Dickkopf-3 maintains the PANC-1 human pancreatic tumor cells in a dedifferentiated state

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Abstract. Pancreatic cancer (PaCa) is the fourth leading cause of cancer deaths in Western societies, with pancreatic ductal adenocarcinomas (PDACs) accounting for >90% of such cases. PDAC is a heterogeneous disease that includes a subset showing overexpression of the secreted glycoprotein Dickkopf-related protein 3 (Dkk-3), a protein shown to be downregulated in various cancers of different tissues. The biological function of Dkk-3 in this subset was studied using the Dkk-3 expressing PANC-1 cell line as a model for PDACs. The influence of Dkk-3 overexpression and knockdown on cellular differentiation and proliferation of PANC-1 was investigated. Confocal microscopy showed that Dkk-3 was expressed in a fraction of PANC-1 cells. While lentiviral-mediated overexpression of *DKK3* did not alter cellular proliferation, knockdown of *DKK3* resulted in significant reduction of cellular proliferation and concomitant induction of cell cycle inhibitors *CDKN2B* ($p15^{INK4b}$), *CDKN1A* ($p21^{CIP1}$) and *CDKN1B* ($p27^{KIP1}$). In parallel, pancreatic epithelial cell differentiation markers *AMY2A*, *CELA1*, *CTRB1*, *GCG*, *GLB1* and *INS* were significantly upregulated. PANC-1 cells differentiated using exendin-4 showed analogous induction of cell cycle inhibitors and differentiation markers. Thus, we conclude that Dkk-3 is required to maintain a highly dedifferentiated and consequently proliferative state in PANC-1, indicating a similar function in the Dkk-3 overexpressing subset of PDACs. Therefore, Dkk-3 represents a potential target for the treatment of Dkk-3-positive subtypes of PaCa to drive cells into cell cycle arrest and differentiation.

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Abbreviations: AMY2A, amylase α2A; *CELA1*, chymotrypsin-like elastase family member 1; *CTRB1*, chymotrypsinogen B1; Dkk-3, Dickkopf-3; *GCG*, glucagon; *GLB1*, galactosidase β1; IEMA, immunoenzymometric assay; *INS*, insulin; KD DKK3, lentiviral-delivered shRNA targeting *DKK3*; KD SCR, lentiviral-delivered shRNA targeting scrambled sequence; OE DKK3, lentiviral overexpression of *DKK3*; OE VEC, lentiviral overexpression of empty control vector; PaCa, pancreatic cancer; PDAC, pancreatic ductal adenocarcinoma; qPCR, quantitative real-time PCR; SA-β-Gal, senescence-associated β-galactosidase

Key words: Dickkopf-3, differentiation, pancreatic cancer, pancreatic ductal adenocarcinoma, proliferation, senescence-associated β-galactosidase

Introduction

Pancreatic cancer (PaCa) is the fourth leading cause of cancer deaths in Western societies. In 2008, there were an estimated 68,500 new cases and 70,200 deaths from the disease in the European Union (1), indicating incidence rates approximately equal to mortality. Clearly PaCa has an extremely poor prognosis, with a five-year relative survival rate of only 5% (2). Because PaCa-specific symptoms occur late in the course of the disease, for the majority of patients curative resections are not usually possible. Median survival from time of diagnosis is 3.5 months for patients who were not resected and 13.3 months for those who underwent resection (3). Thus, novel targets for early detection and therapy of PaCas are urgently needed. A better understanding of the underlying molecular and cellular changes associated with dedifferentiation and proliferation of pancreatic tumor cells is a prerequisite to achieve this purpose.

The secreted glycoprotein Dickkopf-related protein 3 (Dkk-3) is the most divergent member of the human Dickkopf family (4,5) and, in contrast to other family members, does not modulate Wnt signaling (6,7). Dkk-3 has been proposed to represent a novel tumor suppressor since gene expression is downregulated in various tumor cells (8-12), and hypermethylation of its promoter correlates with cancer occurrence (13,14). However, overexpression of Dkk-3 does not influence proliferation of malignant and non-malignant prostate cells (15).

In a cancer-profiling array, Dkk-3 was downregulated in 5 of 7 PaCa samples, but was upregulated in the remaining two (8). Additionally, Dkk-3 expression in tumor epithelial cells was demonstrated in ~17% of pancreatic ductal adenocarcinomas (PDACs) by immunohistochemistry (16), while exocrine or ductal cells of normal adult pancreatic tissue were negative (17), indicating a role for Dkk-3 in a subset of PDACs. Malignant neoplasms arise mainly from exocrine regions of the gland with PDACs accounting for >90% of PaCa cases.

