Acinar-to-Ductal Metaplasia Induced by Transforming Growth Factor Beta Facilitates KRAS\textsuperscript{G12D}-driven Pancreatic Tumorigenesis

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SUMMARY

Acinar-to-Ductal Metaplasia (ADM) is considered the main origin of pancreatic pre-neoplastic lesions that eventually develop into Pancreatic Ductal Adenocarcinoma (PDA). ADM could be a decisive step during tumorigenesis, selecting plastic cells for a more aggressive subsequent tumorigenesis. Our results indicate that TGF\(\beta\), a cytokine overexpressed during early and late pancreatic tumorigenesis, is an inducer of ADM and set up a favorable context for the emergence of oncogene-driven neoplastic lesions. This questions the usual dichotomist vision of TGF\(\beta\) in PDA.

BACKGROUND & AIMS: Transforming growth factor beta (TGF\(\beta\)) acts either as a tumor suppressor or as an oncogene, depending on the cellular context and time of activation. TGF\(\beta\) activates the canonical SMAD pathway through its interaction with the serine/threonine kinase type I and II heterotetrameric receptors. Previous studies investigating TGF\(\beta\)-mediated signaling in the pancreas relied either on loss-of-function approaches or on ligand overexpression, and its effects on acinar cells have so far remained elusive.

METHODS: We developed a transgenic mouse model allowing tamoxifen-inducible and Cre-mediated conditional activation of
a constitutively active type I TGFβ receptor (TβR1CA) in the pancreatic acinar compartment.

RESULTS: We observed that TβR1CA expression induced acinar-to-ductal metaplasia (ADM) reprogramming, eventually facilitating the onset of KRASG12D-induced pre-cancerous pancreatic intraepithelial neoplasia. This phenotype was characterized by the cellular activation of apoptosis and dedifferentiation, two hallmarks of ADM, whereas at the molecular level, we evidenced a modulation in the expression of transcription factors such as Hnf1β, Sox9, and Hes1.

CONCLUSIONS: We demonstrate that TGFβ pathway activation plays a crucial role in pancreatic tumor initiation through its capacity to induce ADM, providing a favorable environment for KRASG12D-driven carcinogenesis. Such findings are highly relevant for the development of early detection markers and of potentially novel treatments for pancreatic cancer patients.

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Materials and Methods

Mouse Strains and Handling

The LSL-TβRIFCA strain was previously generated in the laboratory of Dr Bartholin (TβRI CA is tagged with the hemagglutinin epitope at the C-terminus). We and others have functionally validated the TβRI FCA transgene after successful in vivo targeting in different subcellular compartments or organs such as immune cells 28,30-35 ovaries,36, and uterine tissue.37 In the present study, we only used TβRI FCA males to circumvent the mosaic phenotype occurring in females as a result of random X chromosome inactivation and associated inactivation of the transgene in a proportion of cells, as previously reported for other transgenes located on the X chromosome.38 LSL-KrasG12D 39 Pdx1-Cre,40 Ptf1a-CreERT2,5 and Rosa-CreERT2 41 alleles have been described previously. E2A-Cre 42 allele has been described previously; [E2A-Cre+/–] or [E2A-Cre+/-] mice were crossed with [LSL-TβRIFCA] (R) mice.

E19.5 embryos were removed from [LSL-TβRIFCA] females (R) previously impregnated by [Pdx1-Cre] males (C). To this end, pregnant females (R; n = 11) were euthanized at 19.5 days post coitum, and embryos were collected (n = 92).

Five-week-old mice bearing the Ptf1a-CreERT2 allele along with LSL-TβRIFCA and/or LSL-KrasG12D transgenes were injected with tamoxifen (Sigma-Aldrich #T5648; St Louis, MO; 3 mg per injection) to induce recombination of the LSL-TβRIFCA and LSL-KrasG12D alleles. Animals from the 3-day cohort received 2 injections (day 1 and day 3) and were killed at day 4. Animals from the 3-week, month, and 6-month cohorts received 5 injections (days 1, 3, 5, 7, and 9) and were killed 3 weeks, 2 months, and 6 months after the first injection, respectively. For Rosa26-CreERT2 mice, tamoxifen was injected 7-10 weeks after birth.

Mice were housed and bred in the AniCan specific pathogen-free animal facility of the Centre de Recherche en Cancérologie de Lyon, France. The experiments were performed in compliance with the animal welfare guidelines of the European Union and with French legislation (CECAPP protocol #CLB-2014-008).

Histology and Immunohistochemistry/Immunofluorescence

Histologic (H&E staining) and immunohistochemical experiments were performed as described previously.13,44 For immunohistochemistry experiments, the primary antibodies used were anti-CK19 (Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-INSULIN (Dako A0564; Glostrup, Denmark). The secondary antibodies used were rat immunoglobulin G (H+L) biotinylated (Vector #BA-9400; Vector Laboratories, Burlingame, CA) and guinea pig immunoglobulin G (H+L) biotinylated (Vector #BA-7000). For AMYLASE/CK19 double immunofluorescence, primary antibodies were anti-AMYLASE (Sigma-Aldrich A8273) and anti-CK19 (Developmental Studies Hybridoma Bank) and secondary antibodies used were Rabbit IgG (H+L) Alexa Fluor 647-conjugated (Life Technologies #A-21245 GAR647) and Rat IgG (H+L) Alexa Fluor 594-conjugated (Life Technologies #A-21209 DAR594). For AMYLASE/SOX9 double immunofluorescence, primary antibodies were anti-AMYLASE (Santa-Cruz #166349), anti-SOX9 (Millipore #AB5535) and secondary antibodies used were Rabbit IgG (H+L) Alexa Fluor 647-conjugated (Life Technologies #A-21245 GAR647) and Mouse IgG (H+L) Alexa Fluor 488-conjugated (Life Technologies #A-11001 GAM488), but artificial colors (red for AMYLASE and green for SOX9) were given with the Zeiss software to be consistent with AMYLASE/CK19 double staining. Nuclei were counterstained with DAPI, and images were acquired with a Zeiss Imager M2 AX10 (Carl Zeiss AG, Oberkochen, Germany).

