Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development

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Pancreatic cancer is a disease with an extremely poor prognosis. *Tumor protein 53-induced nuclear protein 1* (*TP53INP1*) is a proapoptotic stress-induced p53 target gene. In this article, we show by immunohistochemical analysis that TP53INP1 expression is dramatically reduced in pancreatic ductal adenocarcinoma (PDAC) and this decrease occurs early during pancreatic cancer development. TP53INP1 reexpression in the pancreatic cancer-derived cell line MiaPaCa2 strongly reduced its capacity to form s.c., i.p., and intrapancreatic tumors in *nude* mice. This anti-tumoral capacity is, at least in part, due to the induction of caspase 3-mediated apoptosis. In addition, TP53INP1$^{-/-}$ mouse embryonic fibroblasts (MEFs) transformed with a retrovirus expressing E1A/ras^{V12} oncoproteins developed bigger tumors than TP53INP1$^{+/+}$ transformed MEFs or TP53INP1$^{-/-}$ transformed MEFs with restored TP53INP1 expression. Finally, TP53INP1 expression is repressed by the oncogenic micro RNA miR-155, which is overexpressed in PDAC cells. TP53INP1 is a previously unknown miR-155 target presenting anti-tumoral activity.

apoptosis | pancreatic cancer | ponasterone A | tumor suppressor | micro RNA

Prognosis of pancreatic ductal adenocarcinoma (PDAC) is the worst among cancers, with only 20% of patients reaching two years of survival. The aggressive nature of the neoplasia, the lack of early detection, and the limited response to available treatments contribute to its high mortality rate. Pancreatic cancer is characterized by modifications in gene expression due to mutations, deletions, and amplifications, as well as alterations in DNA methylation on genes critical for tumor development and progression (1). Detailed knowledge of genes whose expression is altered during pancreatic cancer development and of associated molecular mechanisms may help devising strategies for earlier diagnosis and identifying new therapeutic targets.

TP53INP1 (*tumor protein 53-induced nuclear protein 1*) is a proapoptotic stress-induced p53 target gene (2, 3). TP53INP1 is able to interact with p53 and the homeodomain-interacting protein kinase-2 (HIPK2) within the promyelocytic leukemia nuclear bodies (PML-NBs) modulating p53 transcriptional activity (4). TP53INP1 also interacts physically with the proapoptotic protein kinase C δ upon exposure to genotoxic agents, contributing to the regulation of p53 activity during apoptosis (5). In turn, p53 is able to activate *TP53INP1* transcription. p53 exerts its tumor suppressor function mainly by inducing transcription of target genes involved in cell cycle arrest and apoptosis, as part of the cell response to genotoxic stress (2, 6, 7). Overexpression of *TP53INP1* induces cell cycle arrest and apoptosis in several cell lines, even in the absence of p53. In this case, TP53INP1 is functionally associated with p73 and allows regulation of cell cycle progression and apoptosis, independently from p53 (8). Finally, the E2F1 transcription factor, also a major effector of cell proliferation and apoptosis, is involved in *TP53INP1* transcriptional regulation (9). The *TP53INP1* gene encodes two protein isoforms, TP53INP1α and TP53INP1β, which both induce cell cycle arrest and apoptosis when overexpressed (2). TP53INP1 expression is lost in rat preneoplastic lesions in liver (10, 11) and during gastric cancer progression in human, which correlates with a decreased level of apoptosis in tumor cells and a poor prognosis (12). Altogether, currently available data point to a role of TP53INP1 in cellular homeostasis through its antiproliferative and proapoptotic activities. Therefore, loss of its expression may contribute to deregulation of cell proliferation, a hallmark of oncogenesis.

In this work, we demonstrate that TP53INP1 expression is lost in early stages of pancreatic cancer evolution, that its restoration strongly reduces tumor development, and that TP53INP1 expression is repressed by a mechanism involving miR-155.

Results

TP53INP1 Inactivation Occurs Early in PDAC Development. Expression of TP53INP1 was analyzed by immunohistochemistry in healthy and diseased pancreas tissues by using a specific monoclonal antibody (clone A25-E12). Positive signal was observed in normal tissues, in the epithelial layer ducts as shown in Fig. 1*A*. Regarding benign pancreatic lesion, TP53INP1 expression was detected in 100% of mucinous cystadenoma [Fig. 1*B* and supporting information (SI) Table 1] and intraductal papillary mucinous neoplasms (IPMN) without dysplasia (Fig. 1*C* and SI Table 1). In addition, 97% of chronic obstructive pancreatitis showed an enhanced cytoplasmic TP53INP1 level in both acinar and large interlobular ducts cells (Fig. 1*D* and SI Table 1).

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Abbreviations: PDAC, pancreatic ductal adenocarcinoma; MEF, mouse embryonic fibroblast; PonA, ponasterone A; TP53INP1, tumor protein 53-induced nuclear protein 1; PanIN, pancreatic intraepithelial neoplasia; miRNA, micro RNA.