In the present study, the role of Dkk-3 was investigated in the PANC-1 cell line, an extensively used model for human PDAC (18-23). PANC-1 cells have previously been shown to express *DKK3* at mRNA levels (24). Herein, we demonstrate that Dkk-3 supports proliferation of PANC-1 cells as shown by knockdown of Dkk-3 via lentiviral-delivered shRNA. Dkk-3 knockdown induced the expression of several markers of the differentiated pancreas, indicating that Dkk-3 plays a role in maintaining a dedifferentiated state in the cell line PANC-1 as a prerequisite for proliferation.

Materials and methods

Cell culture and cellular differentiation. PANC-1 cells were purchased from the American Type Culture Collection (ATCC) and were cultured in DMEM containing 4.5 g/l glucose (Lonza), 10% bovine calf serum (BCS, HyClone) and 1% penicillin/streptomycin/L-glutamine (PAA Laboratories). To induce PANC-1 differentiation, cells were seeded in 6-well plates in DMEM/F12 (Sigma-Aldrich) containing 1% BSA and ITS. At day 4 of culture, fresh media were supplemented with 0.3 mM taurine (Sigma-Aldrich), and at day 10, media were replaced by fresh media containing 1.5% BSA, ITS (Sigma-Aldrich), 3 mM taurine (Sigma-Aldrich), 1 mM nicotinamide (Sigma-Aldrich), NEAA (BD Biosciences) and 100 μM exendin-4 (Sigma-Aldrich). Media were replaced every other day. Cells were harvested at day 17. Control cells were maintained in DMEM containing 10% BCS.

Immunofluorescence and confocal microscopy. Cell growth and live confocal imaging was performed in 8 well-chambered coverglasses (Nalge Nunc International). After fixation, the cells were washed in phosphate-buffered saline solution (PBS, three times at RT) and non-specific binding was blocked with 3% BSA/PBS for one hour at room temperature. The primary antibody was goat anti-human Dkk-3 (1 μg/ml, R&D Systems) diluted in 1% BSA/PBS, incubated for 1 h at 37˚C in a humidified chamber. The cells were washed and blocked again as described previously. The secondary antibody (rabbit anti-goat rhodamine, 10 μg/ml; Chemicon) was incubated for 1 h at 37˚C. After the final two washing steps, nuclei were stained with Sytox green nucleic acid stain (Molecular Probes-Invitrogen Life Technologies) 10 nM in PBS, and incubated for 20 min at 37˚C.

Confocal images were captured with an UltraVIEW RS (Perkin-Elmer) mounted on an Olympus IX-70 inverse microscope (Olympus) using a 40x/1.4 oil immersion objective. The results presented are from representative fields. Live confocal microscopy was performed with a microlens-enhanced Nipkow disk-based confocal system UltraVIEW RS (Perkin-Elmer) in combination with the live stains fluorescent wheat germ agglutinin (WGA; Molecular Probes), tetramethylrhodamine methyl ester perchlorate (TMRM; Sigma-Aldrich) and Rhod-2 (Molecular Probes).

Knockdown and overexpression of DKK3 by lentiviral particles. Production of lentiviral particles was carried out according to the manufacturer's protocol (Addgene). For knockdown of *DKK3*, the lentiviral pLKO.1-TRC short hairpin system (Addgene) was applied. The target sequence (5-gagcctgatggag ccttggaccgat) was located within the coding region of *DKK3*. In knockdown experiments, scramble shRNA vector (Addgene plasmid 1864) was used as control. For overexpression, full-length cDNA of *DKK3* was subcloned into the pLenti6 vector (Invitrogen). Empty vector was used as control. Virus particles were produced by cotransfection of HEK293FT cells (Invitrogen) with the lentivirus packaging plasmid (psPAX2, Addgene plasmid 12260), envelope coding plasmid (pMD2.G, Addgene plasmid 12259) and vector construct. The lentivirus-containing supernatants were aliquoted and stored at -80˚C. For viral transduction, cells were seeded in appropriate vessels and left to adhere overnight. Thereafter, medium was replenished and supplemented with virus-containing supernatant at MOI 4 (knockdown) and MOI 0.5 (overexpression), respectively.

Quantitative real-time PCR. mRNA was extracted by the TriFast™ reagent (PeQLAB Biotechology). cDNA first strand synthesis was reverse transcribed from 2 μg total RNA preparation using Reverse Transcription System (Promega) and oligo(dT)15 and random hexamer primers. qPCR was performed by the FastStart DNA Master SYBR-Green I kit and the Light Cycler 480 System (Roche Applied Science) according to manufacturer's instructions. Specificity of PCR products was confirmed by melting curve analysis. Primer sequences are given in Table I. cDNA concentrations were normalized by the housekeeping gene porphobilinogen deaminase (*HMBS*).

