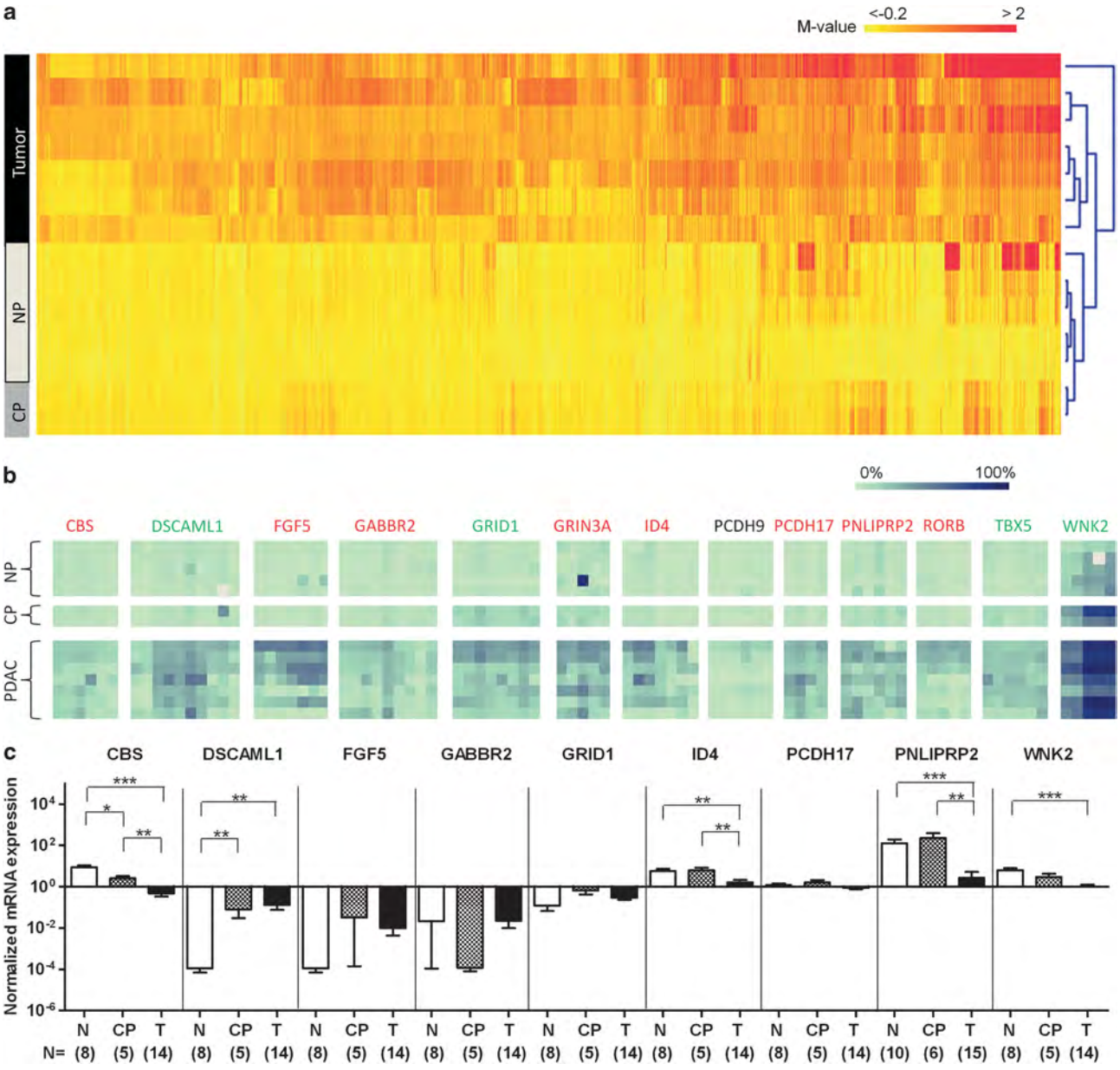

Figure 1. CpG island microarrays revealed hypermethylated and downregulated genes in PDAC. (**a**) Cluster analysis of 7067 probes identified as hypermethylated from the CpG island microarrays data (analysis ii based on methylation threshold). Processed data from 14 arrays were R-based analyzed as described. Only probes showing significant hypermethylation in the tumor versus CP and normal arrays are shown on the heat-map. M-values (log2-intensity ratios) range from $\leqslant -0.2$ (yellow) to $\geqslant 2$ (red). In all, 2.8% of the probes rank below the lower and 1.1% above the upper limit. (**b**) Quantitative DNA methylation analysis of 13 selected hypermethylated islands in the DNAs used for MCIp. Each column represents a group of one to four CpG dinucleotides; each row represents a sample. Methylation percentages span from 0 to 100%. White squares indicate technically unavailable data. Gene names in green showed hypermethylation in PDAC and CP in comparison with NP tissues, those in red were hypermethylated in PDAC only. (**c**) qRT–PCR mRNA expression analysis of 12 genes hypermethylated in PDAC, relative to the average of two housekeeping genes (logarithmic scale). Histograms represent the mean expression of each group of tissues with the corresponding s.e. NP, CP and PDAC tissues are in white, gray and black, respectively. Mann–Whitney tests were used for analyses. The pancreatic tissue samples were obtained from pancreatic ductal adenocarcinoma patients, either from tumor tissue (PDAC) or from adjacent normal pancreatic tissue (NP) or from tissue showing chronic pancreatitis (CP).

