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 RNAi against the tumor suppressors p16

Ink4a

or Trp53 (TP53) using lentiviral gene delivery. Treatment of PDCs with 4OH-tamoxifen increased phosphorylation of ERK downstream of K Ras, and subsequent lentiviral transduction resulted in sustained target gene repression. Double-mutant PDCs were then reintroduced 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 Trp53 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.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in the United States. The 5-year survival rate of all patients suffering from PDAC is 6%, and 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 K ras, 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. To model this, we took advantage of the well-established primary pancreatic ductal cell culture (PDC; refs. 8, 9). Isolation of PDCs from mice that harbor the lox-stop-lox-Kras

G12D

-allele and express Cre-ERT2 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.

Materials and Methods

Isolation of PDCs

Primary PDCs were isolated from mice carrying the genotype Rosa26/ERK\(^{17}\),Lox-Stop-Lox-Kras\(^{G12D}\) (termed Kras-PDCs thereafter) and maintained essentially as described (10). Early passage cells were treated with 4OH-tamoxifen 200 nmol/L (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 minutes. 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). Fifty micrograms were resolved on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in PBS containing 0.05% Tween and 3% non-fat dry milk for 1 hour at room temperature and incubated with anti-pERK (Cell Signaling; 9101S; 1:200), or anti-TRP53 (NCL-p53-CM5p; Novocastra; Leica Biosystems; 1:1,000). Membranes were then subsequently incubated with anti-ERK (BD Biosciences; 610124; 1:1,000) and anti-HER2 (Cell Signaling; 1A9; 1:200), or anti-TRP53 (NCL-p53-CM5p; Novocastra; Leica Biosystems; 1:1,000). Membranes were then subsequently incubated with anti-HER2 (BD Biosciences; 610124; 1:1,000) or anti-HER2 (Cell Signaling; 1A9; 1:200) and anti–β-actin (Sigma Aldrich; Clone AC-74; A5316; 1:5,000). 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; ref. 11). Transfer to PVDF membranes and visualization was conducted as mentioned above using an antibody against KRAS (Merck-Millipore; 1:1,000).

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; ref. 13).

Murine primers were designed to be intron spanning. The following primers were used: p16\(^{ink4a}\) FW: CCCAACCGCCCGGAACCT, P16\(^{ink4a}\) RV: GTGACGTCGGCCATCATCACA, Trp53 FW: AGATCCGCGCCGTAAAC, Trp53 RV: TCTGTCGATCTGGGACATCC, Ppia FW: ATGGTCAACCCCACCGTGT, Ppia RV: TCTGTCGATCTGGGACATCC, p16\(^{ink4a}\) #1 hereafter, and TRCN0000054551, target sequence AGAGTATTTCACCCTCAAGAT, termed shRNA-P16\(^{ink4a}\)#2 hereafter. The clone IDs for shRNATrp53 were: TRCN0000012362, target sequence CTACAAGAAGTGCAACGCA- CAT, termed shRNATrp53 #1 hereafter, and TRCN0000054551, target sequence AGAGTATTTCACCCTCAAGAT, termed shRNATrp53 #2 hereafter. pLKO.1 was used for control. Purified plasmids were tested for integrity before transfection using the restriction enzymes BamHI and NdeI.

For virus production, 6 × 10\(^5\) 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 after transfection, culture media were changed and virus-containing supernatant was collected 24 hours later. Culture media were replaced and collected another 24 hours later. Viral supernatant was stored at −80°C until further use.

Kras-PDCs were transduced with lentiviral constructs as described with only minor modifications (14). Briefly, 4OH-tamoxifen–treated Kras-PDCs were placed in wells of a 6-well plate at a cell number of 3 × 10\(^5\) cells/well and allowed to adhere on plastic overnight. Next day, cells were transduced with viral supernatant containing polybrene 4 μg/mL (Sigma). Twenty-four hours later, PDCs were placed back onto collagen-coated wells and were allowed to adhere for another 24 hours. Upon complete attachment, cells were selected in the presence of puromycin (8 μg/mL) for 10 days (termed Kras-shRNA-PDCs hereafter). Successful depletion of target gene mRNA was confirmed by quantitative RT-PCR. Two different shRNAs per target gene were tested to reduce off-target effects.

Orthotopic transplantation and animal procedures

Immune compromised NOD.Cg-Pkd\(^{+/+}\)Tg129Kras\(^{G12D}\) mice (NOD scid gamma, NSG) were obtained from The Jackson Laboratory. Eight- to 10-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 lacer 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 >1 cm, 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 seconds in hematoxylin, followed by a 5-minute tap water rinse. Counterstaining was performed in Eosin for 30 seconds, and subsequent dehydration was conducted according to standard procedures. For immunohistochemistry, slides were dewaxed and hydrated as above. Antigen retrieval was performed in citrate solution at pH 6.0 for 15 minutes in a microwave at 600 W. 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 Biosystems), 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, the Student t test was used. To analyze survival after orthotopic 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. Because Kras is mutated in over 90% of PDAC cases, we chose in vitro activation of LSL-Kras<sup>G12D</sup> by nuclear translocation of Cre-ER<sup>T2</sup> through the application of 4OH-tamoxifen. Genomic rearrangement and activation of Kras<sup>G12D</sup> were followed by introduction of a well-defined genetic second hit by the virtue of shRNA against Trp53 or p16<sub>ink4a</sub>. These double-mutant PDCs were then reintroduced into the pancreata of NSG mice (Fig. 1).