Dkk-3 quantification in cell culture supernatants by IEMA. PANC-1 cells were seeded at a density of $1x10^5$/well in 6-well plates and left to adhere overnight. Subsequently, medium was replaced and cells were transduced with lentiviral particles. After 72 h, medium was replaced by 1 ml of fresh medium. Levels of Dkk-3 secreted into the medium within 24 h were quantified by a sandwich immunoenzymometric assay (IEMA) based on our own monoclonal antibody, as previously described (25,26).

Cell viability assay. Two thousand cells were seeded in triplicates into 96-well plates (Nunc) in 100 μl culture medium and left to adhere overnight. Thereafter, fresh medium was supplemented with lentivirus particles to transduce cells. Cell viability was determined by a WST-1 cell viability assay (Roche Applied Science) according to the manufacturer's instructions at 24, 72 and 144 h post-transduction (days 1, 3 and 6, respectively). Media were replaced after 48 h.

Relative quantification of DNA synthesis. Two thousand cells were seeded in triplicates into 96-well plates (Nunc) in 100 μl culture medium and left to adhere overnight. Thereafter, fresh medium was supplemented with lentivirus particles to transduce cells. Proliferation rate was analyzed by a BrdU cell proliferation ELISA (Roche Applied Science) according to manufacturer's instructions at 24, 72 and 144 h post-transduction (days 1, 3 and 6, respectively). Media were replaced after 48 h.

Western blot analysis. Total cell extracts were prepared and analyzed by Western blotting as described previously (15). Primary antibodies against PARPα, phospho-p53, $p15^{INK4b}$, $p21^{CIP1}$, $p27^{KIP1}$ (Cell Signaling Technology) and Dkk-3 (R&D

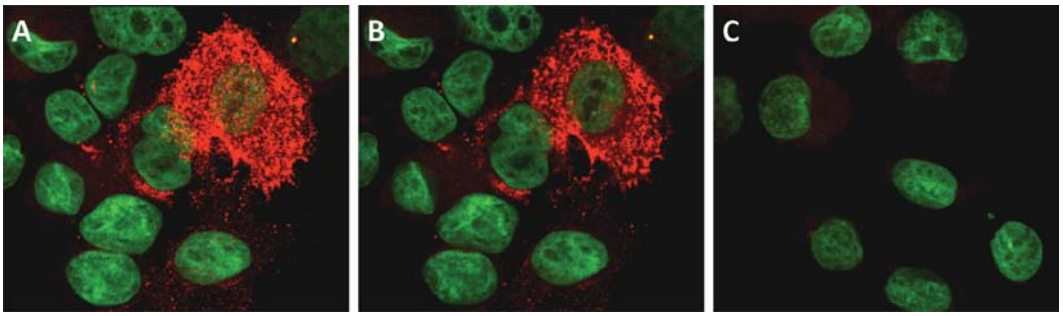

Figure 1. Dkk-3 expression in PANC-1 cells. Confocal immunofluorescence of PANC-1 cells stained with an antibody directed against Dkk-3 (red). Cell nuclei were counterstained with Sytox (green). (A) Confocal z-stack of PANC-1 cells reveal heterogeneous expression of Dkk-3. (B) The staining reaction for Dkk-3 in PANC-1 cells in one confocal plane was distributed in a vesicular pattern in the cytoplasm, while the cell nuclei were not stained. (C) Specificity of the staining was demonstrated by omitting the primary antibody (z-stack).

Table I. Primer sequences.

Gene	Unigene ID	Primer sequences	
		Sense	Antisense
AMY2A	Hs.654437	5-atgtggcctggagacataaa	5-ccattccacttgcgaataac
BAK1	Hs.485139	5-tctggccctacacgtctacc	5-acaaactggcccaacagaac
BAX	Hs.433670	5-tttgcttcagggtttcatcc	5-cagttgaagttgccgtcaga
BCL2	Hs.150749	5-gaggattgtggccttctttg	5-acagttccacaaaggcatcc
BCL2L1	Hs.516966	5-catggcagcagtaaagcaag	5-tgctgcattgttcccataga
CDKN1A ($p21^{CIP1}$)	Hs.370771	5-ggcggcagaccagcatgacagatt	5-gcagggggcggccagggtat
CDKN1B ($p27^{KIP1}$)	Hs.238990	5-aataaggaagcgacctgcaa	5-cgagctgtttacgtttgacg
CDKN2B ($p15^{INK4b}$)	Hs.72901	5-cggggactagtggagaaggt	5-ggtgagagtggcagggtct
CELA1	Hs.348395	5-ggagtgaccagctttgtgtc	5-aaggtcgttggactcaggaa
CTRB1	Hs.610926	5-accgacgtgatgatctgtg	5-gatcttctgcacccaaggta
DKK3	Hs.292156	5-tcatcacctgggagctagag	5-caacttcatactcatcgggg
GCG	Hs.516494	5-cgttcccttcaagacacaga	5-acgcctggagtccagatact
GLB1	Hs.443031	5-tccctccatctacaccaaag	5-tgaaggcctacctgtttcac
HMBS	Hs.82609	5-ccaggacatcttggatctgg	5-atggtagcctgcatggtctc
INS	Hs.272259	5-acgaggcttcttctacacacc	5-agctggtagagggagcagat
TP53 (p53)	Hs.654481	5-gcgcacagaggaagagaa	5-cctcattcagctctcggaac