Quantification of Pancreatic Lesions

Histologic scoring of pancreatic lesions was performed by using 1 representative H&E tissue slide per animal (3-8 animals per condition). Pancreatic lesions were scored from PanIN1 to PanIN3/PDA and were counted. The area of the analyzed tissue was determined by using ImageJ software (National Institutes of Health, Bethesda, MD), and lesion counts were normalized to this area.

RNAseq

TβRIFCA mRNA and Smad7 mRNA were detected in situ by using the RNAscope technology (Advanced Cell Diagnostics, Newark, CA) for the Hu-TβRI (catalog no. 431041) and Mm-Smad7 (catalog no. 429411) probes, respectively.

Cell Culture

The rat pancreatic acinar cell line AR42J (ATCC) was cultured in Dulbecco modified Eagle medium supplemented with fetal calf serum (Lonza Group, Basel, Switzerland) and penicillin/streptomycin (Gibco Laboratories, Gaithersburg, MD). AR42J cells were infected with murine retroviral particles containing a wild-type (pZip-Neo) vector or a K RasG12D -expressing vector (pZip-Neo-K RasG12D; donated by Dr J. Caramel, CRCL, Lyon, France) and further cultured in the presence of Geneticin (PAA Laboratories, Linz, Austria). TGFβ was used at the final concentration of 10 ng/mL.

For proliferation assays, AR42J-WT and AR42J-K RasG12D were seeded in triplicate at 100,000 cells per well onto 12-well plates and treated with TGFβ (10 ng/mL; PeproTech #100-21; Rocky Hill, NJ) for 24 and 48 hours. For each time point, cells were morphologically examined by phase-contrast microscopy and counted by using the trypan blue exclusion method. Kinase inhibitors were used as follows: 5 μmol/L TβRI inhibitor (SB 431542, Sigma-Aldrich #S3417), 10 μmol/L MEK inhibitor (U0126 monooethanolate, Sigma-Aldrich #U120), 2.5 μmol/L JNK inhibitor (Selleckchem #S4901; Houston, TX), 2.5 μmol/L PI3K inhibitor (LY294002, Selleckchem #S1105), and 2.5 μmol/L p38 inhibitor (SB 203580; Enzo Life Sciences, Farmingdale, NY; #BML-E1286-0001). Inhibitors
were added 1 hour before TGFβ treatment (10 ng/mL) and maintained during the 48 hours of TGFβ treatment.

**Immunoprecipitation**

For protein analysis, mouse pancreas tissue was lysed and homogenized in RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 0.5% sodium deoxycholate, 0.1% sodium dodecysulfate, and 1% Nonidet) containing a cocktail of protease and phosphatase inhibitors. After protein quantification, 40 μg protein was used for loading controls, and 2.5 mg protein was used for the immunoprecipitation assay by using the SMAD2/3 antibody (Cell Signaling Technology #3102; Danvers, MA) and protein G Agarose Fast Flow (EMD Millipore). Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and detected by Western blot analysis.

**Western Blot Analysis**

Western blotting was performed as previously described. The primary antibodies used were anti-phospho-SMAD2 (Cell Signaling #3101), anti-SMAD2 (Invitrogen #51-1300; Carlsbad, CA), anti-SMAD3/4 (Epitomics #1676-1; Burlingame, CA), anti-RAS (Santa Cruz Biotechnology), anti-phospho-SMAD2 (Cell Signaling #3101), anti-SMAD4 (Dako #P0448). Peroxidase-labeled anti-rabbit and anti-mouse secondary antibodies were purchased from Dako (mouse immunoglobulin G horseradish peroxidase-conjugated DAKO P0260, rabbit immunoglobulin G horseradish peroxidase-conjugated DAKO P0448).

**Polymerase Chain Reaction and Reverse Transcription Quantitative Polymerase Chain Reaction**

PCR to detect the recombinant T3RfCA allele was performed as described previously. AR42J-WT and AR42J-KRASG12D were treated for 48 hours with 10 ng/mL TGFβ (PreproTech #100-21) before being washed with phosphate-buffered saline (PBS). RNA extraction was performed by using the RNeasy mini kit (QIAGEN #74104; Hilden, Germany), according to the manufacturer’s instructions.

For pancreatic tissues, RNA extraction from frozen tissue was performed by using guanidine thiocyanate enriched lysis solution containing 5 mol/L guanidine thiocyanate (Sigma-Aldrich #G6639), 2.5 mmol/L sodium citrate, 0.5% N-lauryl sarcosine (Sigma-Aldrich #61739), and 1% β-mercaptoethanol. Lysates were centrifuged at 14,000 rpm at 4°C for 5 minutes to eliminate cell fragments. RNA was consecutively purified with the RNeasy mini kit (QIAGEN #74104), according to the manufacturer’s instructions.

