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Detection of Tumor Suppressor Genes in Cancer Development by a Novel shRNA-based method

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Abstract

Pancreatic cancer is one of the deadliest cancers with poor survival rates and limited therapeutic options. To improve the understanding of this disease's biology, a prerequisite for the generation of novel therapeutics, new platforms for rapid and efficient genetic and therapeutic screening are needed. Therefore, a combined *in vitro/in vivo* hybrid shRNA-assay was developed using isolated murine primary pancreatic ductal cells (PDCs), in which oncogenic $Kras^{G12D}$ could be activated in vitro by genomic recombination through 4OH-tamoxifen-induced nuclear translocation of Cre-ERT2 expressed under control of the ROSA26 promoter. Further genetic manipulation was achieved through selective and stable RNA interference (RNAi) against the tumor suppressors $p16^{Ink4a}$ (CDKN2A) or *p53* (TP53) using lentiviral gene delivery. Treatment of PDCs with 4OH-tamoxifen increased phosphorylation of extracelluar signal-regulated kinase (ERK) downstream of KRAS, and subsequent lentiviral transduction resulted in sustained target gene repression. Double-mutant PDCs were then re-introduced into the pancreata of NOD-SCID-gamma (NSG) mice and monitored for tumor growth. Orthotopic implantation of PDCs carrying the activated $Kras^{G12D}$-allele and shRNA against $p16^{Ink4a}$ or *p53* resulted in tumor growth, metastasis and reduced survival of NSG mice. In contrast, $Kras^{G12D}$ alone was not sufficient to induce tumor growth. Implications: The combinatory in vitro/in vivo approach described in this study allows for rapid and efficient identification of genes involved in carcinogenesis and opens new avenues for the development of therapeutic strategies to improve cancer treatment.

Keywords

Pancreatic cancer; shRNA; tumor suppressor; carcinogenesis; genetic screen

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer related deaths in the US. The five-year survival rate of all patients suffering from PDAC is 6%, and

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incidence almost equals mortality rate, underscoring the aggressive behavior of this tumor (1). Most patients suffering from PDAC already present with metastasis, causing the majority of pancreatic cancer associated deaths (2). Although several broad based approaches have been undertaken to shed light on the genetics and biology of pancreatic cancer, only few essential driver mutations have been identified so far (3, 4). One of the most common genetic perturbations in pancreatic cancer is an activating mutation of oncogenic *Kras*, which can be found in more than 90% of PDAC and is thought to represent an initiating event (5). However, single activation of *Kras* in mice results in pancreatic intraepithelial neoplasia (PanIN), but shows only infrequent development of invasive PDAC (6). Thus, additional genetic events are required for the development of invasive PDAC, including loss of the cell cycle regulator $p16^{Ink4a}$ (part of the *Cdkn2a*-locus), and of the tumor suppressor genes *Trp53* and/or *Smad4* (7). Identification of additional genes involved in tumorigenesis will broaden our understanding of pancreatic cancer biology and eventually lead the way to more effective treatments.

Functional characterization of cancer genes can be cumbersome. Although cell culture assays can be easily performed and are highly reproducible, *in vitro* models lack the features of the tumor microenvironment and, thus, may not be suitable to detect gene activities linked to cancer initiation or progression.

The standard approach for investigating candidate cancer genes requires the generation of transgenic and knockout mice that harbor germline alterations in the gene of interest. Although these strains are invaluable tools in the field of cancer research, their generation, maintenance and analysis can be costly and time consuming. Moreover, many data obtained with these models rely on manipulation of cancer genes during embryogenesis, and thus, do not reflect somatic mutations occurring during an individual's life span.