Systems) were used at dilutions of 1:1000, against BAX (Oncogene) at a dilution of 1:500, and against β-actin (Sigma-Aldrich) at a dilution of 1:5000, respectively.

Staining of senescence-associated β-galactosidase activity. For senescence-associated β-galactosidase (SA-β-Gal) activity staining, cells were fixed (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 5 min at room temperature and subsequently incubated in staining solution [40 mM citric acid, 12 mM Na_2HPO_4, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), pH 6.0] for 24 h at 37˚C. Cells were washed in PBS to stop the reaction.

Statistics. Results are expressed as the mean values ± SEM. Statistical differences between treatments were calculated by paired Student's t-test and regarded significant when $P<0.05$ ($^{*}P<0.05$, $^{**}P<0.01$).

Results

Dkk-3 is heterogeneously expressed in PANC-1 cells. To investigate cellular distribution of Dkk-3 protein in PANC-1 cells, confocal immunofluorescence using Sytox green as a counter stain for cell nuclei was performed. PANC-1 cells stained heterogeneously positive for Dkk-3 (Fig. 1A). While the majority of cells had no or only weakly detectable Dkk-3 expression, approximately 10% of the cells were intensely stained. As expected for a secreted protein, Dkk-3 was distributed in a vesicular pattern within the cytoplasm while cell nuclei were negative (Fig. 1B).

DKK3 knockdown reduces proliferation of PANC-1. To investigate the role of Dkk-3 in PANC-1 cells, lentiviral *DKK3* overexpression and knockdown systems were established. The efficiency of the transduction system was confirmed at mRNA and protein levels using quantitative real-time PCR (qPCR)

and IEMA, respectively (Fig. 2A). Lentiviral overexpression of *DKK3* (OE DKK3) increased mRNA levels 586-fold (n=4, P<0.001) compared with empty vector control virus (OE VEC) in PANC-1 cells. The amount of secreted Dkk-3 consistently increased from 18.1 to 5073 ng/ml (280-fold, n=3, P=0.03). Lentiviral-delivered shRNA targeting *DKK3* (KD DKK3) led to an 88% reduction (8.4-fold decrease, n=3, P=0.004) of *DKK3* mRNA level compared with scrambled control (KD SCR), and a 98% reduction (50-fold decrease, n=3, P=0.01) of secreted Dkk-3 protein. Neither cell morphology (visualized with WGA), mitochondrial activity (visualized with TMRM) nor calcium levels (visualized with Rhod-2) showed clear differences between *DKK3* overexpressing and knockdown cells compared to controls (data not shown).

Given the proposed function of Dkk-3 as a tumor suppressor, the effect of *DKK3* overexpression on proliferation of PANC-1 cells was investigated by the established lentiviral transduction system. In agreement with our previous findings in malignant and nonmalignant prostate cells (15), OE DKK3 did not significantly alter cellular proliferation at any time point tested (days 1, 3 and 6 post-transduction) as determined by cell viability (WST-1) and BrdU incorporation (DNA synthesis) assays (Fig. 2B). KD DKK3 did not significantly alter proliferation of PANC-1 cells within 1 or 3 days post-transduction. However, after 6 days, KD DKK3 PANC-1 showed significantly reduced proliferation compared with KD SCR (Fig. 2C; WST-1 signal day 6: KD SCR 0.81±0.02, KD DKK3 0.61±0.05; P=0.04; BrdU signal day 6: KD SCR 1.92±0.07, KD DKK3 1.17±0.18; P=0.02). Of note, KD DKK3 did not attenuate proliferation of Dkk-3 negative cell lines (PC3 prostate cancer cell line, HT-29 colon carcinoma cell line) excluding potential Dkk-3 independent effects of the lentiviral knockdown system (data not shown).