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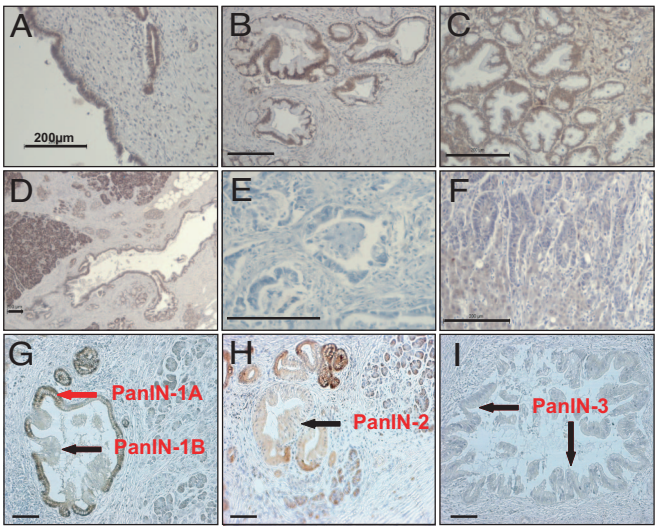

Fig. 1. TP53INP1 expression is lost in PDAC and metastasis. Shown is TP53INP1-positive immunostaining in epithelial cells of large ducts from normal pancreatic tissue (*A*), mucinous cystadenoma (*B*), intraductal papillary mucinous neoplasm (*C*), and in chronic pancreatitis (*D*). TP53INP1 protein was not detected in PDAC (*E*) and in liver metastasis (*F*). (*G–I*). TP53INP1 positive staining in early lesions, PanIN-1A (red arrow) negative staining in PanIN-1B, PanIN-2 and PanIN-3 (black arrow).

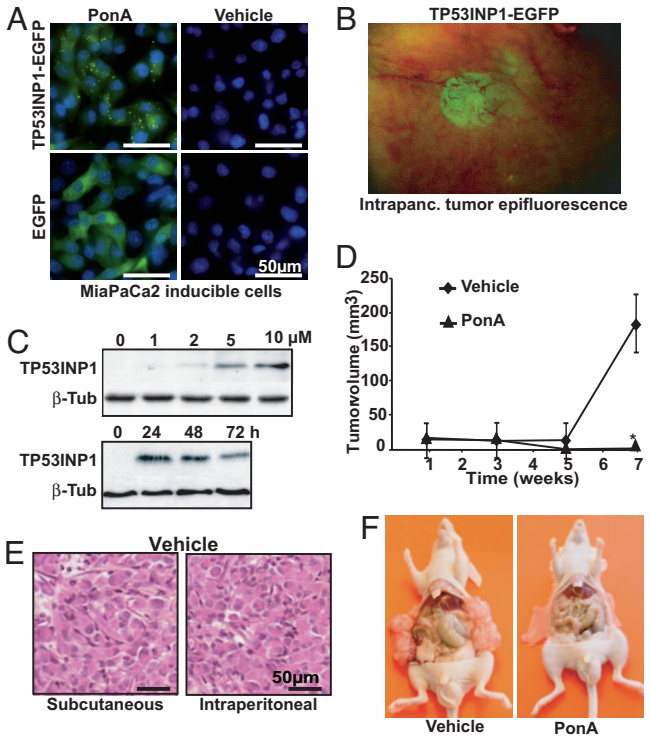

Fig. 2. TP53INP1-inducible expression in MiaPaCa2 cells and inhibition of s.c. and i.p. tumor growth. (*A*) TP53INP1- or EGFP-inducible MiaPaCa2 cells were cultured in the presence of PonA 10 μM or vehicle as control. Sixteen hours later, cells were tested for TP53INP1-EGFP or EGFP expression with an anti-EGFP antibody. (*B*) Cells were injected into the pancreas of mice, and 2 weeks later PonA-releasing pellets were implanted. After 48 h, TP53INP1 induction was visualized by green epifluorescence. (*C*) TP53INP1-inducible MiaPaCa2 cells were incubated with increasing concentrations of PonA for 48 h (*Upper*). Cells were treated for the indicated times with PonA 10 μM and TP53INP1 expression was analyzed by Western blot (*Lower*). β-tubulin level was used as loading control. (*D*) Ten millions of TP53INP1-inducible MiaPaCa2 cells were s.c. injected in mice carrying PonA ($n = 6$) or vehicle ($n = 6$) releasing pellets. Tumoral volume was weekly determined as described in *SI Materials and Methods*. (*E*) Histological analysis of s.c. and i.p. tumors from vehicle-treated mice. (*F*) Ten millions of TP53INP1-inducible MiaPaCa2 cells were i.p. injected in mice carrying PonA ($n = 6$) or vehicle ($n = 6$) releasing pellets. Representative photographs are shown. Values are represented as mean ± SE. *, $P < 0.05$.

Conversely, TP53INP1 expression was markedly reduced or completely lost in the majority of tumoral samples (86%) as shown in Fig. 1*E* and SI Table 1. A heterogeneous staining was observed in the remaining 14% PDAC samples in which only a limited number of positive cells was observed. In addition, TP53INP1 expression was not detected in PDAC metastasis (Fig. 1*F* and SI Table 1).