To obviate these obstacles, we developed an approach in which we combined the ease of *in vitro* genetic manipulation and the power of *in vivo* pancreatic cancer studies. In this model, we took advantage of the well-established primary pancreatic ductal cell culture (PDCs) (8, 9). Isolation of PDCs from mice that harbor the lox-stop-lox-$Kras^{G12D}$-allele and express Cre-ER^{T2} under control of the ubiquitous ROSA26-promoter allowed us to induce genetic recombination and subsequent activation of $Kras^{G12D}$ *in vitro*. We hypothesized that additional depletion of the tumor suppressor genes $p16^{Ink4a}$ or *Trp53* in the context of *Kras*-activation in PDCs will lead to accelerated tumor growth and invasive PDAC. Genetic silencing was achieved by lentiviral delivery of shRNA, and orthotopic implantation of these resulted in tumor growth. However, *Kras* activation on its own was not sufficient to induce tumor growth. The method described in this study will simplify the identification and validation of new cancer genes with high reliability and without the need for tedious mouse models.

Material and Methods

Isolation of Pancreatic ductal cells

Primary pancreatic ductal cells (PDCs) were isolated from mice carrying the genotype ROSA26Cre-ER^{T2};Lox-Stop-Lox-$Kras^{G12D}$ (termed *Kras*-PDCs thereafter) and maintained

essentially as described (10). Early passage cells were treated with 4OH-tamoxifen 200 nM (Sigma) or vehicle for 10 days.

Western Blot Analysis

PDCs were collected from collagen by digestion with collagenase type 2 (Worthington) at a final concentration of 1 mg/ml at 37 °C for 15 min. Upon complete digestion, cells were pelleted by centrifugation and washed with ice cold PBS. The final pellet was lysed and protein concentration was normalized using Bradford reagent (Biorad). 50 μg were resolved on a 10% sodium-dodecylsulfate-(SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (PVDF). Membranes were blocked in PBS containing 0.05% Tween and 3% non-fat dry milk for 1 h at room temperature and incubated with anti-pERK (Cell Signaling 4370) 1:1000, anti-P16INK4a (M-156, Santa-Cruz sc-1207)1:200 or anti-TRP53 (NCL-p53-CM5p, Novocastra, Leica Bisosystems) 1:1000. Membranes were then subsequently incubated with anti-ERK (BD Biosciences 610124) 1:1000 and anti-β-Actin (Sigma-Aldrich, Clone AC-74, A5316) 1:5000. Visualization was performed using IRDye 680 (anti-rabbit) or IRDye 780 (anti-mouse) secondary antibodies on an Odyssey Infrared Imaging System (all LiCor).

RAS activation assay

Detection of activated KRAS was performed essentially as described using a Raf-RBD-pulldown assay (Cytoskeleton) (11). Transfer to PVDF membranes and visualization was conducted as mentioned above using an antibody against KRAS (Merck-Millipore, 1:1000).

Quantitative Real-Time PCR

Total RNA was extracted using the RNeasy kit (Qiagen). Synthesis of cDNA using random hexamers and MMLV-based reverse transcriptase (Life Technologies) was achieved as previously described (12). Quantitative analysis was carried out on StepONEplus real-time PCR system (Applied Biosystems, Life Technologies) and the amount of target gene was normalized to the endogenous reference *Ppia* (Cyclophilin A) (13).

Murine primers were designed to be intron spanning. The following primers were used: $p16^{Ink4a}$ FW: CCCAACGCCCCGAACT, $P16^{Ink4a}$ RV: GTGAACGTTGCCCATCATCA, *Trp53* FW: AGATCCGCGGGCGTAAAC RV: TCTGTAGCATGGGCATCCTTT *Ppia* FW: ATGGTCAACCCCACCGTGT, *Ppia* RV: TTCTGCTGTCTTTGGAACTTTGTC.

Lentiviral constructs, virus generation, target cell transduction and selection

Glycerol stocks containing the desired lentiviral constructs were obtained from Open Biosystems (now part of GE Healthcare) and grown according to the manufacturer's instructions. The clone IDs for shRNA$p16^{Ink4a}$ were: TRCN0000077814, target sequence GTGATGATGATGGGCAACGTT, termed shRNA$p16^{Ink4a}$ #1 hereafter, and TRCN0000077813, target sequence CATCAAGACATCGTGCGATAT, termed shRNA$p16^{Ink4a}$ #2 hereafter. The clone IDs for shRNA*Trp53* were: TRCN0000012362, target sequence CTACAAGAAGTCACAGCACAT, termed shRNA*Trp53* #1 hereafter, and TRCN0000054551, target sequence AGAGTATTTCACCCTCAAGAT, termed

shRNA*Trp53* #2 herafter. pLKO.1 was used for control. Purified plasmids were tested for integrity prior to transfection using the restriction enzymes *BamHI* and *NdeI*.