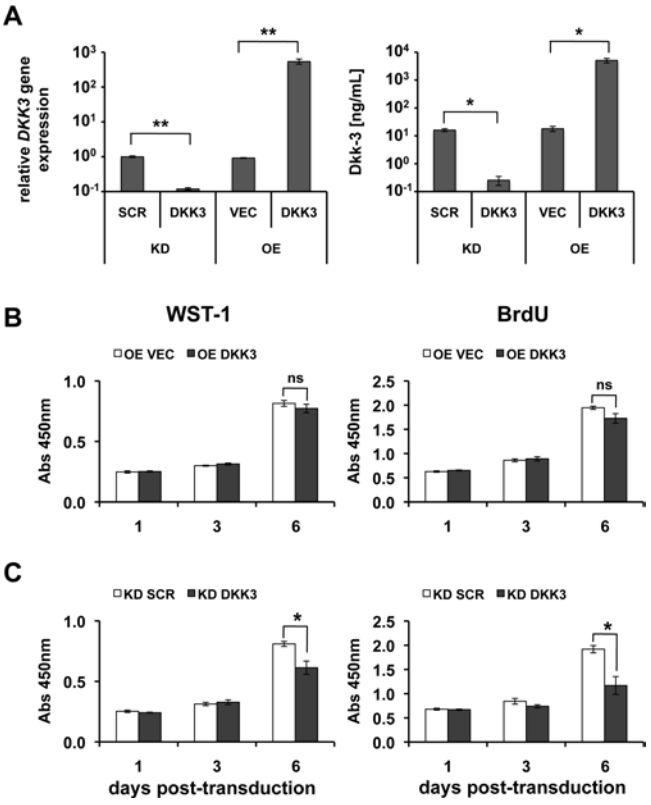

Figure 2. Influence of *DKK3* overexpression and knockdown on PANC-1 cellular proliferation. (A) Efficiency of *DKK3* overexpression and knockdown. *DKK3* mRNA levels and Dkk-3 protein levels determined in cell culture supernatants by IEMA of PANC-1 cells transduced with lentiviruses delivering shRNA targeting *DKK3* (KD DKK3), shRNA targeting scrambled control (KD SCR), full length *DKK3* (OE DKK3) or empty vector control (OE VEC), respectively. Knockdown lentiviruses (KD DKK3 and KD SCR) were applied at a MOI of 4, overexpression lentiviruses (OE DKK3 and OE VEC) at a MOI of 0.5. *DKK3* gene expression levels were normalized using the housekeeping gene *HMBS* and are shown relative to KD SCR PANC-1. (B) Lentiviral overexpression of *DKK3* (OE DKK3) did not affect proliferation of PANC-1 cells as determined by cell viability (WST-1) and DNA synthesis (BrdU incorporation) assays performed on day 1, 3 or 6 post-transduction. (C) *DKK3* knockdown (KD DKK3) significantly reduced proliferation of Dkk-3 expressing PANC-1 cells on day 6 post-transduction. Results represent mean values ± SEM of three independent experiments. Statistical differences were calculated by paired Student's t-test and regarded significant when P<0.05 (*P<0.05, **P<0.01).

Elevated p27 levels in DKK3 knockdown cells. To gain insight into the molecular mechanisms underlying the reduced proliferation of PANC-1 upon KD DKK3, expression levels of several genes related to apoptosis were analyzed. KD DKK3 PANC-1 revealed no significant changes in expression levels of *BAK1*, *BCL2L1* and *TP53* (p53) compared with KD SCR PANC-1 (Fig. 3A). While mRNA levels of the anti-apoptotic BCL2 was slightly, but not statistically significantly, elevated (3.1-fold, P=0.06), the pro-apoptotic *BAX* was significantly downregulated (-1.5-fold, P=0.013). However, at protein level, neither BAX nor the apoptosis markers PARPα and phospho-p53 were regulated, indicating that KD DKK3 did not induce apoptosis in PANC-1 cells (Fig. 3B).

Additionally, expression levels of several cell cycle-dependent kinase inhibitors were analyzed. *CDKN2B* ($p15^{INK4b}$; 8.1-fold, P=0.03), *CDKN1A* ($p21^{CIP1}$; 2.9-fold, P=0.04) and *CDKN1B* ($p27^{KIP1}$; 3.4-fold, P=0.003) were significantly induced in KD DKK3 compared with KD SCR PANC-1 (Fig. 3C). The induction of $p27^{KIP1}$ was confirmed at the protein level by Western blot analysis, while protein levels of $p15^{INK4b}$ and $p21^{CIP1}$ were not altered (Fig. 3D).

DKK3 knockdown induces differentiation of PANC-1 cells. $p27^{KIP1}$ plays an important role in the switch of proliferation to differentiation in a variety of cell types, and loss of $p27^{KIP1}$ is associated with a poorly differentiated phenotype in several malignancies (27), including PDAC (28). Thus, a panel of differentiation markers was analyzed to evaluate whether the observed reduced proliferation of KD DKK3 PANC-1 was associated with differentiation. First, activity of SA-β-Gal, a marker mainly correlated with senescence but also upregulated during differentiation (29), was assessed. In KD DKK3 PANC-1, an increased number of SA-β-Gal-positive cells compared with KD SCR cells was observed (Fig. 4A). Quantification of stained cells demonstrated that the increase was significant (Fig. 4B; KD SCR: 2.6% SA-β-Gal positive, KD DKK3: 12.2%, P=0.002). Moreover, mRNA levels of *GLB1*, the enzyme responsible for SA-β-Gal staining, were significantly elevated in KD DKK3 PANC-1 (Fig. 4C; 5.0-fold, P=0.004).