After RT (ThermoFisher Scientific, Waltham, MA) by using random primers, qPCR was performed by using the MESA GREEN qPCR MasterMix Plus for SYBR Assay ROX (Eurogentec, Liège, Belgium; #RT-SY2X-06+WOU) and the following primers:

| Gene | Forward | Reverse |
|------|---------|---------|
| Ela1 | 5′-GGTGCTCTGATGAGACAGATG-3′ | 5′-CGCTATTCTGACTAAGCTTTG-3′ |
| Mist1 | 5′-CCGTTAAGCCGATGTTCC-3′ | 5′-CTCCGGACTGGAGATCCG-3′ |
| Cpa1 | 5′-ACCTTGTGGACACAGCATT-3′ | 5′-ACATCATTGGAGATCCG-3′ |
| Sox9 | 5′-GGTGTGAACAGAAGTTAAGG-3′ | 5′-GGTCACTATTGGACAGCAG-3′ |
| Hnf1β | 5′-ATCCAATGCTGCTGTTGC-3′ | 5′-GCCATACATGCGACAGCAG-3′ |
| Serpine-1 | 5′-ACATCCTGCTGATTCC-3′ | 5′-ATCACTTGGATCGCGG-3′ |
| TfRfCA | 5′-TGGTGAACAGAAGTTAAGG-3′ | 5′-AGCATATTCCTACAGC-3′ |
| Actb | 5′-GCGAGGAAGAGATGATT-3′ | 5′-ACGCACGTCGACAGTC-3′ |
| Bmf | 5′-AGATGTGATGATGATT-3′ | 5′-ATCTGGGCTGTTGTGG-3′ |

**RAS Activity Assay**

GTP-bound RAS (activated RAS) was measured in cell lysates incubated with beads coated with RAF1-RBD (Upstate; EMD Millipore). The experiment was performed by using the active RAS detection kit (Cell Signaling Technology #8821).

**Cell Death Assays**

For the caspase-3 activity assay, AR42J-WT and AR42J-KRASG12D cells were treated with 10 ng/mL TGFβ for 12 hours. Caspase-3 activity was determined by using the CASPASE-3/CPP32 Fluorimetric Assay Kit, according to the manufacturer’s instructions (Gentaur Biovision, Kampenhout, Belgium). For the annexin V assay, AR42J-WT and AR42J-KRASG12D cells were treated with 10 ng/mL TGFβ for 48 hours (PreproTech). Cells were harvested in PBS and incubated with annexin V-fluorescein isothiocyanate and propidium iodide (BD Pharmingen, San Diego, CA), according to the manufacturer’s instructions, and analyzed by flow cytometry (FACS Calibur cytometer; BD Biosciences). For the TUNEL cell death assay, AR42J-WT and AR42J-KRASG12D (24 hours of TGFβ treatment) cells or tissue sections were fixed in formaldehyde before being permeabilized with PBS-0.2% Triton X-100 and incubated in TdT buffer, 1 mmol/L CoCl2 (Sigma-Aldrich #15862-1 mL-F) for 5 minutes. Then they were incubated with TdT enzyme (Roche #11767305001; Basel, Switzerland) for 1 hour at 37°C. The reaction was
stopped by incubating the samples with 300 mmol/L NaCl, 2.5 mmol/L sodium citrate for 15 minutes. After washing with PBS, unspecific sites were saturated with 2% bovine serum albumin, 10 mmol/L PBS. After washing with PBS, slides were incubated with streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, PA) diluted 1/200 in 2% bovine serum albumin in PBS. Finally, the nuclei were counterstained with DAPI (Sigma-Aldrich), and images were acquired with a Zeiss Imager M2 AX10.

**Results**

**Transforming Growth Factor Beta-induced Cell Growth Inhibition In Vitro Is Enhanced in Acinar Cells Expressing KRAS^{G12D}**

We initially explored the combined effects of KRAS activation and TGFβ treatment on rat AR42J pancreatic acinar cells.\(^{45,46}\) infected either with a wild-type retroviral vector (AR42J-WT cells) or with a KRAS^{G12D} retroviral vector (AR42J-KRAS^{G12D} cells). We ascertained that KRAS^{G12D} was present and functional in AR42J-KRAS^{G12D} cells via Western blot analysis, which revealed a significant increase in the level of active GTP-bound RAS protein compared with AR42J-WT cells (Figure 1A). Examination of the cells by phase-contrast microscopy revealed that AR42J-KRAS^{G12D} cells treated with TGFβ grew to a lower cell density than the other cell populations (Figure 1B). This growth-inhibition effect was confirmed by counting cells, highlighting a clear decrease in the number of AR42J-KRAS^{G12D} cells treated with TGFβ (Figure 1C). Furthermore, this decrease was much stronger than that observed for AR42J-WT cells. Functionally, although these latter cells were poorly responsive to TGFβ with respect to their capacity to phosphorylate SMAD2 (P-SMAD2), AR42J-KRAS^{G12D} cells displayed a drastic increase in P-SMAD2 on TGFβ treatment, as evidenced by Western blot analysis (Figure 1D). As expected, the levels of SMAD2/3 and SMAD4 were affected neither by KRAS activation nor by TGFβ treatment. Taken together, these results demonstrate that the activation of KRAS potentiates TGFβ cell growth inhibition and SMAD pathway activation in rat pancreatic acinar cells.