For virus production 6×10^6 293T cells in a 10 cm dish were transfected with 12 μg lentiviral construct, 4,2 μg pMD2G-VSVG and 7,8 μg pCMV-dR8.74 using Lipofectamine 2000 (Life Technologies). Twenty-four hours post transfection culture media was changed and virus-containing supernatant was collected 24 h later. Culture media was replaced and collected another 24 h later. Viral supernatant was stored at -80 °C until further use.

Kras-PDCs were transduced with lentivral constructs as described with only minor modifications (14). Briefly, 4OH-tamoxifen-treated *Kras*-PDCs were placed in wells of a six-well plate at a cell number of 3×10^5 cells/well and allowed to adhere on plastic over night. Next day, cells were transduced with viral supernatant containing ploybrene 4 μg/ml (Sigma). Twenty-four hours later PDCs were placed back onto collagen coated wells and were allowed to adhere for another 24 h. Upon complete attachment, cells were selected in the presence of puromycin 8 μg/ml for ten days (termed *Kras*-shRNA-PDCs hereafter). Successful depletion of target gene mRNA was confirmed by quantitative RTPCR. Two different shRNAs per target gene were tested to reduce off-target effects.

Orthoptopic transplantation and animal procedures

Immunocompromised NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (NOD scid gamma, NSG) were obtained form The Jackson Laboratory. Eight to ten week old animals were anaesthetized using a combination of Medetomidine, Midazolam and Fentanyl. A total of 5×10^5 *Kras*-shRNA-PDCs in a volume of 20 μl were injected into the pancreata of NSG-mice as described (12). Briefly, a small left abdominal incision was made and the pancreas was retrieved by gently dislodging the spleen. Tumor cells were injected into the pancreas in an area adjacent to the spleen using a micro liter syringe with a 27 gauge needle. Successful injection was confirmed by an intrapancreatic bleb. The peritoneal layer was sutured with Ethilon 5-0 (Johnson and Johnson) and the cutaneous wound was closed using wound clips. We injected three mice per shRNA. Animals were investigated weekly for tumor growth, development of ascites and weight loss. Animals were euthanized upon palpable local tumor growth > 1cm, development of ascites or loss of body weight > 20%. If none of these occurred, animals were euthanized after a period of 26 weeks. All animal procedures were in agreement with the Government of Upper Bavaria (protocol 55.2-1-54-2532-117-13).

Histology

Mice were euthanized and organs were removed and fixed overnight in 4% paraformaldehyde. Organs were then embedded in paraffin, sectioned at 2.5 μm and mounted on glass slides. Following standard dewaxing and hydration procedures, staining was performed for 30 s in Hematoxylin, followed by a 5 min tap water rinse. Counterstaing was performed in Eosin 30 s, and subsequent dehydration was conducted according to standard procedures. For immunohistochemistry, slides were dewaxed and hydrated as above. Antigen retrieval was perfomed in citrate solution at pH 6.0 for 15 min in a microwave at 600W. The following antibodies were used: anti-P16INK4a (1:100, Santa Cruz, F-12, sc-1661) and anti-TRP53 (1:300, NCL-p53-CM5p, Novocastra, Leica

Bisosystems), followed by secondary biotin-conjugated antibodies. Peroxidase conjugated streptavidin was used with 3,3'-diaminobenzidine tetrahydrochloride (DAB, VectorLabs) as a chromogen for detection. Hematoxylin was used for counterstaining. Pictures were then recorded on an AxioImagerA1 microscope with an AxioCam color camera using AxioVision 4.3 software (all Carl Zeiss).

Statistical Analysis

Statistics were performed using graph pad prism. For expression analysis, student's t-test was used. To analyze survival after orthtopic implantation of *Kras*shRNA-PDCs, Log-Rank (Mantle-Cox) analysis was applied.