The current multistep progression model for PDAC follows the hyperplasia-dysplasia-invasive adenocarcinoma sequence. In pancreas, the dysplastic lesions are termed pancreatic intraepithelial neoplasia (PanIN) and are graded from PanIN-1A to PanIN-3 depending on the intensity of architectural and cellular atypia (1, 13, 14). We looked at which of these stages TP53INP1 expression was lost by examining the PanIN in the vicinity of 43 invasive PDAC. From 69 PanINs observed (SI Table 1), 24 were graded as PanIN-1A, 29 as PanIN-2, and 16 as PanIN-3. We found that TP53INP1 was expressed in all early PanIN-1A lesions (Fig. 1*G* and SI Table 1). By contrast, TP53INP1 expression was detected in only 13 of the 29 PanIN-2 (Fig. 1*H* and SI Table 1) and in none of the PanIN-3 lesions (Fig. 1*I* and SI Table 1). These results suggest that TP53INP1 expression is partially lost at the PanIN-2 stage and completely in PanIN-3 lesions.

Restored TP53INP1 Expression in Pancreatic Cancer Cells Inhibits Tumoral Growth *in Vivo*. To analyze the role of TP53INP1 in pancreatic cancer development, we developed a TP53INP1-inducible MiaPaCa2 cell line expressing controlled levels of TP53INP1 fused to EGFP or of EGFP alone. In this system, TP53INP1 expression is activated by ponasterone A (PonA). Vehicle-treated cells do not show any signal upon direct fluorescence microscopic or Western blot analysis, indicating that there is no leakage of TP53INP1 or EGFP expression. On the contrary, after treating the cells with PonA, we observed rapid TP53INP1 induction (Fig. 2 *A* and *C*). MiaPaCa2 cell line was chosen because it does not express endogenous TP53INP1 as evidenced by RT-PCR and Western blot analysis (data not shown).

In the absence of PonA, the TP53INP1-inducible MiaPaCa2 cell line generates tumors after s.c., i.p., or intrapancreatic injections in *nude* mice. After PonA treatment, epifluorescence of the EGFP-tag allowed controlling that TP53INP1 was indeed expressed in tumors (Fig. 2*B*). To assess the significance of TP53INP1 restoration in tumoral development, inducible MiaPaCa2 cells were injected s.c. or i.p. in *nude* mice carrying vehicle or PonA implants ($n = 6$). In the absence of PonA, mice injected s.c. developed tumors after 7 weeks, with a mean volume of 185 ± 56 mm^3, whereas none of PonA-treated mice developed tumors (Fig. 2*D*). In the same way, TP53INP1-inducible cells injected i.p. developed tumors that extended to the peritoneal cavity in 83% of vehicle-treated mice ($n = 6$) whereas no tumors appeared in PonA-treated mice (Fig. 2*F*). To assess the influence of PonA itself on tumoral development, EGFP-inducible MiaPaCa2 cells were injected s.c. or i.p. in *nude* mice treated or not with PonA-releasing implants. We observed that these two treatments do not induce significant differences in tumor development (data not shown). This result indicates that PonA does not have any effect on this model of tumoral development.

Finally, to study the role of TP53INP1 restoration in the pancreatic environment, inducible MiaPaCa2 cells were injected

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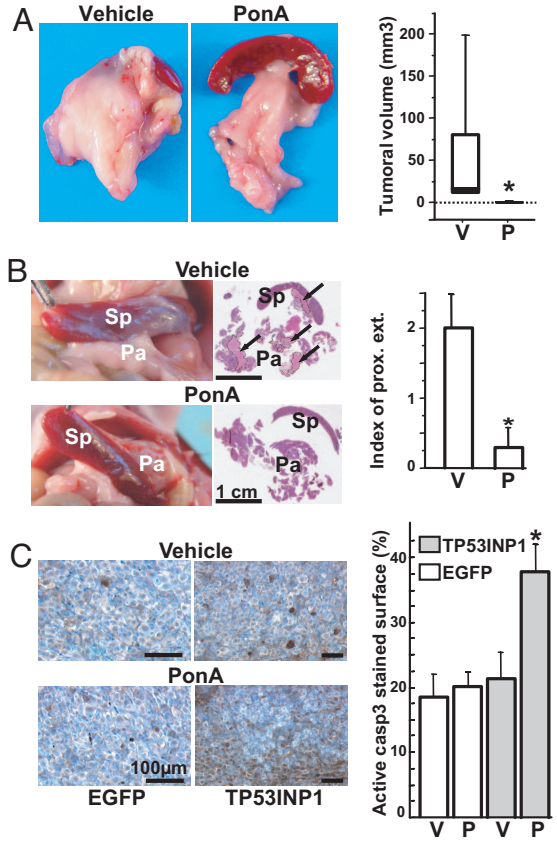