**KRAS^{G12D} Sensitizes Acinar Cells to Transforming Growth Factor Beta–induced Apoptosis and Dedifferentiation In Vitro**

Next, we speculated that the inhibition of AR42J-KRAS^{G12D} pancreatic acinar cell growth in the presence of TGFβ was due to apoptosis.\(^{47}\) Microscopic examinations revealed that TGFβ-treated AR42J-KRAS^{G12D} cells were characterized by the presence of poorly refringent cells (Figure 1B, inset) and by an increase in the number of floating cells (data not shown), evoking the presence of apoptotic cells. The highest rates of apoptosis were observed in AR42J-KRAS^{G12D} cells treated with TGFβ, as demonstrated by caspase-3 activity (Figure 2A), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Figure 2B), and annexin-V (Figure 2C) assays. TGFβ-induced apoptosis mainly occurs via the mitochondrial pathway by downregulating antiapoptotic factors of the BCL-2 family (BCL-xl) and by positively regulating proapoptotic factors of the BCL-2 family.\(^{23}\) Proapoptotic Bmf (BCL2-modifying factor) was previously shown to be upregulated after TGFβ treatment in the normal murine mammary epithelial cell line NMuMG.\(^{48}\) Reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments performed on total RNA prepared from AR42J-WT and AR42J-KRAS^{G12D} cells showed an increase of Bmf mRNA expression after TGFβ treatment (Figure 2D). This activation is compromised in the presence of SB431542, an inhibitor of TβRI kinase activity. These observations are consistent with the increase in apoptosis observed by caspase-3, TUNEL, and annexin-V assays.

Phase-contrast micrographs revealed that TGFβ-treated AR42J-KRAS^{G12D} cells (and to a lesser extent AR42J-WT cells) acquired a spindle-like shape after TGFβ treatment (Figure 3A). RT-qPCR did not reveal significant changes in response to TGFβ in EMT markers such as Snai1, Zeb1, Vimentin, and Cdh2 in AR42J-WT and AR42J-KRAS^{G12D} cells (data not shown). In contrast, a decrease in the expression of acinar markers (Ela1, Gpa1, and Mist1) and a marked increase in the expression of ductal/dedifferentiation/progenitor markers (Hes1, Hnf1β, Sox9) were observed in AR42J-KRAS^{G12D} cells treated with TGFβ (Figure 3B). These morphologic and transcriptional changes are reminiscent of ADM. Among the genes we tested and known to be positively correlated to ADM, we focused our interest on Hnf1β because it was the best TGFβ responder in experiments presented in Figure 3B. TGFβ signaling is mediated by both the canonical SMAD pathway and non-canonical pathways (MAPK, PI3K, RH0-RAC).\(^{22,23}\) AR42J-WT cells were cultured in the presence of TGFβ along with kinase inhibitors of both the canonical and non-canonical TGFβ pathways. RT-qPCR revealed that Hnf1β activation depends on SMAD and MEK pathways but not on JNK, P38, or PI3K pathways (Figure 3C).

These results demonstrate that TGFβ treatment and KRAS activation cooperate to compromise AR42J rat acinar cell differentiation by increasing both apoptosis and ductal-like reprogramming, two hallmarks of ADM in vivo.

**Activation of Transforming Growth Factor Beta Signaling in the Mouse Embryonic Pancreas Severely Compromises the Development of the Acinar Compartment**

We then analyzed whether the activation of TGFβ signaling (alone or in combination with KRAS activation) could compromise the homeostasis of pancreatic acinar cells in vivo. To achieve this, we used a mouse strain ([LSL-TβRI^{CAG}) also called R] containing a constitutively active TGFβ type I receptor (TβRI), previously generated in the laboratory of Dr Bartholin by using a knock-in strategy.\(^{28,30}\) Transgene expression under the control of the ubiquitous CAG (human cytomegalovirus enhancer and chicken β-actin) promoter is repressed by a floxed transcriptional Stop (LSL, Lox-Stop-Lox), which can be excised in the presence of Cre recombinase. E2A-Cre allows recombination early during embryonic development before the E5 uterine wall implantation stage. We never
obtained \([E2A-Cre; LSL-T\betaRI^{CA}]\) mice (Figure 4), strongly suggesting that the expression of \(T\betaRI^{CA}\) at an early stage of development was embryonically or prenatally lethal. To restrict TGF\(\beta\) gain-of-function to the whole organism of mice postpartum, we generated \([\text{Rosa-Cre}^{ERT2}; LSL-T\betaRI^{CA}]\) mice that were subsequently treated with tamoxifen to induce ubiquitous Cre-mediated recombination, the Rosa-26 locus being active in all adult lineages (Figure 5A). All \([\text{Rosa-Cre}^{ERT2}; LSL-T\betaRI^{CA}]\) mice \((n = 4)\) injected with tamoxifen at the age of 7–10 weeks were euthanized 4 or 5 days after treatment because of the rapid onset of a highly detrimental phenotype, whereas all of the wild-type mice survived \((n = 5)\) (Figure 5B). Histologic examination of the internal organs revealed that the pancreas was the most impaired organ, with many apoptotic figures (Figure 5C), clearly indicating a deleterious effect of T\(\betaRI\) activation in the pancreas, which could explain the severe phenotype of these animals. To test this hypothesis, we expressed T\(\betaRI^{CA}\) in all of the pancreatic epithelial lineages by crossing \([LSL-T\betaRI^{CA}]\) (R) and \([\text{Pdx1-Cre}]\) (C) mice, which drives Cre recombinase expression in embryonic progenitors (at E8.5) of all pancreatic epithelial
lineages (Figure 5D). Histologic analysis revealed that [Pdx1-Cre; LSL-TβR1CA] embryos (CR) presented pancreatic defects characterized by a massive reduction in the number of mature acinar cells (hematoxylin-eosin staining) along with abnormally abundant ductal structures (shown by CK19 expression) (Figure 5E). No obvious defect in the endocrine compartment was observed (using INSULIN staining as a marker) (Figure 5E). These latter results show that pancreatic activation of TGFβ signaling at an early stage of development affects normal pancreatic development by impacting the acinar cell compartment.
To circumvent the afore-mentioned embryonic developmental defects, we investigated the effects of TβRICA expression starting after birth. To that end, we used the tamoxifen-inducible Pft1a-CreERT2 mouse allele (CER), which limits the expression of Cre recombinase to adult acinar cells (Figure 6A). Mice were injected with tamoxifen at 5 weeks of age. Excision of the floxed transcriptional Stop in the pancreas of CER(+TAM) mice was validated by PCR on genomic DNA (Figure 6B).