Results

Development of an *in vitro Kras* activation method

To obtain a strictly genetically defined model without contaminating stromal cells we decided to isolate a purely ductal cell population from the pancreas for further *in vitro* manipulation. Since *Kras* is mutated in over 90% of PDAC cases, we chose *in vitro* activation of LSL-$Kras^{G12D}$ by nuclear translocation of Cre-ER^{T2} through the application of 4OH-tamoxifen. Genomic re-arrangement and activation of $Kras^{G12D}$ was followed by introduction of a well-defined genetic second hit by the virtue of shRNA against *Trp53* or $p16^{Ink4a}$. These double-mutant PDCs were then re-introduced into the pancreata of NSG mice (Figure 1).

Indirect and direct assessment of *Kras*-activation by determination of the phosphorylation status of extracelluar signal-regulated kinase (ERK) and by using a Raf-RBD-GST-pulldown assay clearly demonstrated an increase of acitvated *Kras* in 4OH-tamoxifen treated *Kras*-PDCs (Figure 2 A and B).

Stable expression of shRNA leads to long-term gene regulation

As lentiviral transduction results in stable integration of the lentiviral genome into the host genome, we evaluated for sustained gene silencing after lentiviral infection. To that end, we tested target gene expression ten days after withdrawal of puromycin. Indeed, we observed long-term gene silencing of $p16^{Ink4a}$ and *Trp53* in all shRNA constructs used as demonstrated by assessment of target gene expression by qRT-PCR (Figure 3 A and C). Additional western blot analyis confirmed significant reduction of protein expression of $P16^{INK4a}$ and TRP53, respectively. (Figure 3 B and D).

Depletion of $p16^{Ink4a}$ in *Kras*-PDCs results in tumorigenesis

Tumor growth did not occur in animals that received *Kras*-shRNA-*Control*-PDCs over a time period of 26 weeks. In particular, the histological examination of control pancreata did not reveal any PanINs or other atypical or premalignant cell formation. By contrast, stable knock down of $P16^{Ink4a}$ resulted in rapid local tumor growth in five out of six animals in total. Interestingly, implantation of *Kras*shRNA-$p16^{Ink4a}$ #1-PDCs resulted in tumor growth in two out of three animals, whereas *Kras*-shRNA-$p16^{Ink4a}$ #2-PDCs led to development of pancreatic tumors only in all animals. In addition, the median survival in animals receiving

*Kras*shRNA-*p16*^{Ink4a} #1-PDCs was 160.0 days and 105.0 days in those receiving *Kras*-shRNA-*p16*^{Ink4a} #2-PDCs. However, gross anatomy as well as histological findings did not differ between the two shRNAs against *p16*^{Ink4a}. Immunohistological staining for P16^{INK4a} did not yield any signal in tumors that developed from *Kras*-shRNA-*p16*^{Ink4a}-PDCs, indicating sustained gene silencing by both shRNAs directed against *p16*^{Ink4a}. PanINs from three month old *Ptf1a-Cre*; LSL-*Kras*^{G12D} animals served as positive control (Figure S1 A). Of note, the tumors did not show the classical, desmoplastic architecture typical for PDAC but rather displayed a tumor cell rich growth with only sparse duct formation *in vivo* and almost no stromal reaction (Figure 4 A and B). Macroscopic liver metastasis did not occur upon depletion of *p16*^{Ink4a}. However, micro-metastasis could be found in one animal out of five mice that developed tumors upon depletion of *p16*^{Ink4a} (Figure S2).

Loss of *Trp53*leads to tumor growth

To address the question whether increased tumor growth is specific to the loss of *p16*^{Ink4a} in the setting of *Kras*-activation, we asked if depletion of another tumor suppressor, *Trp53,* would result in tumorigenesis as well. In line with the findings mentioned above, implantation of *Kras*-shRNA-*Trp53*-PDCs resulted in tumor development in five out of six animals in total. Two out of three animals receiving *Kras*-shRNA-*Trp53* #1-PDCs and all animals receiving *Kras*-shRNA-*Trp53* #2-PDCs developed pancreatic tumors. The median survival was 140 and 111 days, respectively. Loss of TRP53 was demonstrated by the absence of nuclear staining compared to TRP53 positive PanINs of three month old *Ptf1a-Cre*; LSL-*Kras*^{G12D} animals (Figure S1 B), demonstrating downregulation of TRP53 in these tumors. Interestingly, the macroscopic and histologic findings were similar to those seen in animals that have been implanted with *Kras*-shRNA-*p16*^{Ink4a}-PDCs, with tumors containing densely arranged tumor cells but almost no stromal reaction (Figure 5 A and B). Macrosopic liver metastasis occurred in three out of five tumor bearing animals and was confirmed by histology (Figure S2).