Subsequently, expression levels of marker genes of differentiated pancreatic epithelium were analyzed by qPCR in KD DKK3 and KD SCR PANC-1, respectively. Pancreatic amylase (*AMY2A*; 2.8-fold, P=0.008), elastase 1 (*CELA1*; 7.8-fold, P=0.017), chymotrypsinogen B1 (*CTRB1*; 9.3-fold, P=0.013),

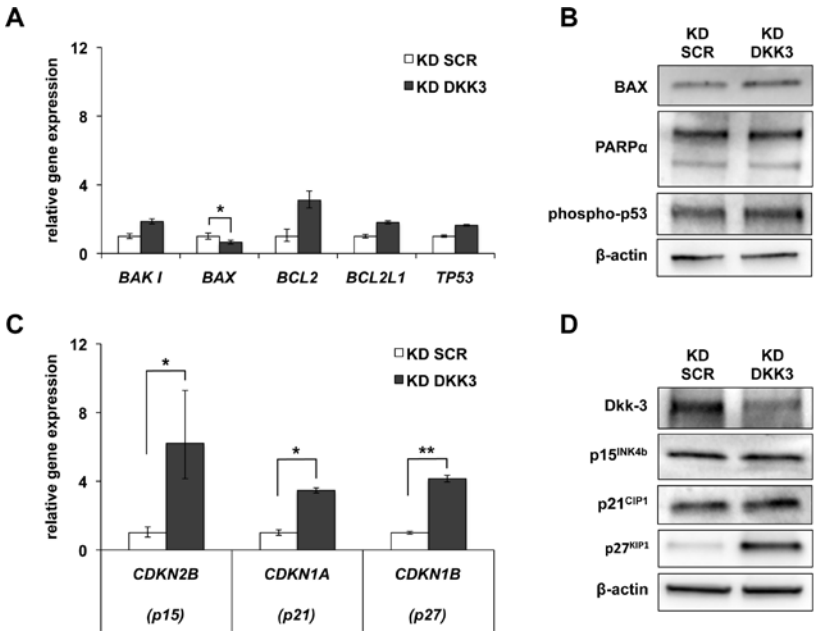

Figure 3. *DKK3* knockdown induced cyclin-dependent kinase inhibitor expression in PANC-1 cells. (A) Expression of the apoptosis-related genes *BAK1*, *BCL2*, *BCL2L1* and *TP53* was not affected by lentiviral-delivered shRNA targeting *DKK3* (KD DKK3) compared with scrambled control (KD SCR) in PANC-1 cells. While BAX mRNA levels were slightly reduced by KD DKK3, protein levels were comparable to KD SCR (B). Protein levels of PARPα and phospho-p53 were not altered. (C) The cyclin-dependent kinase inhibitors *CDKN2B* ($p15^{INK4b}$), *CDKN1A* ($p21^{CIP1}$) and *CDKN1B* ($p27^{KIP1}$) were significantly induced in KD DKK3 compared with KD SCR. (D) Induction of $p27^{KIP1}$ was confirmed at protein levels by Western blot analysis while $p15^{INK4b}$ and $p21^{CIP1}$ protein levels were not affected. β-actin served as loading control. (A and C) Bars represent mean ± SEM of four independent experiments.