Fig. 3. TP53INP1 restoration inhibits tumoral growth in a pancreatic cancer model *in vivo*. Eighteen millions of inducible MiaPaCa2 cells were injected into the pancreas of *nude* mice ($n = 7$ for each group) one day after s.c. implantation of pellets releasing vehicle as control (V) or PonA (P). Assessment of pancreatic tumor growth and spreading was done 19 days after cells injection. (*A*) (*Left* and *Center*) Representative photographs of pancreatic tumors. (*Right*) Intrapancreatic tumoral volume (tumor sizes were measured as described in *SI Materials and Methods*). (*B*) (*Left*) Representative images showing the spreading of pancreatic tumors in PonA and vehicle treated animals (Sp, spleen; Pa, pancreas). (*Center*) Representative images of HES-stained pancreas and spleen from vehicle and PonA-treated animals. Tumor surface is outlined in black and indicated by arrows. (*Right*) Representation of the proximal extension index. The index was determined as described in *SI Materials and Methods*. (*C*) (*Left* and *Center*) Representative images of pancreatic tumors active caspase-3 immunohistochemistry on vehicle and PonA-treated animals with TP53INP1-inducilbe MiaPaCa2 or EGFP-inducible MiaPaCa2 tumors. (*Right*) The percentage of tumor surface containing active caspase-3. Values are represented as mean ± SE. *, $P < 0.05$.

into the pancreas of *nude* mice implanted s.c. with PonA or vehicle releasing pellets ($n = 7$). Nineteen days after cells injection, we observed that all vehicle-treated mice developed intrapancreatic tumors whereas only 43% of the PonA-treated mice were positive for intrapancreatic tumor. Mean size of tumors was 55 ± 36 mm^3 or 0.5 ± 0.3 mm^3 for vehicle or PonA-treated mice respectively (Fig. 3*A*). Moreover, tumors expressing TP53INP1 showed significantly less extrapancreatic tumoral extension to proximal (Fig. 3*B*) and distal organs, indicating that TP53INP1 expression affects the growth and the spreading of pancreatic cancer cells.

To analyze the putative proapoptotic effect of TP53INP1 expression in these tumors, we measured the presence of active caspase-3 by immunohistochemistry in intrapancreatic tumors. TP53INP1-inducible PonA-treated tumors showed a positive area for active caspase-3 significantly higher than vehicle-treated tumors (38 ± 4% vs. 21.5 ± 4%). On the other hand EGFP-inducible tumors treated with vehicle or PonA showed levels of activated caspase-3 similar to TP53INP1-inducible tumors treated with vehicle (18 ± 4% and 20.5± 2% vs. 21.5 ± 4%) (Fig. 3*C*). These results suggest that apoptosis contributes to the inhibition of tumor development observed with TP53INP1-expressing cells and that this difference is not due to PonA itself.

E1A/ras-TP53INP1−/− Mouse Embryonic Fibroblasts (MEFs) Promote the Formation of Bigger Tumors Than E1A/ras-TP53INP1+/+ MEFs. To confirm the role of TP53INP1 in another cellular model, MEFs were derived from E14.5 $TP53INP1^{+/+}$ or $TP53INP1^{-/-}$ mouse embryos. MEFs were then infected with a retrovirus allowing the expression of *E1A* and ras^{V12} oncogenes to transform them (E1A/ras-MEFs). E1A/ras-$TP53INP1^{+/+}$ and $TP53INP1^{-/-}$ MEFs expressed equivalent high levels of ras as estimated by Western blot analysis (data not shown). It is noteworthy that the lack of TP53INP1 expression significantly increases the growth in E1A/ras-MEFs (Fig. 4*C*). To restore TP53INP1 expression, E1A/ras-$TP53INP1^{-/-}$ MEFs were transduced with the MSCV-TP53INP1-Myc retrovirus. Endogenous and restored TP53INP1 levels were controlled by Western blot analysis. (Fig. 4*B* and SI Fig. 6). Interestingly, TP53INP1 reexpression reduced significantly the growth rate of E1A/ras-$TP53INP1^{-/-}$ MEFs (Fig. 4*C*).

The tumorigenic properties of E1A/ras-$TP53INP1^{+/+}$ MEFs, E1A/ras-$TP53INP1^{-/-}$ MEFs and E1A/ras-$TP53INP1^{-/-}$ MEFs reexpressing TP53INP1 were assessed. We checked their ability to grow in an anchorage independent medium *in vitro* and to form s.c. tumors in *nude* mice *in vivo*. In soft-agar assays, E1A/ras-$TP53INP1^{-/-}$ MEFs formed bigger colonies than E1A/ras-$TP53INP1^{+/+}$ MEFs or E1A/ras-$TP53INP1^{-/-}$ MEFs reexpressing TP53INP1 (Fig. 4*D*). Similarly, E1A/ras-$TP53INP1^{-/-}$ MEFs developed bigger tumors in all *nude* mice than E1A/ras-$TP53INP1^{+/+}$ MEFs when injected s.c. (Fig. 4 *E* and *F Left*). Moreover, E1A/ras-$TP53INP1^{-/-}$ MEFs reexpressing TP53INP1 developed smaller tumors than E1A/ras-$TP53INP1^{-/-}$ MEFs (Fig. 4 *E* and *F Right*). These results are in agreement with the results obtained with TP53INP1-inducible MiaPaCa2 cells and support the idea that TP53INP1 plays a role in the prevention of tumor establishment and/or development.