shRNA mediated gene silencing results in decreased survival

In total, five out of six animals receiving *Kras*-shRNA*p16*^{Ink4a}-PDCs or *Kras* shRNA*Trp53*-PDCs developed tumors. These animals display a significantly shorter survival when compared to animals that received *Kras*-shRNAControl. However there was no difference between animals implanted with *Kras*shRNA*p16*^{Ink4a}-PDCs or *Kras*-shRNA*Trp53*-PDCs (Figure S3).

Discussion

The data presented here demonstrate a stepwise manipulation of adult pancreatic ductal cells to model PDAC *in vivo*. First, we report isolation of an already well-defined pancreatic cell population that can be genetically altered by *ex vivo* recombination events through transient nuclear translocation of Cre-ER^{T2} (15), thereby activating oncogenic *Kras*. Second, we show sustained long-term gene silencing in *Kras*-PDCs using selective shRNAs against the tumor suppressor genes *p16*^{Ink4a} or *Trp53*. Third, we clearly demonstrate that *in vitro* modeling of genetic pathways that have been implicated in pancreatic cancer development and progression to lead to malignant pancreatic tumors *in vivo*. One main advantage of the

system used is the rapid generation of the desired cell line carrying the shRNA against the gene of interest within a few weeks. In addition, cell lines can be produced in parallel and the impact on pancreatic cancer biology of various genes can be studied simultaneously. An overall reduction of time consuming and expensive generation of germline-altered animal models and subsequent breedings and genotyping, not to mention long-term backcrossing, will emerge as a consequence.

Although the advantage of shRNA has been widely used in screening assays, many of the studies performed so far use either cells from animals that have already undergone embryonic loss of tumor suppressor genes (16, 17), cells with introduction of more than one genetic lesion prior to the transduction with shRNA (17, 18) or use already established and immortalized cancer cell lines (19, 20). The use of cells that are derived from embryonic tissue and/or harbor constitutive activation of oncogenes might lead to interaction with various developmentally activated but otherwise inactive pathways, thus resulting in cell fate decisions and phenotypes that do not occur upon sporadic oncogene activation in somatic cells. This is especially true for pancreatic cancer research as most animal models utilize *Pdx1*- of *Ptf1a*-driven Cre, which leads to oncogene activation or tumor suppressor deletion in all functional compartments of the pancreas due to their early promoter activity on days E8.5 and E9.5, respectively (21, 22). Also, sensitizing cells by more than one genetic alteration may lead to over interpretation of a newly identified tumor suppressor gene's impact as cells may be “supersensitized” to only minor oncogenic events. Third, long-term cultured cancer cell lines carry numerous genetic and epigenetic changes and do only partly reflect the cell of origin. In contrast, our model is designed to recapitulate truly somatic oncogene activation as we were able to avoid germline-activation of oncogenic *Kras*. In addition, one additional genetic hit was sufficient to induce tumor growth. It is important to realize that pancreatic loss $p16^{Ink4a}/p19^{Arf}$ on its own does not result in PanIN or PDAC development in mice (23). Although *Trp53*-/- animals are prone to develop malignancies, these are mostly lymphomas and soft tissue tumors. The development of epithelial cancers in these animals is rare (reviewed in (24)), and we do not know about any report of development of PDAC upon pancreas specific *Trp53*-deletion. Thus, our model more closely resembles a truly sequential second-hit carcinogenesis as initially proposed by Knudson in 1971 (25).