(Figure 4a and Supplementary Figure 6A). Methylation levels were measured throughout the *WNK2* promoter-associated CpG island (Supplementary Figure 6B). Hypermethylation was most pronounced in amplicon A1m, and we thus focused further analyses on this amplicon. Both acute and chronic pancreatitis mouse models as well as the acinar cell *in vitro* model (Figure 5b) did not show any methylation changes neither 48 h after caerulein treatment nor after 5 days of cell growth in suspension, respectively. In contrast, hypermethylation was observed in DNA from pancreatic tissues of the KPC versus wild-type mice (Figure 5e). *WNK2* mRNA expression was quantified by qRT–PCR in the described mouse models. A decreased expression was observed in both models for pancreatitis (Figure 5c) as well as in the KPC mouse model (Figure 5f). Taken together, these results

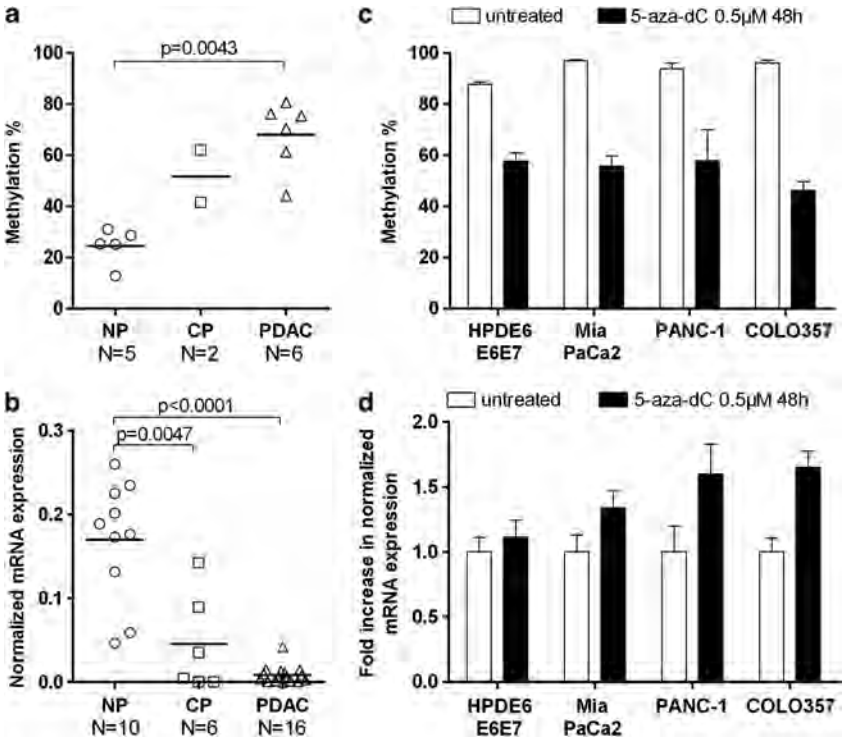

Figure 2. *WNK2* is hypermethylated and downregulated in pancreatic cancer. (**a**) Quantitative DNA methylation analysis of *WNK2*-positive probes in pancreatic tissues. The average methylation percentage for each sample from Figure 1b is shown. Horizontal bars represent mean methylation values for each group. (**b**) *WNK2* qRT–PCR mRNA expression analysis in pancreatic tissues, relative to the average of three housekeeping genes. (**c**) Average methylation percentage of *WNK2* quantified by MassARRAY in three pancreatic cancer and HPDE6-E6E7 normal cell lines, untreated and 5-aza-dC treated. Histograms represent the average value of two technical replicates, with the corresponding s.d. (**d**) Normalized *WNK2* mRNA expression in 5-aza-dC-treated cell lines relative to the expression in untreated cells. Histograms represent the average ± s.d. of three technical replicates. The pancreatic tissue samples were obtained from pancreatic ductal adenocarcinoma patients, either from tumor tissue (PDAC) or from adjacent normal pancreatic tissue (NP) or from tissue showing chronic pancreatitis (CP).