miR-155 Interacts with the TP53INP1 mRNA 3′ UTR. TP53INP1 expression is strongly decreased in pancreatic cancer cells. Because TP53INP1 expression seems to be important for pancreatic cancer development, we decided to analyze the molecular mechanism by which TP53INP1 expression is lost. We compared the methylation status and the presence of mutations in the TP53INP1 promoter in PDAC and peritumoral pancreas (PTP). No differences were found (data not shown). Then, we quantified the TP53INP1 mRNA level by RT-PCR in 11 samples known to be negative for TP53INP1 immunostaining and in their corresponding (TP53INP1-positive) peritumoral regions. To our surprise, TP53INP1 mRNA levels were similar in the PADC and in the PTP whereas the protein was present in PTP and almost undetectable in PADC (Fig. 5*A*). These findings suggested that TP53INP1 down-regulation is not due to a transcriptional modulation. We therefore made the hypothesis that loss of TP53INP1 expression could be due to down-regulation by a miRNA. Bioinformatic approaches were used to identify potential micro RNA (miRNA) targets in the TP53INP1 mRNA 3′ UTR. Interrogation of the TargetScan database (15) revealed, with the highest scores, the presence of 4 putative sites for miRNA targeting corresponding to miR-155, miR-190, miR-182, and miR-504. However, we focused our analysis on miR-155 because it exhibits a high degree of complementarity with the 1217–1243 region in the TP53INP1 3′ UTR (Fig. 5*B*), it is overexpressed in pancreatic cancer cells (16, 17) and it has been described to be oncogenic (18, 19). miR-155 level was measured by RT-PCR on PDAC and PTP. As was already observed by

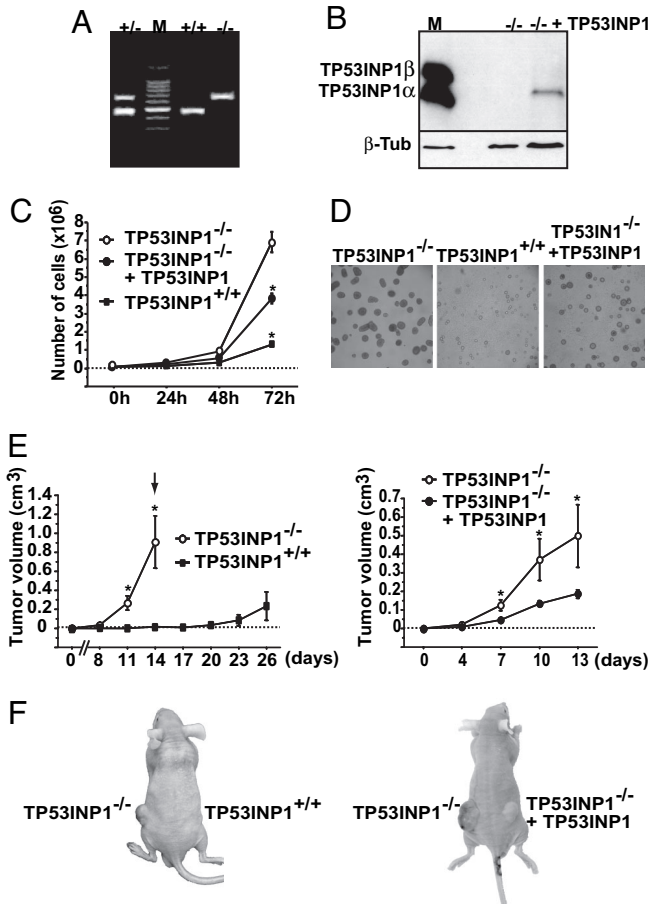

Fig. 4. TP53INP1 reexpression reduces the growth rate of E1A/ras-TP53INP1$^{-/-}$ MEFs *in vitro* and *in vivo*. (*A*) TP53INP1 genotyping of primary MEFs determined by PCR. (*B*) Western blot showing the TP53INP1 restoration in E1A/ras-TP53INP1$^{-/-}$ MEFs. TP53INP1 restoration was obtained by transduction of E1A/ras-TP53INP1$^{-/-}$ MEFs with a retrovirus expressing TP53INP1 (TP53INP1$^{-/-}$ plus TP53INP1). β-tubulin (β-tub) was used as control. Total cellular extract containing TP53INP1α and β transfected proteins were migrated in parallel (M) (*C*) Growth curves for E1A/ras-MEFs. (*D*) Growth in soft-agar for E1A/ras-MEFs. (*E*) Subcutaneous tumoral growth in *nude* mice. One million cells were s.c. injected. Tumoral volume was determined as described in *SI Materials and Methods*. (*Left*) Tumoral growth for E1A/ras-TP53INP1$^{-/-}$ MEFs ($n = 6$) versus E1A/ras-TP53INP1$^{+/+}$ MEFs ($n = 6$). The arrow corresponds to the excision of indicated tumors. (*Right*) Tumoral growth for E1A/ras-TP53INP1$^{-/-}$ MEFs ($n = 6$) versus E1A/ras-TP53INP1$^{-/-}$ MEFs reexpressing TP53INP1 ($n = 5$). (*F*) Representative images of s.c. tumors. Values are represented as mean ± SE. *, $P < 0.05$.