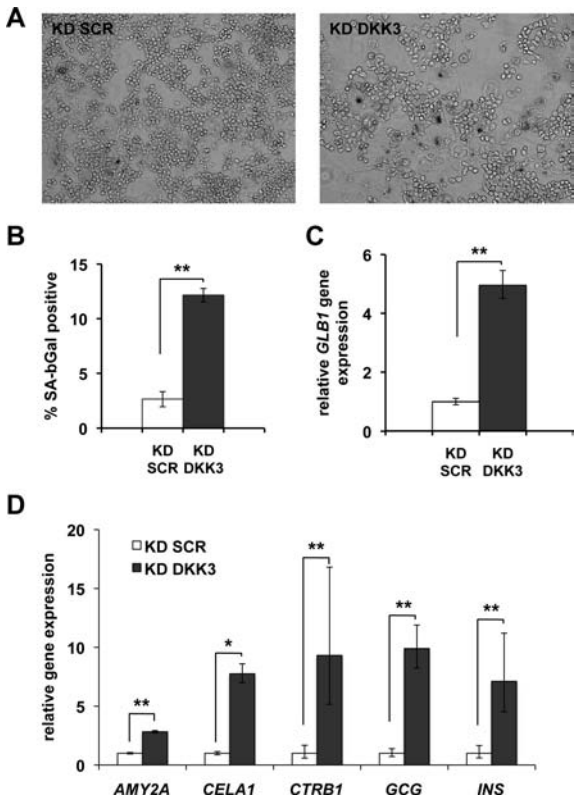

Figure 4. *DKK3* knockdown induced expression of epithelial cell differentiation markers in PANC-1 cells. (A) *DKK3* knockdown by lentiviral-delivered shRNA (KD DKK3) led to an increase of senescence-associated β-galactosidase (SA-β-gal)-positive PANC-1 cells on day 6 post-transduction. (B) Quantification of SA-β-gal stained-cells demonstrated that the observed increase was significant (P<0.01). (C) Gene expression of the SA-β-gal-active enzyme *GLB1* was significantly increased in KD DKK3 PANC-1, confirming the results of the SA-β-gal assay. (D) Markers for differentiated pancreatic epithelial cells were increased in KD DKK3 PANC-1. Data represent mean ± SEM of three independent experiments.

glucagon (*GCG*; 9.9-fold, P=0.007) and insulin (*INS*; 7.1-fold, P=0.003) were significantly induced in KD DKK3 cells (Fig. 4D).

To determine whether KD DKK3-induced differentiation of PANC-1 cells was similar to the formation of islet-like cell aggregates (ICAs), previously described (30), ICA formation was induced by switching PANC-1 cells to serum-free conditions in the presence of exendin-4. Induction of differentiation markers was comparable to KD DKK3 (Fig. 5A; *AMY2A*: 2.5-fold, P=0.049; *CELA1*: 2.7-fold, P=0.12; *CTRB1*: 5.7-fold P=0.014; *GCG*: 14.6-fold, P=0.005; *INS*: 5.7-fold, P=0.0003). Moreover, expression of *GLB1* was significantly elevated (6.4-fold; P=0.032) and exendin-4-differentiated cells showed high SA-β-Gal activity (Fig. 5B), confirming its suitability as differentiation marker. We also analyzed expression levels of the above-mentioned cell cycle-dependent kinase inhibitors, and while *CDKN2B* mRNA levels were not significantly altered in exendin-4 induced differentiation (1.5-fold, P=0.188), *CDKN1A* (1.8-fold, P=0.014) and *CDKN1B* (2.9-fold, P=0.005) levels were significantly increased (Fig. 5C). This expression pattern was confirmed at protein level (Fig. 5D). Interestingly, *DKK3* mRNA levels were slightly increased in differentiated PANC-1 cells (1.5-fold, P=0.024) and protein levels were comparable to control cells, indicating that while KD DKK3 induces differentiation markers in PANC-1, reduced Dkk-3 protein levels are not a prerequisite for differentiation.

Discussion

The PANC-1 cell line serves as a model for Dkk-3 expressing PDAC due to its expression DKK3 at mRNA levels (24). By confocal immunofluorescence, Western blot analysis and an IEMA based on our monoclonal antibody, Dkk-3 protein