In contrast to the human disease and most genetically engineered mouse models of pancreatic cancer, the tumors described in this study lack the classical stromal component and show a more dedifferentiated phenotype. This observation may be due to various reasons. First, we injected ductal cells that have undergone genetic maipulation. However, the induction of a stromal cell response is known to take place early in PDAC development, so that this critical phase might be missed in our model (26). Second, NOD scid gamma mice used in this study are depleted for B- and T-cells, which are also believed to play an important role during the generation of a stromal response (27). Third, injection of a cell suspension might not reflect the hypoxic conditions naturally occurring in a solid tumor, thereby reducing levels of secreted factors that usually foster development of a stromal reaction (28). Intriguingly, tumors described in this study closely resemble those seen in mouse models that *a priori* lack the stromal compartment (29, 30). However, we argue that

orthotopic implantation is preferred over subcutaneous tumor xenograft models as the latter completely lack tumor cell interaction with neighboring cells at the naturally occurring site of origin of PDAC. This might not only be important in tumor initiation processes but also during the course of metastasis.

Because *p16*^{Ink4a} and *Trp53* act non-redundantly through either the control of cell cycle regulation or DNA damage repair mechanisms (31), we argue that our model will be an expandable and powerful tool to screen for new tumor suppressor genes and will broaden our understanding of cancer biology. Moreover, the principle of stepwise in vitro acquisition of genetic hits in primary pancreatic ductal cells may be transferrable to other techniques of gene modulation, including genome editing using CRIPSR/Cas9, as it has already been described for liver cancer (32).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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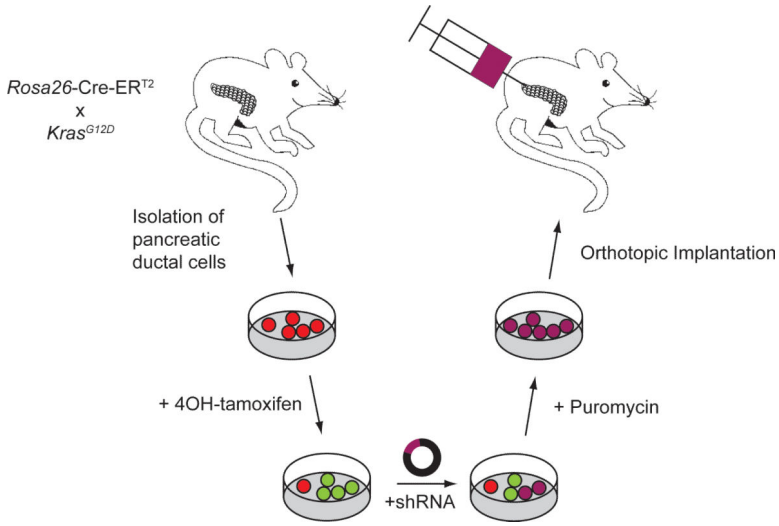

Figure 1.

Approach to generate double-mutant primary pancreatic ductal cells. PDCs were harvested from ROSA26CreER^{T2};*Kras*^{G12D} animals and treated with 4OH-tamoxifen to induce recombination. A second genetic hit was subsequently introduced by infection with lentiviral particles containing empty control vector or short hairpin RNAs directed against *p16*^{Ink4a} or *Trp53*. Upon selection for viral integration by puromycin, double-mutant PDCs were injected orthotopically into recipient mice (n=3 per lentiviral construct).

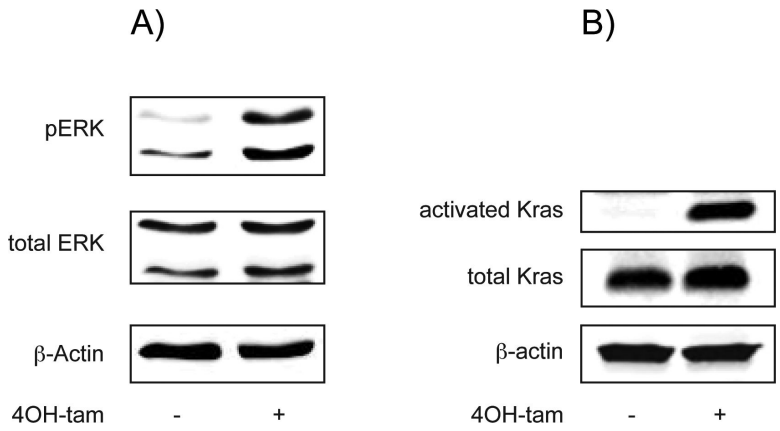

Figure 2.