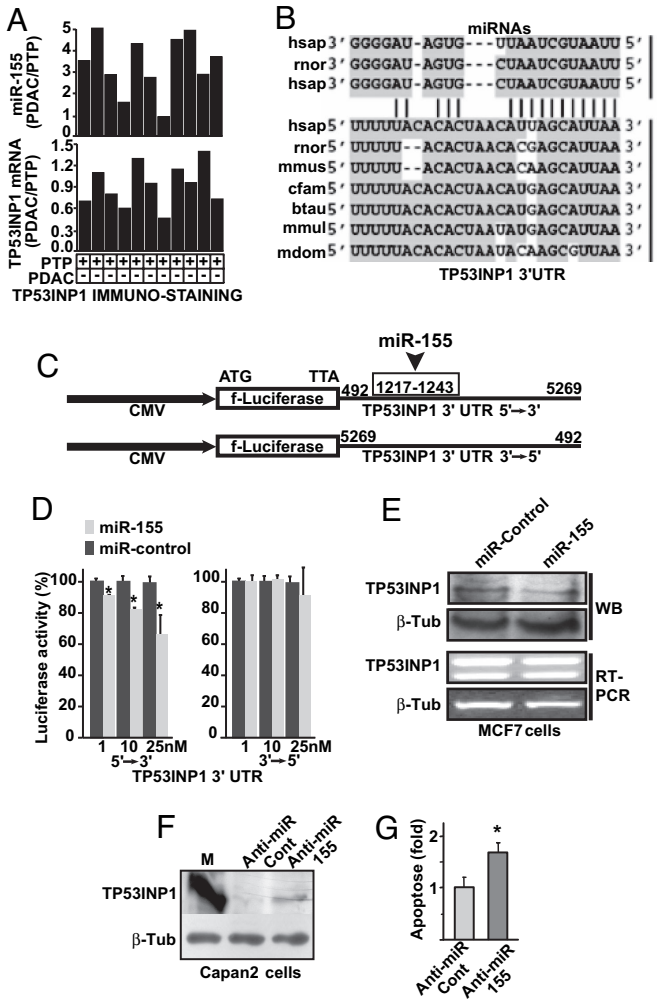

Fig. 5. TP53INP1 is a miR-155 target. (*A*) Data represent mRNA and miR-155 level ratios between PDAC and peritumoral pancreas (PTP). TP53INP1 immunohistochemical analysis are presented below the graph: +, positive staining; −, negative staining. (*B*) Alignment of mouse, rat and human miR-155 and the TP53INP1 3′ UTR in different species. (*C*) Representation of the pMIR-TP53INP1 vectors used in the Luciferase assay. (*D*) 293T cells were cotransfected with pMIR-TP53INP1 3′ UTR constructs in the 5′→3′ or 3′→5′ orientation, and the indicated concentrations of miR-155 or miR-control. Luciferase activity was measured, 48 h after transfection. (*E*) MCF7 cells were transfected with 50 nM miR-155 or miR-control. After transfection with a p53-expressing vector and gamma irradiation (30 Gy), TP53INP1 level was evaluated by Western blot and RT-PCR. β-tubulin level was used as control. Values are represented as mean ± SE. *, $P < 0.05$. (*F*) Capan2 cells were transfected with Anti-miR-155 or Anti-miR-control and gamma-irradiated (30 Gy), TP53INP1 level was evaluated by Western blot. β-tubulin level was used as control. Capan2 cells transfected with a plasmid allowing the expression of TP53INP1 were migrated in parallel as molecular weight control (M). (*G*) Apoptosis was measured in Capan2 cells 24 h after transfection with Anti-miR-155 or Anti-miR-control.

others (16, 17) miR-155 was overexpressed in the majority of PDAC samples (Fig. 5*A*). To validate experimentally computational data, the TP53INP1 3′ UTR (i.e., 4777 bp) was subcloned down-stream of the f-Luciferase ORF in the direct (5′→3′) and in the reversed orientation (3′→5′) as control (Fig. 5*C*). These reporter constructs were cotransfected in 293T cells with either, the pEGFP-N1 plasmid (to normalize transfection efficiency) and the miR-155 or a miR-control (nontargeting RNA oligonucleotide). Interestingly, Luciferase activity was markedly reduced only in the cells cotransfected with the miR-155 and the TP53INP1 3′ UTR in the direct orientation (Fig. 5*D Left*). The Luciferase activity drop was dose-dependent and showed a 9% reduction in the presence of only 1 nM of miR-155, reaching a reduction of 33% when concentration was raised to 25 nM. No significant reduction of Luciferase activity was observed in controls (Fig. 5*D Right*). It was concluded that miR-155 could target the TP53INP1 3′ UTR. To validate this result with endogenous TP53INP1 expression, MCF7 cells were transfected with miR-155 and the TP53INP1 protein level was evaluated by Western blot 36 h later. To induce a detectable expression of TP53INP1 in MCF7, cells were transfected with a p53-expressing vector (pcDNA3-p53) and 24 h later they were irradiated at 30 Gy (gamma irradiation) as described by Okamura (6). miR-155 or miR-control was cotransfected with pcDNA3-p53. As shown in Fig. 5*E* mir-155 inhibits the expression of endogenous TP53INP1. To investigate whether inhibition of miR-155 allows the reexpression of TP53INP1 and induces cell death, pancreatic

Capan2 cells were transfected with an oligonucleotide able to inhibit the miR-155 activity (anti-miR-155) or a control oligonucleotide. As expected, cells transfected with anti-miR-155 reexpressed TP53INP1 (Fig. 5*F*) and showed a significant increase in apoptosis (Fig. 5*G*). Altogether, these results demonstrate that TP53INP1 is a target of miR-155.