RT-qPCR experiments revealed a significant enrichment in TβRI mRNA in CER(+TAM) pancreata compared with R pancreata, which was correlated with upregulation of the TGFβ-target gene Serpine-1 (Figure 6C). Immunoprecipitation assays and Western blot analyses revealed a marked accumulation of P-SMAD2 in the pancreas of CER(+TAM) compared with wild-type mice (Figure 6D) (as expected the level of total SMAD2 in the lysates or in the immunoprecipitates did not differ between wild-type and CER(+TAM) mice). Next, we ascertained that the expression of the TβRI transgene was restricted to the pancreatic acinar compartment by performing...
RNAscope (Figure 6E). It is worth noting that 100% of the acinar cells expressed the transgene, and that transgene expression was not observed in other lineages (ducts and islets). In addition, the detection of Smad7 mRNA by RNAscope suggested that the canonical TGFβ-SMAD pathway was activated in vivo in the presence of TβRICA. These results clearly indicate that the conditional TβRICA transgene is restricted to pancreatic epithelial cells on tamoxifen treatment and is correlated with the activation of the SMAD pathway in vivo.

### Acinar-to-Ductal Metaplasia in Response to TβRICA Expression

Activating mutations of KRAS (eg, KRASG12D) are found in >90% of human PDAs and are sufficient to trigger carcinogenesis when targeted in mice.49 We generated mice expressing TβRICA (R) and KrasG12D (K) transgenes either alone or in combination under the control of Ptf1a-CreERT2 (CER), and we performed histologic analyses of pancreata collected at different time points after tamoxifen injection (3 days, 3 weeks, 2 months, and 6 months). As early as 3 days after tamoxifen treatment (Figure 7A), CERR(+TAM) and CERRKR(+TAM) pancreata presented a severe reduction in the acinar compartment associated with increased apoptosis (Figure 7B; H&E, black arrowheads; TUNEL). CERRK(+TAM) were normal and CERRKR(+TAM) undistinguishable from CERRR(+TAM) pancreata. Amylase is a marker of acinar cells, whereas CK19 and SOX9 are ductal markers. SOX9 is also an early positive regulator of ADM. Interestingly, we observed in CERRR(+TAM) and CERRKR(+TAM) pancreata displayed double-positive CK19+/amyrase+ or SOX9+/amyrase+ acinar cells (Figure 7B, immunofluorescence, white arrowheads), a feature of acinar cells undergoing early ADM reprogramming to form ductal-like structures.50 These “ducts” consisted of simple cuboidal epithelial cells and were characterized by the expression of stemness markers and ductal markers. These double-positive acinar cells (CK19+/amyrase+, SOX9+/amyrase+) most likely correspond to a regenerative process accompanying acinar cell loss. Three weeks after tamoxifen treatment (Figure 8), CERRK(+TAM) pancreata were still normal. However, compared with the analysis performed 3 days after tamoxifen injection, we observed a drastic loss in the acinar compartment (amyrase+), concomitantly with a large increase in the number of duct-like structures (CK19+), when TβRICA was expressed (CERRR(+TAM)), independently of oncogenic KRAS activation (eg, KRASG12D).

### Tumorigenesis Induced by KRASG12D Is Enhanced by TβRICA

Two and 6 months after tamoxifen treatment (Figure 9A and B), we observed in CERRR(+TAM) pancreata a massive loss of acinar tissue replaced by adipose tissue, a phenomenon known as acinar fatty infiltration (AFI). In CERRK(+TAM) pancreata, PanINs and ADM lesions were clearly distinguishable without clear evidence of AFI. Interestingly, CERRKR(+TAM) double mutant pancreata presented both AFI and PanINs. Precise quantitative and qualitative analysis of epithelial lesions (Figure 9C) revealed at 2 months a 3-fold increase in the number of PanIN1 in CERRR(+TAM) compared with CERRK(+TAM) and the absence of PanIN2 in CERRKR(+TAM), whereas these lesions were detectable in CERRR(+TAM). At 6 months the analysis showed the presence of PanIN3/PDA only in CERRR(+TAM) (absence in CERRK(+TAM)), with three-fourths of CERRKR(+TAM) mice developing PDA compared with 0 of 5 CERRK(+TAM) mice (Fisher’s exact test, P = .0476; data not shown).