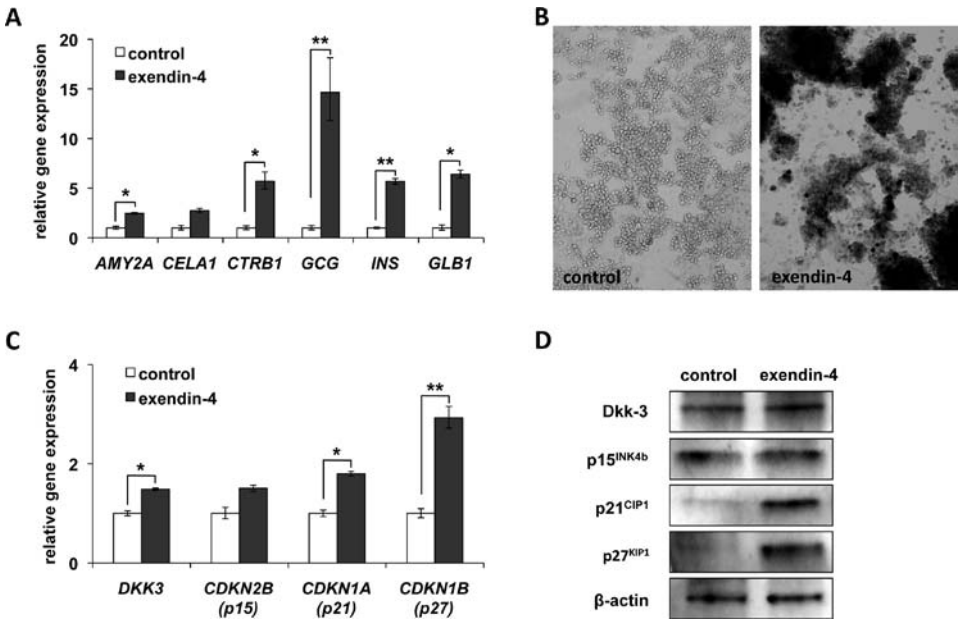

Figure 5. Marker profile of exendin-4 differentiated PANC-1 cells. (A) Pancreatic epithelial cell differentiation markers were increased in exendin-4-treated cells. (B) Elevated expression of *GLB1* was confirmed at enzyme activity levels using staining for senescence-associated β-galactosidase activity. Differentiation induced by exendin-4 was accompanied by elevated *CDKN1A* ($p21^{CIP1}$) and *CDKN1B* ($p27^{KIP1}$) mRNA (C) and protein (D) levels, while *CDKN2B* ($p15^{INK4b}$) was not significantly changed compared with control-treated cells. *DKK3* mRNA levels were found slightly increased in differentiated cells, whereas protein levels remained comparable with control. Data represent mean ± SEM of three independent experiments.

expression was confirmed in PANC-1 cells and supernatants. Dkk-3 was heterogeneously distributed with a subpopulation of PANC-1 showing intense immunofluorescent staining. We previously reported similar results in the islets of Langerhans from normal adult human pancreas, where Dkk-3 was located only in a subpopulation of β cells (17). The role of Dkk-3 in PANC-1 cells was investigated by generating lentiviral transduction systems to specifically overexpress or knockdown *DKK3*, and those results verified the efficiency of the transduction system.

While OE DKK3 did not affect proliferation, KD DKK3 significantly reduced proliferation of PANC-1 cells, indicating that Dkk-3 was required to maintain the proliferative state of this cell line. KD DKK3 did not induce apoptosis, but led to a significant induction of cell cycle inhibitors and concomitantly increased gene expression levels of pancreatic epithelial cell differentiation markers, indicating that Dkk-3 is necessary for the maintenance of an undifferentiated state of PANC-1 cells. Our findings are surprising, since in other cell types, Dkk-3 has been shown to support differentiation. Depletion of Dkk-3 disrupted acinar morphogenesis of the prostate epithelial cell line RWPE-1 (31). Moreover, Dkk-3 supported capillary formation of peripheral blood-derived endothelial colony-forming cells (32). Taken together, Dkk-3 is either a key molecule stabilizing the differentiated as well as the undifferentiated state of cells, or has a distinct role in different cell types and cells of different origins.

In PANC-1 cells, Dkk-3 supports a highly proliferative, poorly differentiated state, both characteristics of progenitor or transit amplifying cells. As recently reported, Dkk-3 is strongly expressed at the base of colon crypts, a region known to contain proliferating epithelial precursor cells (33). Additionally, in skin hair follicles, Dkk-3 was preferentially expressed in the keratinocyte stem cell-containing bulge (34,35). Dkk-3 has also been reported to represent a stemness gene in mesenchymal stem cells, where expression of Dkk-3 is reduced during differentiation (36). In a recent study, Dkk-3 overexpression was demonstrated in subsets of hepatoblastomas (11 of 14 cases) and hepatocellular carcinomas (14 of 72 cases), whereas the non-cancerous counterpart samples were negative for Dkk-3 (37). The authors suggested that Dkk-3 expression may distinguish a more immature or dedifferentiated subset of cancer. The question is whether the observed Dkk-3-positive PANC-1 fraction reflects a more progenitor-like subpopulation. If so, they provide an ideal model system to address further questions regarding the afore-mentioned hypothesis.

PDAC is a heterogeneous disease with several potential cellular origins (38,39). In this context, the Dkk-3 overexpressing subset of PDACs might represent a subtype of PDACs with stem/progenitor cell origin.

At any rate, Dkk-3 expression distinguishes subsets of PDACs, which might require different treatments. Induction of ER stress by overexpression of Dkk-3 has been suggested as therapy for various cancers (40,41). However, the requirement for Dkk-3 in PANC-1 cells to maintain their dedifferentiated phenotype indicates that this approach is inappropriate at least for Dkk-3 overexpressing PDACs. On the contrary, for this type of PaCa, Dkk-3 represents a potential target for functional neutralization in order to change the phenotype of the cancer cells to a more differentiated, and consequently less proliferative state.

In summary, KD DKK3 significantly reduced proliferation of PANC-1 and induced expression of epithelial cell differentiation markers. The results indicate that Dkk-3 maintains the tumor cells in a highly dedifferentiated state as a prerequisite for proliferation in the Dkk-3 overexpressing PDACs. Thus, Dkk-3 represents a potential target in this subset of PaCas.

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