Discussion

The development of invasive PDAC involves the deregulation of numerous genes and the subsequent transformation of cells in noninvasive precursor lesions into cancer cells (1). However, in this cancer, the morphological modifications that occur during tumor progression have poor diagnostic value (20, 21) and finding markers that could help identifying high-risk lesions would be extremely useful. Here we show that TP53INP1 is present in nonmalignant human pancreatic lesions, is significantly or completely lost in the majority of primary PDAC and is absent in metastasis. The fact that TP53INP1 is lost or, at least, strongly reduced in a large proportion of tumors and that such reduction correlates with tumor progression suggests that TP53INP1 level reduction might be an indicator of pancreatic malignancy. We propose to include TP53INP1 in the list of PDAC markers known to be involved in pancreatic adenocarcinoma evolution (*K-ras*, *p53*, *DPC*, *PSCA*, *epithelial apomucins*, *Cyclin D1*, and *PMP22*) (22–24). To date, distinguishing before surgery lesions that could degenerate into invasive malignancy from benign lesions remains a problem to the clinician (25). TP53INP1 departs from previous markers because its expression is almost ''all or none'' between benign and precancerous pancreatic lesions, which makes TP53INP1 immunohistochemistry a previously undescribed tool to distinguish low risk from high-risk pancreatic affections. Assessing TP53INP1 presence before surgery by fine-needle aspiration (26) or brush cytology (27) should allow differential diagnosis of benign features (strong TP53INP1 level) from malignant transformation at an early stage (absence or low level of TP53INP1).

On a functional standpoint, the possibility that TP53INP1 repression contributes to PDAC formation is extremely interesting. Significant reduction or loss of TP53INP1 expression was also detected during the progression of adenocarcinoma of the stomach (12), colon (unpublished results) and in pancreatic and intestinal endocrine tumors (S.G., unpublished data). Also, we recently showed that mice deficient for *TP53INP1* presented with exacerbated colitis-associated carcinogenesis (28). These observations suggest that, besides pancreas, loss of TP53INP1 expression might be a general feature of carcinoma development which, as such, deserves being investigated further in other epithelial tumors.

In this work we used a model of PonA-dependent conditional TP53INP1 expression to show that a pancreatic cell line (MiaPaCa2), which do not express TP53INP1 and can form tumors in *nude* mice, loose that capacity if TP53INP1 is reexpressed (Figs. 2 and 3). That observation was extended to E1A/ras-MEFs which, upon injection into *nude* mice, also proliferate to form tumors. When MEFs were derived from TP53INP1$^{-/-}$ mice, tumors grew significantly faster than when they were obtained from wild type mice or when TP53INP1 had been reexpressed (Fig. 4). Obtaining similar results with two very different models of xenografted tumors strongly supports the idea that TP53INP1 expression possesses tumor suppressor properties.

Pancreatic cancer is an epigenetic and genetic disease. Studies on the mechanisms by which genes are inactivated during pancreatic cancer progression revealed that they involved mutations (e.g., *K-ras*, *p53*) or alterations of DNA methylation (e.g., *p16*, *cyclin D2*) (1). Our results suggest that, contrary to these genes, *TP53INP1* is neither mutated nor hypermethylated during pancreatic cancer. Surprisingly, TP53INP1 mRNA levels in tumors and in adjacent normal tissue were similar, whereas the protein, present in normal tissue, was undetectable in PDAC. This observation points toward a translational or posttranslational regulation of TP53INP1 expression.

Recently, miRNAs have been described as small, siRNA-like molecules, encoded in the genome and regulating gene expression by binding specific mRNAs and modulating their translation. Several reports have shown that the expression levels of some miRNAs are modified during tumor progression, suggesting links between miRNAs and cancer (29, 30). For this reason we investigated the possibility that miRNAs are involved in TP53INP1 loss. A computer search for miRNA targets in the TP53INP1 3′ UTR sequence revealed the presence of a region with significant complementarity with miR-155. That 3′ UTR region is extremely conserved among different species (Fig. 5) suggesting a functional role. A remarkable feature of miR-155 is its conservation during evolution, as shown by the high degree of similarity between the mouse, rat and human orthologs (Fig. 5). Therefore, miR-155 seems to be a functional miRNA and not an artifact of *in silico* genomics. This statement is supported by data reported above (Fig. 5) showing that miR-155 could indeed inhibit TP53INP1 expression.