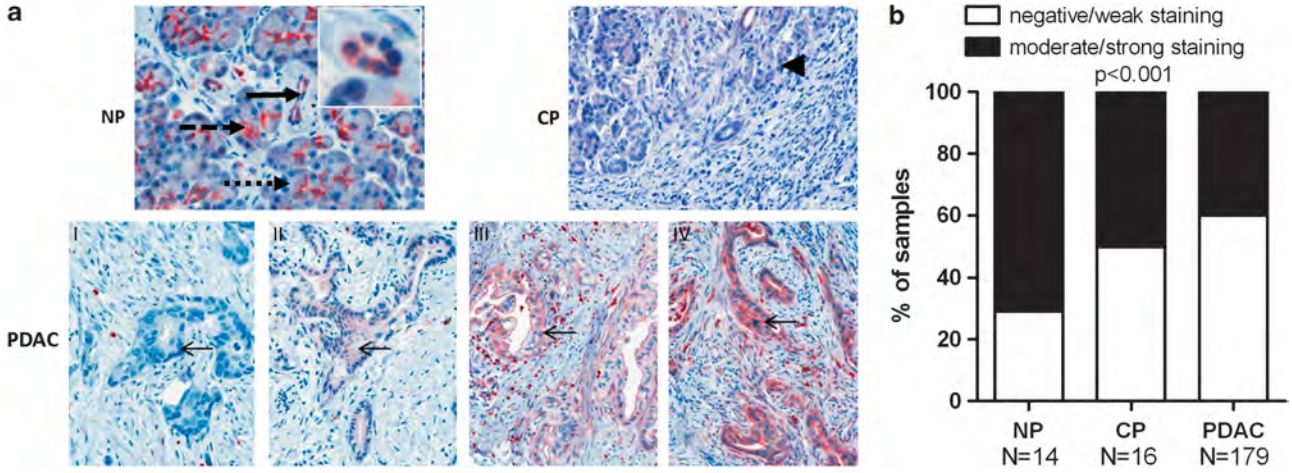

Figure 3. Expression of WNK2 in non-neoplastic pancreas, chronic pancreatitis and pancreatic cancer. (**a**) TMA immunohistochemistry for WNK2. Red color indicates positive staining. In non-neoplastic pancreatic tissue (NP), small ducts (black arrow, insert) display cytoplasmic WNK2 positivity as is also seen in centroacinar cells (dashed line arrow), whereas there is no staining in acinar cells (dotted line arrow) and islets (not shown). In chronic pancreatitis (CP), perilobular and intralobular fibrosis is seen, as well as scattered lymphocytes. Within inflammatory altered lobules, acinar cells are transformed into WNK2-positive ductal cells (arrowhead). In pancreatic ductal adenocarcinoma (PDAC), the duct-like atypical epithelial tumor cell formations (small arrow), embedded in desmoplastic stroma, frequently show negative immunoreaction for WNK2. Representative sections of PDAC are shown, depicting negative reaction for WNK2 (I) as well as weak (II), moderate (III) and strong (IV) cytoplasmic staining reaction. Within the desmoplastic stroma of PDAC, WNK2-positive inflammatory cells were observed to a various degree. As a comparable stroma is not seen in the non-neoplastic tissues, the WNK2 expression levels decreasing from normal to PDAC tissue obviously reflect the expression found in the ductal epithelial cells. In contrast, the desmoplastic stroma including its inflammatory cells seems to have no significant impact. (**b**) WNK2 protein expression on TMAs. χ^2-test shows differences in WNK2 expression between the three tissues, that is, WNK2 is more expressed in NP than CP and PDAC ($P<0.001$). The pancreatic tissue samples were obtained from pancreatic ductal adenocarcinoma patients, either from tumor tissue (PDAC) or from tissue showing chronic pancreatitis (CP) or from non-neoplastic sections adjacent to inflammatory sites (NP).

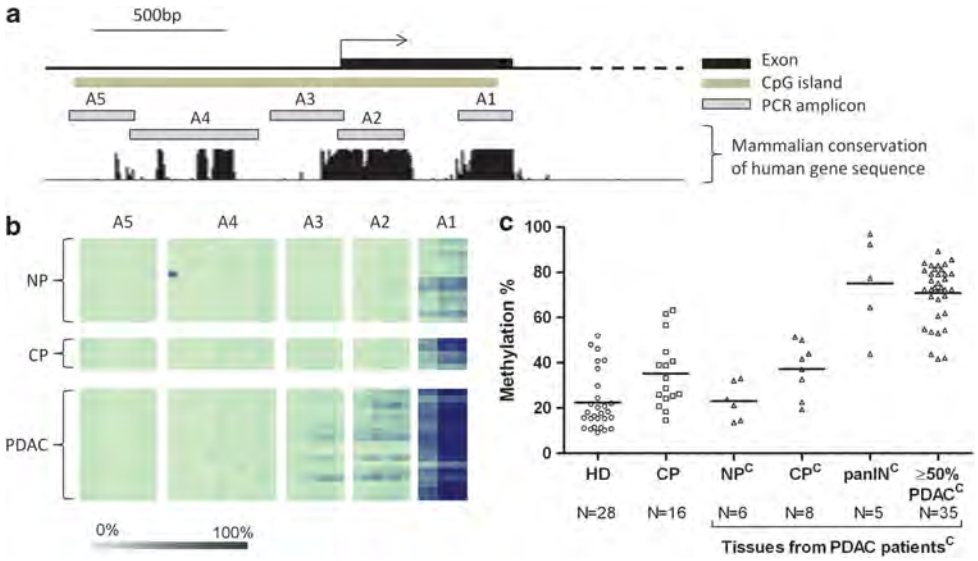

Figure 4. Analysis of *WNK2* promoter methylation in pancreatic tissues. (**a**) Scheme of *WNK2* promoter with location of CpG island and PCR amplicons (A1–A5). Amplicon A1 was initially screened (Figures 1–3). Arrow indicates the predicted transcription start site. Mammalian conservation using PhastCons is shown with black bars. (**b**) Quantitative DNA methylation analysis of *WNK2* promoter-associated CpG island with MassARRAY (cf explanations in Figure 1b). DNA samples included 13 NP, five CP and 17 PDAC tissues. (**c**) Quantitative DNA methylation analysis of A1 amplicon in pancreatic tissues. Tissues from PDAC patients (annotated with C) (NP^c, CP^c, $PanIN^c$ and $PDAC^c$) were classified according to their histology, that is, tumor and inflammatory cell content. Horizontal bars, mean methylation percentage within each group of tissues. HD, healthy donor. Mann–Whitney *U*-test results are given in Supplementary Table 7. The pancreatic tissue samples were obtained from pancreatic ductal adenocarcinoma patients, either from tumor tissue (PDAC) or from adjacent normal pancreatic tissue (NP) or from tissue showing chronic pancreatitis (CP). In addition, 28 samples of normal pancreatic tissue from healthy donors (HD) were obtained through an organ donor procurement program whenever there was no suitable recipient for pancreas transplantation.

suggest that *WNK2* hypermethylation is an early event in PDAC carcinogenesis that is not observed in pancreatitis.