Validation of KRAS activation in 4-OHtamoxifen treated PDCs.

A) Western blot analysis reveals increased levels of pERK in 4OH-tamoxifen treated PDCs as compared to control as a functional readout for *Kras* activation.

B) Direct evidence for KRAS-activation by 4OH-tamoxifen by detection of active KRAS using a Raf-RBD-assay, followed by western blot analysis. Note the absence of active endogenous KRAS in non-treated cells and comparable levels of total KRAS.

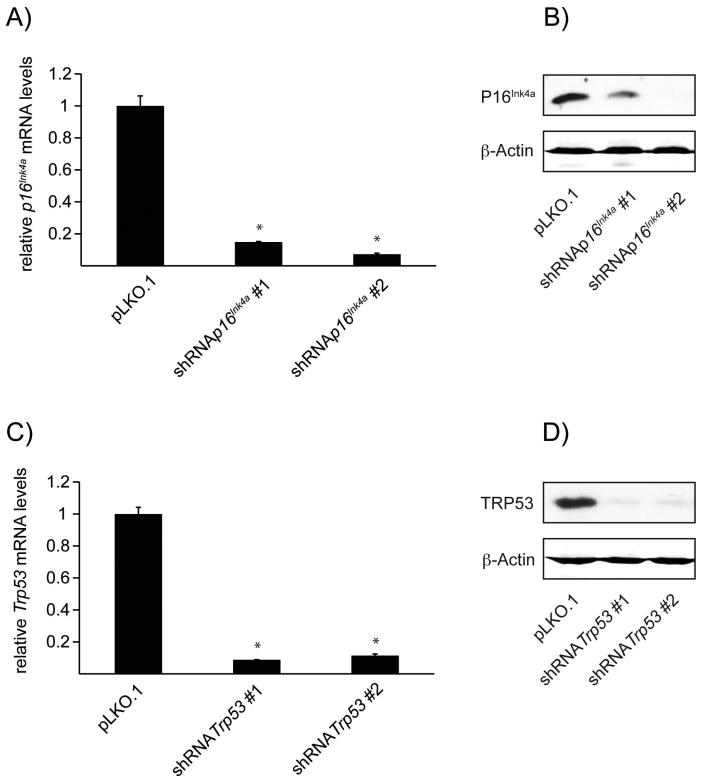

Figure 3.

Transduction with shRNA results in long-term gene silencing.

A) Quantitative RT-PCR of *p16*^{Ink4a}. 4OH-tamoxifen treated PDCs have been transduced with lentiviral particles containing either pLKO.1 empty control vector or shRNA against *p16*^{Ink4a}. Ten days after 4OH-tamoxifen withdrawal PDCs show significant decrease of target gene mRNA in both experimental groups vs. control. * = $p<0.05$, n=3.

B) Western blot analysis of P16^{INK4a} expression. 4OH-tamoxifen treated PDCs have been transduced with lentiviral particles containing either pLKO.1 empty control vector or shRNA against *p16*^{Ink4a}. Ten days after 4OH-tamoxifen withdrawal PDCs show significant decrease of P16^{INK4a} protein expression in both experimental groups vs. control.

C) Quantitative RT-PCR of *Trp53*. 4OH-tamoxifen treated PDCs have been transduced with lentiviral particles containing either pLKO.1 empty control vector or shRNA against *Trp53*. Ten days after 4OH-tamoxifen withdrawal PDCs show significant decrease of target gene mRNA in both experimental groups vs. control. * = $p<0.05$, n=3

D) Western blot analysis of TRP53 expression. 4OH-tamoxifen treated PDCs have been transduced with lentiviral particles containing either pLKO.1 empty control vector or shRNA against *Trp53*. Ten days after 4OH-tamoxifen withdrawal PDCs show significant decrease of TRP53 protein expression in both experimental groups vs. control.