Interestingly, miR-155 is up-regulated in different neoplasms such as Burkitt lymphoma (31), classical Hodgkin disease, primary mediastinal, diffuse large-cell lymphoma (32), B cell chronic lymphocytic leukemia (33) and in lung (34) and breast cancer (35). Costinean *et al*. (36) have recently shown in a transgenic mouse model that selective overexpression of miR-155 in B cells induces early B cell polyclonal proliferation followed by high-grade lymphoma-pre-B leukemia. This report shows that a miRNA can induce by itself a neoplastic disease and, consequently, be considered as an oncogene (18, 19). In addition, two studies have recently shown that miR-155 is overexpressed in PDAC. Szafranska *et al*. (17) monitored alterations of miRNA expression in pancreatic cancer and observed a 10-fold increase in miR-155 expression. Independently Lee *et al*. identified a miRNA signature in pancreatic cancer in which miR-155 is up-regulated 14 times in PDAC and is among the 20 miRNAs whose expression is altered the most between normal and cancer (16).

The fact that up-regulation of this miRNA is associated with lymphoma, leukemia and with several solid cancers suggests a broad oncogenic function. Data not shown might indicate that one or more of its targets could be genes linked to a process that is commonly lost in all cancers, irrespective of their origin. The mechanism by which miR-155 acts as an oncogene is unknown. An interesting suggestion is that it could knock down expression of anti-tumoral proteins (37). TP53INP1, by its anti-tumoral activity, could be one of these target genes. This hypothesis is very exciting because TP53INP1 is a previously undescribed miR-155 target with anti-tumoral activity.

Materials and Methods

Histological, Immunohistochemical, and Immunocytofluorescence Analyses. Anti-TP53INP1 monoclonal antibody (clone A25-E12) was used as primary antibody (6 μg/ml, overnight incubation) for immunostaining in paraffin-embedded sections from patient samples. Detection was done by using Rat ABC Staining system (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. Slides were then counterstained with hematoxylin (Vector Laboratories, Burlingame, CA) and mounted by using Eukitt solution. For negative control experiments, anti-TP53INP1 was either replaced by saline or preincubated with recombinant TP53INP1 protein (10 μg/ml). The proportion of positive cells was analyzed.

TP53INP1- and EGFP-inducible MiaPaCa2 cells were plated on glass coverslips. After a 16-h treatment with 10 μM PonA or vehicle, cells were fixed in PBS 4% formaldehyde, permeabilized in 0.2% Triton X-100, and incubated with a mouse monoclonal

anti-GFP antibody (1/800) (Roche, Indianapolis, IN; clones 7.1 and 13.1). The secondary antibody was anti-mouse FITC-conjugated IgG (1/1,000) (Santa Cruz Biotechnology). Finally, coverslips were mounted in ProLong Gold antifade reagent with DAPI (Molecular Probes, Leiden, The Netherlands).

Cell Culture and Treatment. MiaPaCa2, Capan2, 293T, MEFs, and MCF7 cells were maintained at 37°C and 5% CO_2 in DMEM Glutamax medium (Invitrogen, Groningen, The Netherlands) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells' gamma irradiation was performed at 30 Gy with an IBL 437 Irradiator from CIS BIO International.

TP53INP1$^{+/+}$ and TP53INP1$^{-/-}$ MEFs. *TP53INP1* deficient mice construction is described in ref. 28. TP53INP1$^{-/-}$ and TP53INP1$^{+/+}$ MEFs were obtained from embryos derived from homozygous breeding at 14.5 days postcoitum (E14.5) according to standard protocol (38), cultured in DMEM Glutamax medium containing 10% FCS, and used at early passages. TP53INP1 genotypes of cultured MEFs were determined by PCR (Fig. 4*A*). MEFs were transformed by transduction with the pBabe-E1A/ras^{V12} retroviral vector (details in *SI Materials and Methods*).

Measurement of Luciferase Activity. 293T cells were transfected with a mixture of 0.2 μg of Luciferase reporter plasmid construct, 0.2 μg of pEGFP plasmid, and the indicated concentration of miRNA. The miR-155 and miRNA Negative Control #1 (miR-control) were obtained from Ambion (Austin, TX). After 48 h, cells were washed and lysed with Reporter lysis buffer (Promega, Madison, WI), and firefly Luciferase activity was determined by using the Luciferase assay system (Promega) and a luminometer/fluorimeter (Xenius, SAFAS S.A, Monaco). The Luciferase reporter activity was normalized for transfection efficiency with the EGFP fluorescence.

Intrapancreatic Tumor Epifluorescence. TP53INP1-EGFP expression was verified in pancreas by EGFP epifluorescence with a Leica fluorescent MZFL3 binocular microscope (Leica, Wetzlar, Germany). Pancreatic tissue was excised, briefly washed in phosphate saline buffer, and directly observed.

Immunohistochemistry of Active Caspase-3 in Intrapancreatic Tumors. After deparaffinization and blocking of nonspecific binding, sections were incubated with an anti-human active-caspase-3 antibody (1:200; Promega). Immunoperoxidase procedure was performed by using a Vectastain goat anti-rabbit kit (Vector Laboratories). Active caspase-3 labeled surface was quantified with an Olympus BX61 automated microscope (×10 objective) by using Samba 2050 image analyzer (Samba Technologies, Meylan, France).

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