WNK2 has tumor suppressor properties in PDAC

WNK2 has been shown to inhibit cell proliferation by negatively regulating the activation of the MEK1/ERK1/2 MAP kinase pathway in human cancer cells[21] (Supplementary Figure 7). Therefore, we evaluated the unphosphorylated and phosphorylated forms of ERK1/2 (pERK1/2) protein by immunohistochemistry on TMAs (Figure 6). A significant decrease in ERK1/2 was accompanied by an increase in pERK1/2 from NP to CP to PDAC tissues (Figures 6b,d, P is <0.0001 for each protein, illustrated by representative micrographs in Figures 6a–c). This shows that lower WNK2 protein expression (Figure 3b) is associated with higher pERK1/2 in PDAC (Figure 6d).

Stable overexpression of WNK2 was performed in PANC-1 cells. In comparison with cells transfected with an empty vector, overexpression leads to a 49-fold increase in *WNK2* mRNA (Figure 7a, left panel). Western blotting confirmed the overexpression of WNK2 (Figure 7a, right panel). Flow cytometry analysis of the cells 3 h and 6 h after BrdU incorporation showed that the proportion of BrdU staining was lower for cells overexpressing WNK2 than for those transfected with the empty vector (Figure 7b). Further, cell proliferation analyses using colony-forming assays (Figure 7c, $P=0.0022$) and SRB assays (Figure 7d, $P=0.029$ for 6-day time point) showed significantly reduced proliferation of cells with WNK2 overexpression versus empty vector control cells. Taken together, these results suggest a moderate but real repressive cell growth effect of WNK2 overexpression.

DISCUSSION

Applying CpG island microarray analysis on methylation-enriched DNA of PDAC samples, we showed that 3.8% of 27.800 interrogated CpG islands were significantly hypermethylated in PDAC versus NP and CP tissues. Previous studies by Omura *et al.*[6] and Vincent *et al.*[7] used methylated CpG island amplification coupled with CpG island microarray analysis to search for aberrant methylation in PANC-1 versus HPDE6-E6E7 cell lines and in PDAC versus NP tissues, respectively. About 90 and 62% of the genes that were identified with our assay in pancreatic tissues were unique and did not overlap with these respective previous analyses. Of the 12 hypermethylated genes examined here in more details, only four were associated with a decreased expression in PDAC. The *ID4* gene had already been identified as hypermethylated and downregulated in PDAC by Vincent *et al.*[7] The other three genes, *CBS*, *PNLIPRP2* and *WNK2*, are novel epigenetic targets that had not been described before as being aberrantly methylated in PDAC. It is noteworthy that only three out of 11 genes with promoter hypermethylation in PDAC were also downregulated in PDAC, suggesting that promoter hypermethylation alone is not sufficient to silence the expression of the still active genes. Vincent *et al.*[7] observed an even lower correlation between hypermethylation and downregulated expression in their microarray studies and found that only 8% of 1206 hypermethylated genes were downregulated in pancreatic cancer cells compared with HPDE6-E6E7 cells.

We focused our further analyses on the tumor suppressor WNK2, a cytoplasmic serine-threonine kinase. So far, aberrant methylation of this gene has been reported in brain tumors[21,22] but not in PDAC. We show here that *WNK2* is silenced by hypermethylation in PDAC versus NP tissues, at both mRNA and protein levels. The link between methylation and expression was further supported by studies in pancreatic normal and tumor cell lines, where demethylation of the *WNK2* promoter with 5-aza-dC leads to an increase in *WNK2* mRNA expression. *WNK* genes other than *WNK2* did not display aberrant promoter methylation in PDAC samples in our microarray methylation data. However, one should keep in mind that other WNK family members might