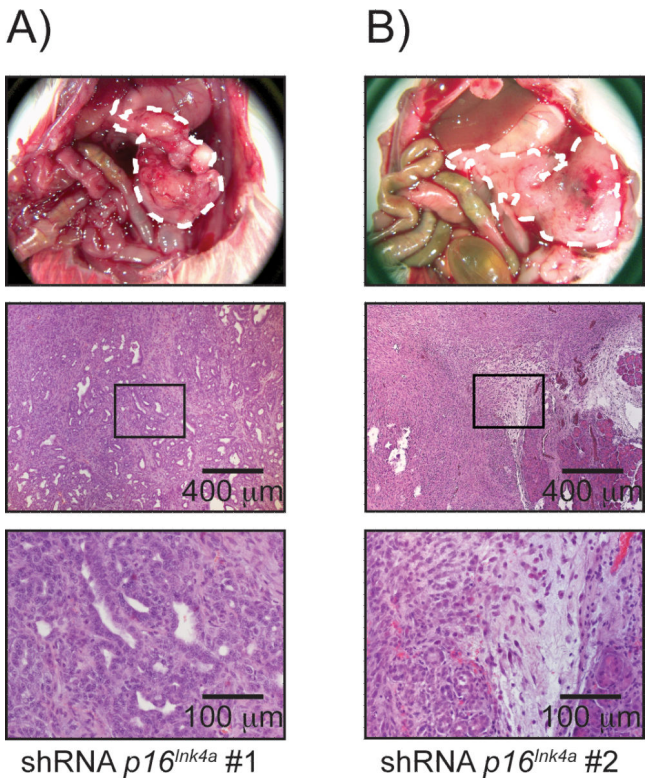

Figure 4.

Loss of *p16*^{Ink4a} results in PDAC formation

Animals receiving *Kras*-shRNAControl-PDCs did not develop tumors. Animals injected with *Kras*-shRNA*p16*^{Ink4a}-PDCs develop tumors and die due to their tumor burden. A) and B): Gross anatomy shows tumor growth within the anatomical site of injection and histology confirms tumor growth (magnification 50× and 200×).

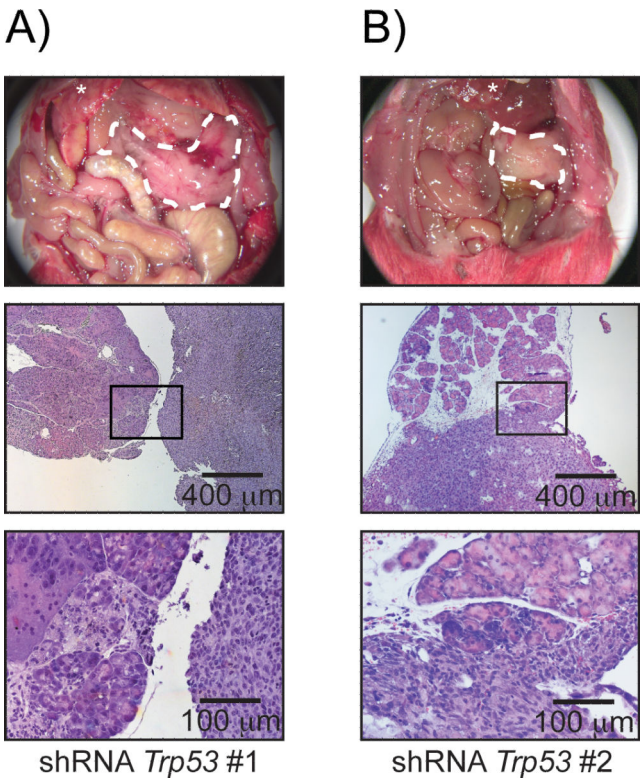

Figure 5.

Loss of *Trp53* results in PDAC formation

Animals receiving *Kras*-shRNAControl-PDCs did not develop tumors. Animals injected with *Kras*-shRNA*Trp53* develop tumors and die due to their tumor burden. A) and B) Gross anatomy shows tumor growth within the anatomical site of injection and histology confirms tumor growth (magnification 50× and 200×).