In double mutants, a strong desmoplastic reaction associated with a drastic reduction of the acinar compartment was observed at 2 and 6 months. Immunohistochemistry analysis of pancreata at 2 months and 6 months after tamoxifen induction confirmed the ductal nature of the lesions and demonstrated that TβRICA expression in CERRR(+TAM) mice does not further affect endocrine islets compared with CERRK(+TAM), which we have previously shown to present disorganized islets architecture51 (Figure 10).

Our results demonstrate that in adult mice (1) activation of TGFβ signaling induces ADM by disrupting acinar cell homeostasis, leading to a drastic increase in the number of ductal structures at the expense of acinar structures; and (2) activation of TGFβ signaling in combination with oncogenic KRAS activation leads to the early onset of pre-neoplastic lesions that can naturally evolve toward high-grade/locally invasive lesions.

### Discussion

Tumorigenesis can be divided into 3 consecutive steps. The priming stage corresponds to biological processes (such as cellular stress, inflammation, dedifferentiation) that predispose normal cells to further transformation by creating a propitious cellular state or microenvironment. The initiation stage is represented by all genetic and epigenetic events sufficient for the acquisition of the transformed phenotype.
(such as KRAS activating mutations). The progression/metastatic stage is characterized by mechanisms conferring aggressiveness and invasive properties to cancer cells (such as inactivation of TP53 or SMAD4 tumor suppressors). Bioactive TGFβ, which is found in normal and pathologic pancreas tissues, plays a crucial role in both normal tissue homeostasis and pancreatic diseases. The TGFβ paradox in cancer consists in the observation that TGFβ behaves either as a tumor suppressor or as a tumor promoter depending on the cellular context, especially in the 3 above-described stages. The studies conducted so far to address the multifaceted role of TGFβ in pancreatic cancer in vivo mostly relied on loss-of-function approaches (receptor inactivation, SMAD inactivation) and ligand gain-of-function (effect is not restricted to cancer cells). These strategies did not provide a clear answer as to the precise role of TGFβ signaling in pancreatic epithelial cells. In the present study, we developed a conditional and inducible TGFβ gain-of-function mouse model expressing a constitutively active type 1 TGFβ receptor (TβRICA) in the pancreas. We demonstrated that cell-autonomous expression of TβRICA in the epithelial pancreatic compartment could severely compromise acinar cell homeostasis by inducing early ADM reprogramming. This phenotype was associated with the activation of both a proapoptotic program and a ductal-like differentiation program. Lately, the predominant role of necroptosis (a

Figure 5. Expression of TβRICA in mouse embryo compromises development of acinar compartment. (A) Breeding strategy to target TβRICA expression in whole adult body by using tamoxifen-inducible Rosa26-CreERT2 allele. (B) Overall survival (Kaplan-Meier analysis) of wild-type (WT) and [Rosa26-CreERT2; LSL-TβRICA] mice after tamoxifen injection. Log-rank (Mantel-Cox) test. **P = .0047. (C) Histology of pancreata prepared from WT and [Rosa26-CreERT2; LSL-TβRICA] mice 5 days after tamoxifen injection. White arrows, apoptotic cells. Scale bars, 200 μm. (D) Breeding strategy to target TβRICA expression in all epithelial pancreatic lineages from embryonic day 8.5 (E8.5) by using Pdx1-Cre allele. (E) Histology of pancreata prepared from WT and [Pdx1-Cre; LSL-TβRICA] (CR) E19.5 embryos. Scale bars, 200 μm. mag, magnification; TAM, tamoxifen.
programmed form of necrosis death) in acinar cell death was reported in severe experimental mouse pancreatitis.\textsuperscript{52}

We also demonstrated that in the presence of mutated KRAS\textsuperscript{G12D}, TGF\textbeta\textsuperscript{-}induced ADM reprogramming facilitates the onset of PanINs. This work represents a demonstration that the activation of cell-autonomous TGF\textbeta\textsuperscript{-}signaling compromises pancreatic acinar cell identity and eventually potentiates KRAS\textsuperscript{G12D}-driven tumor initiation.