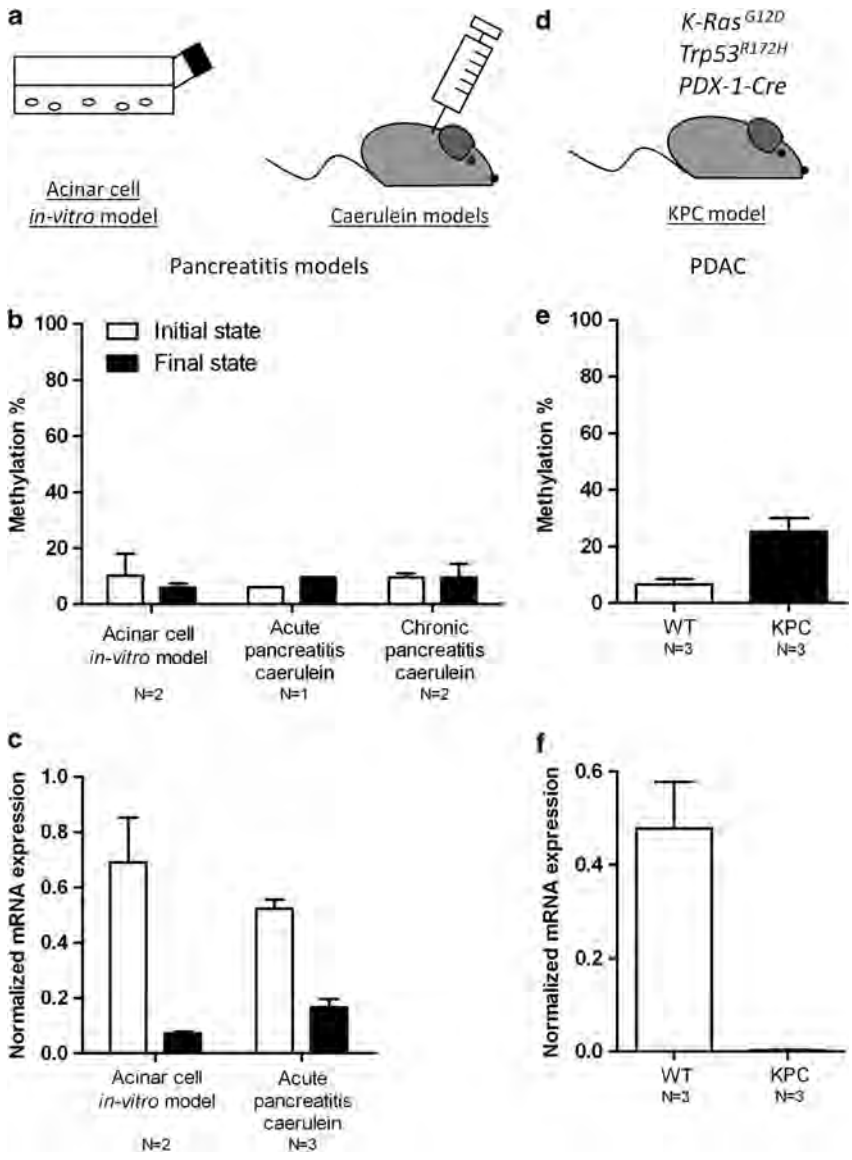

Figure 5. *WNK2* methylation and expression in pancreatitis and PDAC mouse models. (**a**, **d**) Schematic representation of mouse models. (**b**, **c**) MassARRAY and qRT–PCR expression analysis in the pancreatitis models. Histograms represent the average value ± s.d. of the samples. RNA from the chronic pancreatitis caerulein model was not available. Cells were analyzed after 5 days after growth in suspension (acinar cell model) and after 48 h/4 weeks (acute/chronic pancreatitis models). (**e**, **f**) MassARRAY and qRT–PCR expression analysis in the PDAC model. Normalized mRNA expression is 0.002 ± 0.001.

influence WNK2 activity,[23–26] and that the levels of these proteins might modulate the cellular response to changes in WNK2 expression.

Methylation screening of the whole *WNK2* promoter-associated CpG island revealed that only its 3′ part is hypermethylated in PDAC. This is in agreement with findings from brain tumor studies.[21,22] The absence of aberrant methylation in the 5′ region of this CpG island suggests that methylation of this region is not required for silencing, or that histone deacetylation downregulates the expression of *WNK2* in this part of the island, as seen in glioma.[21] In this study, Hong *et al.*[21] used promoter–reporter assays in HEK293T cells to show strong promoter activity in the 5′ region of this CpG island. The correlation between *WNK2* expression and the methylation of the 3′ region in our study suggests that the aberrantly methylated 3′ region may harbor a binding site for an enhancer transcription factor that cannot bind to methylated DNA in PDAC, leading to *WNK2* downregulation.

WNK2 hypermethylation seems to be an early event in PDAC carcinogenesis. The observed degree of *WNK2* methylation in PanIN lesions was comparable to PDAC tissues with high tumor cell content. Pancreatic tumors showed also a higher degree of *WNK2* methylation in a *K-Ras*-mutated mouse model for PDAC than in wild-type controls, corroborating our observations in human PDAC. PDAC pathogenesis is characterized by a change in population lineages, that is, an increase in ductal cells, which normally represents only about 4% of the normal pancreas in volume,[27] and a decrease in acinar cells. It is therefore the question whether the observed changes in WNK2 expression and promoter methylation could be attributed to changes in cell lineages. Our data show that WNK2 was expressed in ductal cells but not in acinar cells of NP tissue. In PDAC, it is hypermethylated and frequently not expressed in the duct-like cancer cells. Acinar atrophy due to PDAC and accompanying chronic inflammation thus does not seem to contribute to the observed WNK2