Indirect and direct assessment of Kras activation by determination of the phosphorylation status of ERK and by using a Raf–RBD–GST-pulldown assay clearly demonstrated an increase of activated Kras in 4OH-tamoxifen–treated Kras-PDCs (Fig. 2A 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 10 days after withdrawal of puromycin. Indeed, we observed long-term gene silencing of p16<sub>ink4a</sub> and Trp53 in all shRNA constructs used as demonstrated by assessment of target gene expression by qRT-PCR (Fig. 3A and C). Additional Western blot analysis confirmed significant
reduction of protein expression of P16\textsuperscript{INK4a} and TRP53, respectively (Fig. 3B and D).

Depletion of p16\textsuperscript{INK4a} in Kras-PDCs results in tumorigenesis

Tumor growth did not occur in animals that received Kras\textsuperscript{-shRNA-Control-PDCs} over a time period of 26 weeks. In particular, the histologic examination of control pancreata did not reveal any PanINs or other atypical or premalignant cell formation. By contrast, stable knockdown of Kras\textsuperscript{-shRNA-#1-PDCs} resulted in rapid local tumor growth in 5 of 6 animals in total. Interestingly, implantation of Kras\textsuperscript{-shRNA-p16\textsuperscript{INK4a}-#1-PDCs} resulted in tumor growth in 2 of 3 animals, whereas Kras\textsuperscript{-shRNA-p16\textsuperscript{INK4a}-#2-PDCs} led to development of pancreatic tumors in all animals. In addition, the median survival in animals receiving Kras\textsuperscript{-shRNA-p16\textsuperscript{INK4a}-#1-PDCs} was 160.0 days and 105.0 days in those receiving Kras\textsuperscript{-shRNA-p16\textsuperscript{INK4a}-#2-PDCs}. However, gross anatomy as well as histologic findings did not differ between the two shRNAs against p16\textsuperscript{INK4a}. Immunohistological staining for P16\textsuperscript{INK4a} did not yield any signal in tumors that developed from Kras\textsuperscript{-shRNA-p16\textsuperscript{INK4a}-PDCs}, indicating sustained gene silencing by both shRNAs directed against p16\textsuperscript{INK4a}. PanINs from 3-month-old Ptf1a-Cre; LSL-Kras\textsuperscript{G12D} animals served as positive control (Supplementary Fig. S1A).

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 \textit{in vivo} and almost no stromal reaction (Fig. 4A and B). Macroscopic liver metastasis did not occur upon depletion of p16\textsuperscript{INK4a}. However, micrometastasis could be found in 1 animal of 5 mice that developed tumors upon depletion of p16\textsuperscript{INK4a} (Supplementary Fig. S2).

Loss of Trp53 leads to tumor growth

To address the question whether increased tumor growth is specific to the loss of p16\textsuperscript{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\textsuperscript{-shRNA-Trp53-PDCs} resulted in tumor development in 5 of 6 animals in total. Two of 3 animals receiving Kras\textsuperscript{-shRNA-Trp53 #1-PDCs} and all animals receiving Kras\textsuperscript{-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 with TRP53-positive PanINs of 3-month-old Ptf1a-Cre; LSL-Kras\textsuperscript{G12D} animals (Supplementary Fig. S1B), demonstrating downregulation of TRP53 in these
An shRNA-Based Method for Detection of Tumor Suppressors

Figure 4. Loss of p16\textsuperscript{inva} results in PDAC formation. Animals receiving Kras-shRNA-Control-PDCs did not develop tumors. Animals injected with Kras-shRNAp16\textsuperscript{inva}-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, \texttimes50 and \texttimes200).

Figure 5. Loss of Trp53 results in PDAC formation. Animals receiving Kras-shRNA-Control-PDCs did not develop tumors. Animals injected with Kras-shRNATrp53-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, \texttimes50 and \texttimes200).

the gene of interest within a few weeks. In addition, cell lines generation of the desired cell line carrying the shRNA against the tumor suppressor genes p16\textsuperscript{inva} or Trp53. Third, we clearly demonstrate that in vitro modeling of genetic pathways that have been implicated in pancreatic cancer development and progression leads 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 before 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- or 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.

tumors. Interestingly, the macroscopic and histologic findings were similar to those seen in animals that have been implanted with Kras-shRNA-p16\textsuperscript{inva}-PDCs, with tumors containing densely arranged tumor cells but almost no stromal reaction (Fig. 5A and B). Macroscopic liver metastasis occurred in 3 of 5 tumor-bearing animals and was confirmed by histology (Supplementary Fig. S2).

shRNA-mediated gene silencing results in decreased survival

In total, 5 of 6 animals receiving Kras-shRNAp16\textsuperscript{inva}-PDCs or Kras-shRNATrp53-PDCs developed tumors. These animals display a significantly shorter survival when compared with animals that received Kras-shRNA-Control. However, there was no difference between animals implanted with Kras-shRNA-p16\textsuperscript{inva}-PDCs or Kras-shRNATrp53-PDCs (Supplementary Fig. S3).

Discussion

The data presented here demonstrate a stepwise manipulation of adult PDCs 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-ERT\textsuperscript{2} (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\textsuperscript{inva} or Trp53. Third, we clearly demonstrate that in vitro modeling of genetic pathways that have been implicated in pancreatic cancer development and progression leads 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
In addition, one additional genetic hit was sufficient to induce tumor growth. It is important to realize that pancreatic cancer loss of p16Ink4a/p19Arf on its own does not result in PanIN or PDAC development in mice (23). Although Trp53<sup>−/−</sup> 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 ref. 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 Knudsen 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 gene manipulation. 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<sup>ink4a</sup> and Trp53 act nonredundantly 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 PDCs may be transferrable to other techniques of gene modulation, including genome editing using CRISPR/Cas9, as it has already been described for liver cancer (32).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. von Burstin, S. Diensch
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Writing, review, and/or revision of the manuscript: J. von Burstin, M. Reichert, A.K. Rustgi, R.M. Schmid
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Schneider
Study supervision: R.M. Schmid

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