**Cell-autonomous Transforming Growth Factor Beta Activation Induces Acinar-to-Ductal Metaplasia Reprogramming**

Three days after T\textbeta\textsuperscript{RIFCA} induction in adult mouse pancreatic acinar cells, we observed a drastic reduction in acinar tissue as attested by decreased amylase expression in the pancreata of mice expressing the transgene. This observation corroborates previous studies, which reported a repressive role for TGF\textbeta\textsuperscript{-} on the fate of acinar cells. Indeed, it was demonstrated that TGF\textbeta\textsuperscript{-} could inhibit the formation of acinar tissue \textit{ex vivo} in cultures of pancreatic embryonic buds.\textsuperscript{53} In \textit{vivo}, by using a transgenic mouse model expressing a type II TGF\textbeta\textsuperscript{-}dominant-negative mutant receptor under the regulation of the metallothionein 1 (Mt1) promoter, TGF\textbeta\textsuperscript{-} was shown to be essential for the maintenance of the acinar compartment homeostasis.\textsuperscript{54} In the present study, we observed that targeted activation of TGF\textbeta\textsuperscript{-} signaling in acinar cells \textit{in vivo} efficiently compromised acinar identity. At the microscopic level, we observed that the loss of acinar tissue was associated with massive
**Figure 7.** TβRI	extsuperscript{CA} expression in acinar cells induces apoptosis and ductal-like differentiation 3 days after induction. (A) Diagram of experimental design for 5-week-old mice injected with tamoxifen and euthanized 3 days later. (B) H&E staining, TUNEL assay, and immunofluorescence of amylase, CK19, and SOX9. Black arrowheads, apoptotic cells; white arrowheads, CK19/amylase and SOX9/amylase double-positive cells; WT, wild-type; [Ptf1a-Cre	extsuperscript{ERT2}; LSL-TβRI	extsuperscript{CA}], CERR; [Ptf1a-Cre	extsuperscript{ERT2}; LSL-KrasG12D], CERK; [Ptf1a-Cre	extsuperscript{ERT2}; LSL-TβRI	extsuperscript{CA}; LSL-KrasG12D], CERKR. TAM, tamoxifen. Scale bars, 50 μm.
apoptosis and with the appearance of new highly abundant ductal structures of small diameter. This phenotype is reminiscent of ADM, a process involved in pancreas replenishment after tissue injury.\textsuperscript{1,6,11} During this process and as observed in the model presented here, the acinar cells are reprogrammed into a ductal-like cell population, displaying features of progenitor cells, with the ability to regenerate the different lineages in the injured pancreas. Recently, Liu et al.\textsuperscript{55} have shown that TGF$\beta$ could convert primary human acinar cells to ductal-like cells \textit{in vitro}, which corroborates our current \textit{in vivo} demonstration that TGF$\beta$ induces ADM. Although the signals triggering ADM

\textbf{Figure 8.} $T_{\beta}RI^{CA}$ expression in acinar cells leads to regenerative ADM 3 weeks after induction. (A) Diagram of experimental design for 5-week-old mice injected with tamoxifen and euthanized 3 weeks later. (B) H&E staining and immunofluorescence of amylase and CK19. WT, wild-type; [Ptf1a-Cre\textsuperscript{ERT2}; LSL-Kras\textsuperscript{G12D}; LSL-T$\beta$R\textsuperscript{CA}], C\textsuperscript{ERR}; [Ptf1a-Cre\textsuperscript{ERT2}; LSL-Kras\textsuperscript{G12D}], C\textsuperscript{ERK}; [Ptf1a-Cre\textsuperscript{ERT2}; LSL-T$\beta$R\textsuperscript{CA}; LSL-Kras\textsuperscript{G12D}], C\textsuperscript{ERKR}. TAM, tamoxifen. Scale bars, 100 \textmu m.
A

\[ Ptf1a-Cre^{ERT2} \times LSL-Kras^{G12D}; LSL-T\beta R1^CA \]

B

|               | WT (TAM) | C_{ERR} (TAM) | C_{ERK} (TAM) | C_{ERK/R} (TAM) |
|---------------|----------|---------------|---------------|-----------------|
| 2 months POST TAM | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| 6 months POST TAM | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |

C

|               | PanIN1 | PanIN2 | PanIN2 | PanIN3/PDA |
|---------------|--------|--------|--------|------------|
| 2 months POST TAM | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| 6 months POST TAM | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |
in vivo are poorly known, we demonstrate herein that TGFβ signaling activation is one of these signals.

Acinar-to-Ductal Metaplasia Reprogramming Induced by Transforming Growth Factor Beta Activation Facilitates KRASG12D-mediated Carcinogenesis

ADM has largely been documented to represent the first step in pancreatic tumor development by providing a suitable surrounding for the malignant transformation of cells in response to oncogene-driven mutations such as KRASG12D. Here we report a significant increase in both the number and the grade of PanIN lesions in the pancreas of mice expressing both TβRI CA and KrasG12D transgenes in comparison with age-matched KrasG12D mice. PanINs were never observed in the presence of TβRI CA alone. This observation supports the idea that KRASG12D generates a persistent ADM (known as for acinar-to-ductal reprogramming), facilitating the subsequent onset of PanINs. Active TGFβ thus appears to be crucial to potentiate KRASG12D transforming properties through its capacity to confer a ductal-like phenotype to acinar cells that possess progenitor properties and that are more sensitive to transformation. It is worth underlining that the phenotype we observed in the pancreas of TβRI CA mice resembles that of pancreatitis, a pathology predisposing to PDA.57 In aging mice we observed that ADM was resolved by AFI, which represents the normal evolution of chronic pancreatitis when the inflammatory stress ceases. Pancreatitis characterized by ADM has also been reported to be tightly dependent on TGFβ activation. Indeed, it was shown by others that inhibition of TGFβ in different mouse models, by using a dominant-negative type II TGFβ receptor,58-60 by overexpressing the inhibitory SMAD7,61 or by using halofuginone to inhibit TGFβ-induced collagen deposition,62 could compromise cerulein-induced pancreatitis. Pancreatitis has also been shown to be reversible in the absence of activated KRAS and irreversible in the presence of activated KRAS,63 demonstrating that KRASG12D is able to harness the pancreatitis phenotype to facilitate the development of PanINs. The presence of activated KRAS would then prevent spontaneous resorption of the phenotype, driving cells toward persistent ADM and PanINs. Our findings, together with these previous studies, demonstrate that TGFβ activation in the pancreas induces ADM, providing a propitious environment, with features reminiscent of pancreatitis, for the onset of KRASG12D-induced PanINs. Hence, cell-autonomous activation of TGFβ

Figure 9. (See previous page). TβRI CA expression in acinar cells accelerates KRASG12D-induced tumorigenesis several months after induction. (A) Diagram of experimental design representing 5-week-old mice injected with tamoxifen and euthanized 2 or 6 months later. (B) H&E staining. WT, wild-type; [Ptf1a-CreERT2; LSL-TβRI CA] (CER); [Ptf1a-CreERT2; LSL-KrasG12D] (CERK); [Ptf1a-CreERT2; LSL-TβRI CA; LSL-KrasG12D] (CERKR). Scale bars, 100 μm. (C) Quantification of pancreatic epithelial lesions at different grades and observed in CERK and CERKR pancreata 2 months and 6 months after TAM injection. TAM, tamoxifen.