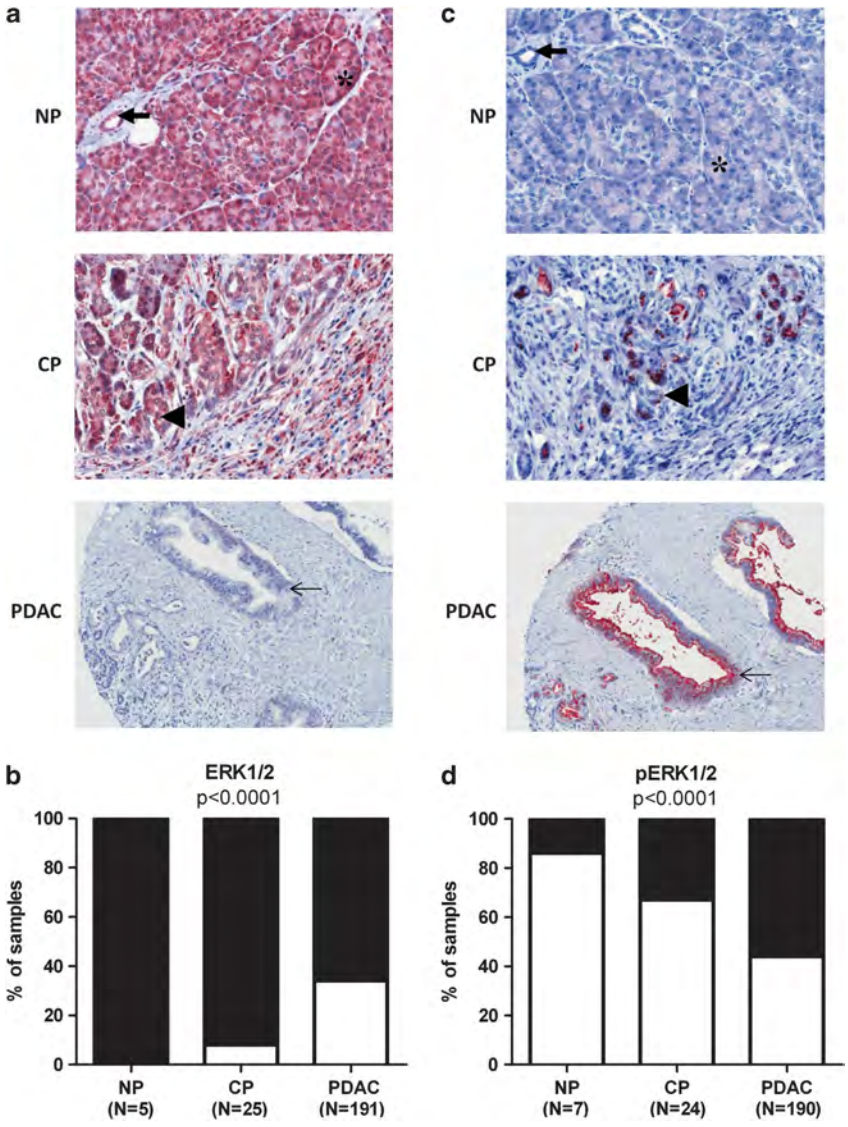

Figure 6. Expression of WNK2 downstream targets on TMAs. Serial sections of the corresponding tissues were used to immunohistochemically analyze the expression of the WNK2 downstream targets ERK1/2 (**a**, **b**) and pERK1/2 (**c**, **d**). As a result, a frequently intense cytoplasmic staining was detected for ERK in ductal and acinar cells in NP and CP (**a**, **b**). On the contrary, there was no significant pERK positivity in most NP tissues, whereas immunohistochemistry revealed moderate-to-strong cytoplasmic positivity in ducts in CP (**c**). The depicted PDAC (WNK2-positive, compare Figure 3a) displayed no significant immunoreactivity for ERK (**a**), but strong cytoplasmic positivity for pERK (**c**). For further details refer to Figure 3. χ^2-test shows differences in ERK and pERK expressions between the three tissues ($P<0.001$). The pancreatic tissue samples were obtained from pancreatic ductal adenocarcinoma patients, either from tumor tissue (PDAC) or from tissue showing chronic pancreatitis (CP) or from non-neoplastic sections adjacent to inflammatory sites (NP).

expression downregulation in PDAC, on the contrary to the example reported for PNLIPRP2.[28] The cell of origin for PDAC has long been debated and still remains an open question.[29] The hypermethylation profile of WNK2 might thus be considered as either an early event in PDAC development or as a marker of PDAC progenitor cells.

WNK2 hypermethylation and downregulation were also observed in patients with CP, although at lower levels than in patients with PDAC. This is in agreement with studies of pancreatic juice DNA,[15] in which methylation levels of specific marker genes were often higher in CP patients than in control donors but lower than in PDAC patients. Investigation of *WNK2* DNA methylation in mouse models for pancreatitis was performed to understand the changes in *WNK2* methylation following inflammation of pancreatic tissues. No change in *WNK2* methylation could be observed in the models for pancreatitis relative to controls. It is, however, noteworthy that *WNK2* mRNA expression decreased in the pancreatitis models compared with controls. This suggests that the decrease in *WNK2* expression occurs before the more stable silencing by promoter DNA methylation. The initial decrease in expression could be mediated via other epigenetic mechanisms, for example, the binding of specific miRNAs or histone modifications. It remains to be determined whether the mechanisms regulating *WNK2* expression are the same in mouse and human pancreatic tissues.

Our data support a functional role of WNK2 in PDAC pathogenesis. WNK2 overexpression in a pancreatic cancer cell line reduced cell growth, supporting the hypothesis that WNK2 acts as a potential tumor suppressor in pancreatic tissues. This has already been observed in brain tumor cells.[21,22] In our study, immunohistochemistry data in human pancreatic tissues showed that the levels of WNK2 and pERK1/2 proteins were inversely

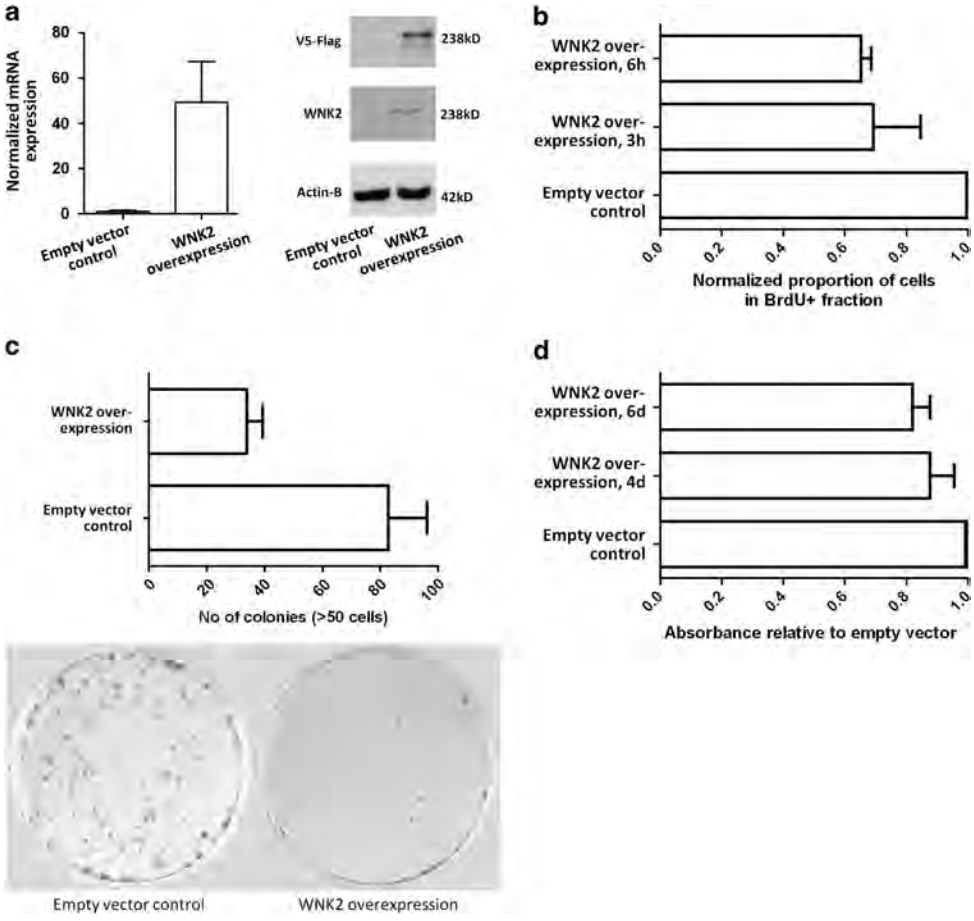

Figure 7. Effects of WNK2 overexpression in PANC1 cells. (**a**) Left panel, normalized *WNK2* mRNA expression in cells stably overexpressing WNK2, relative to cells transfected with the empty vector. Histograms represent the average value ± s.d. of three technical replicates. Right panel, western blots showing WNK2 protein in the cells described in left panel, detected with WNK2 or V5-Tag antibodies, with Actin-B as an internal loading control. (**b**) Results from BrdU incorporation flow cytometry assay. Proportion of PANC1 cells described in (**a**) with BrdU staining (cells which went through S-phase). Cell proliferation was determined by BrdU incorporation for 3 and 6 h, respectively. Proportions were normalized to levels in cells transfected with the empty vector. Histograms represent the average value ± s.d. of three technical replicates. (**c**) Results from colony formation assay. Cells were grown in six-well tissue culture plates for 10 days. Colonies containing >50 cells were counted. Photographs of representative wells are shown. Histograms represent the average value ± s.d. of six determinations. The WNK2 overexpressing cells show reduced colony-forming ability ($P = 0.0022$, Mann–Whitney test). (**d**) Cell growth was determined by sulforhodamine B (SRB) staining. Cells were seeded at a density of 4.000 cells/well for 4 days and of 1.000 cells/well for 6 days. Absorbance was measured at 515 nm, and results are expressed as absorbance relative to the empty vector control cells. Histograms represent the average value ± s.d. of four technical replicates. WNK2 overexpression at the 6-day time point shows significantly reduced relative absorbance ($P = 0.029$, Mann–Whitney test).

correlated. Moniz *et al.*[19,20] observed that WNK2 inhibits cell proliferation *in vitro* by negatively regulating the activation of the MEK1/ERK1/2 MAP kinase pathway. They showed that WNK2 depletion leads to ERK1/2 phosphorylation, a master regulator of the G1-S-phase transition[30] that is activated through phosphorylation by MEK1. Our data are in line with these *in vitro* studies and show that WNK2 downregulation is accompanied by an increase in pERK1/2 both in PDAC and pancreatitis, which may be responsible for enhanced cell proliferation. This has to be elucidated in future studies.

WNK2 hypermethylation and expression revealed no correlation with patient survival (data not shown). Operated patients with PDAC have a low 5-year survival rate (about 25%).[31] It is thus difficult to observe an effect of the expression of *WNK2* on short patient survival time periods. Moreover, our sample set was only composed of patients who underwent surgery and who thus had similar expected survival rates.

Taken together, our genome-wide profiling of CpG island methylation combined with a defined gene selection strategy identified *WNK2* as a potential tumor suppressor gene, which is epigenetically silenced via promoter methylation early in PDAC development.

MATERIALS AND METHODS

Patients and tissue specimens

Pancreatic tissue specimens ($N = 98$) were obtained from the tissue bank of the National Center of Tumor Diseases (Heidelberg) and the Department of General, Visceral, and Transplantation Surgery, University of Heidelberg. Together, the sample set was composed of 34 NP, 24 CP, five PanIN and 35 PDAC tissues. Samples were divided into a discovery and a replication sample set. Because of the limited availability of materials, not all experiments could be performed with each sample (for further details, see Supplementary Table 1). All specimens were histologically classified according to established criteria by an expert in pancreatic pathology (FB). Patients did not receive treatment before surgery. Written and oral informed consent was obtained from all subjects. The study was approved by the institutional ethics committee (301/2001, 206/2005 and 207/2005).

Mouse models relevant for PDAC pathogenesis

Three murine models were used in our study, (i) caerulein-induced pancreatitis in CD1 mice,[32–34] (ii) an *in vitro* cell culture model mimicking pancreatitis, with acinar cells derived from CD1 mice[35,36] and (iii) the *PDX-1-Cre;Trp53^{R172H};LSL-K-Ras^{G12D}* mouse model,[37] which recapitulates PDAC development (see Supplementary Materials and Methods and Figures 5a–d). Time points for analysis in the pancreatitis models have been carefully chosen to ensure a 'peak' change as judged by histological analysis, based on published observations as well as our own results. Initial and final states of the three murine models are as follows: (i) For the *in vitro* model, freshly isolated acinar cell fraction and cultured cell fraction. (ii) For caerulein-induced acute pancreatitis, untreated normal mouse pancreas and 48 h after start of caerulein injections, corresponding to the peak in pancreatitis. (iii) For caerulein-induced CP, untreated normal mouse pancreas and 10 weeks after start of caerulein injections. Further specification is given in Supplementary Materials and Methods and Supplementary Figure 8).

All animal experiments were approved by the ethical Committee of the Free University Brussels and the Garvan Institute for Medical Research, and performed according to the guidelines for Ethical Conduct in the Care and Use of Animals, developed by the Council for International Organizations of Medical Sciences.

DNA and RNA isolation from human and mouse pancreatic tissues, cell lines, 5-aza-2′-deoxycytidine (5-aza-dC) treatment in cell lines

Detailed descriptions on DNA and RNA isolation, cell lines and treatment of the cells with 5-aza-dC are available in Supplementary Materials and Methods.

Enrichment of methylated DNA by methyl-CpG immunoprecipitation (MCIp)

DNA from five NP, two CP and seven PDAC tissues were used for the MCIp experiment (discovery set). All samples were from male patients. Methyl-CpG-binding domain (MBD)-Fc protein was produced and MCIp was performed as described previously[18] with minor modifications. Briefly, 60 μg of MBD-Fc protein was coupled to 150 μl nProtein A Sepharose beads (GE Healthcare, Neu Isenburg, Germany) at 4 °C overnight. After binding of 2-μg sonicated DNA mixed with 2 pg of a mixture of *in vitro* methylated and unmethylated synthetic DNA of 630 bp containing 47 CpG dinucleotides, DNA elution was performed with increasing NaCl concentrations (300–1000 mM) using Ultra-free-CL centrifugal filter devices (Merck-Millipore, Billerica, USA). Low and high salt concentrations were used to enrich for lowly and highly methylated DNA, respectively. Quantitative PCR (qPCR) of *SYCP3*, a gene expected to be methylated in human tissues, was used to monitor the enrichment of methylated DNA (for primers see Supplementary Table 6). Proper enrichment of the *in vitro* methylated and unmethylated synthetic DNAs was monitored by qPCR. Highly methylated sample and reference (pool of DNAs from 11 NP tissues including male and female patients) DNAs were labeled with Alexa Fluor 5 and 3 (BioPrime Total Genomic Labeling System, Invitrogen, Karlsruhe, Germany), respectively, and co-hybridized on a 244K Human CpG Island microarray (Agilent technologies, Palo Alto, CA, USA).

CpG island microarray analysis

Scanned raw data were processed and normalized using the R statistical environment as described previously.[38] In order to find genes specifically hypermethylated in PDAC, CP samples were considered as normals. After data processing, three parallel strategies were followed for candidate gene selection, (i) using the 10.000 probes with the highest log-intensity ratios (M-values), (ii) calculating methylation thresholds by fitting log-normal mixture models to probes and using a statistical testing procedure to identify candidate CpG islands and (iii) performing matched-pair analyses (details in Supplementary Materials and Methods).

Quantitative DNA methylation analysis

Genomic DNA was bisulfite treated using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). After bisulfite conversion and PCR amplification of a region of interest, DNA methylation status was quantitatively assessed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, as described previously (Epityper assay, Sequenom, Hamburg, Germany[39]). Amplicons were designed to cover the positive regions derived from microarray analyses (primers are given in Supplementary Table 4).

mRNA expression analysis, immunohistochemistry on TMAs, immunoblotting, BrdU incorporation assay, colony formation assay and SRB assay

Detailed descriptions are available in Supplementary Materials and Methods.

Statistical analysis of quantitative DNA methylation data and mRNA expression data

Statistical analysis, unless otherwise specified, was performed using the Mann–Whitney–*U*-test. χ^2-test was performed to test the differences in immunohistochemistry staining between the different groups of samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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