Figure 10. KRASG12D and TGFβ activation cooperate to accelerate pancreatic tumorigenesis without affecting endocrine compartment. Immunohistochemical detection of CK19 and INSULIN in mice of indicated genotypes 2 and 6 months after tamoxifen induction. [Ptf1a-CreERT2; LSL-TβRI CA] (CER); [Ptf1a-CreERT2; LSL-KrasG12D] (CERK); [Ptf1a-CreERT2; LSL-TβRI CA; LSL-KrasG12D] (CERKR). Scale bars, 200 μm.
signaling in pancreatic acinar cells may represent a crucial step in pancreatitis, which is also known to predispose to PDA, thus providing a physiopathologic relevance for the results described in the present study.

**Simultaneous Transforming Growth Factor Beta Signaling and KRAS Activation Cooperate to Induce Apoptosis and Ductal Reprogramming of Acinar Cells**

We demonstrated *in vitro* and *in vivo* that the simultaneous activation of TGFβ and KRAS could cooperate to induce apoptosis and ductal reprogramming of acinar cells, two cellular events classically observed in pancreatic ADM. Importantly, in the tamoxifen-inducible model, 100% of pancreatic epithelial cells expressed the TβRII transgene *in vivo*, thus indicating that the deleterious phenotype observed in the presence of the KRASG12D transgene resulted from a cell-autonomous functional interaction. Although our observations indicate that TGFβ activation induces direct ADM reprogramming, the mechanisms dictating cell fate, *i.e.*, apoptotic death versus survival associated with the acquisition of an ADM phenotype, remain to be uncovered. Indeed, the mechanisms that allow a proportion of acinar cells to escape apoptosis and pursue their route toward ADM remain to be identified. As suggested by a recent study, the fate of pancreatic cancer cells is tightly controlled by factors downstream of TGFβ (SNAIL and SOX4). David et al. showed that TGFβ-induced EMT resulted in apoptosis activation and tumor suppressive activity. Hence, in the transformed pancreas, TGFβ-induced apoptosis reduces the number of cancer cells, thus substantiating its tumor suppressive effect at this stage, whereas in the normal pancreas (the present work), TGFβ induced apoptosis and promotes ADM, thereby proving its oncogenic effect. Whether this model proposed by David et al can be transposed to untransformed cells to study tumor initiation needs to be tested.

On the basis of the use of AR42J rat acinar cells stably expressing KRASG12D and treated with TGFβ, we were able to show that KRAS activation sensitized acinar cells to both TGFβ-induced apoptosis and dedifferentiation. At the molecular level, we evidenced in this model a cooperative effect of both pathways to modulate the expression of Hnf1β, Sox9, and Hes1. The combination of these 3 transcription factors is a hallmark of the formation of progenitor cells during development, which is in accordance with the well-documented role of TGFβ as a promoter of stemness. Indeed, SOX9 and HNF1β are the first transcription factors that commit endoderm cells to pancreatic progenitors during organogenesis. They are markers of the ductal tree, re-expressed during ADM. Moreover, the adult SOX9 has been shown to be a master positive regulator of the ADM process. Indeed, the ectopic expression of SOX9 in acinar cells activates the expression of ductal markers and increases KRASG12D-induced ADM, and conversely, inactivation of SOX9 in KRASG12D-expressing adult acinar cells compromises the onset of PanINs, confirming its critical role in the transition toward a ductal phenotype. In the present study, we reported that the *in vivo* co-expression of TβRII and KRASG12D was able to induce the onset of amylase/ SOX9 double-positive cells. This feature is known to be associated with the regenerative process after acinar cell loss, stemming from double-positive cells for acinar and ductal markers. The precise role of SOX9 in this model remains to be explored and whether Hes1 and Hnf1β are direct transcriptional targets of SMAD proteins. How KRASG12D potentiates their activation is currently under investigation in our laboratory.

**Physiopathologic Relevance and Therapeutic Implications**

Our observation that TGFβ behaves as a tumor promoter may seem paradoxical in light of other studies showing that inactivation of TGFβ signaling by inactivating Smad4 or TβRII accelerates the progression of KRASG12D-induced lesions. This is most likely a consequence of both the gain-of-function approach we used and the stage at which TGFβ signaling was impaired. Indeed, in our model through its capacity to induce ADM, activation of TGFβ signaling resulted in an increased KRASG12D-induced tumor initiation. This can be explained by the effect of TGFβ on the priming stage of tumor progression by committing acinar cells to an ADM differentiation program favorable to transformation. In contrast, TβRII or Smad4 homozygous deletions facilitate the progression of KRASG12D-induced preexisting PanINs. This observation is consistent with the loss of SMAD4 at late stages and widespread metastatic human diseases. Furthermore, we recently demonstrated that the activation of TGFβ signaling was sufficient to induce the onset of ovarian tumors, strongly supporting our current findings that this signaling pathway plays an active role in promoting tumor initiation. Overall, this work sheds new light on another level of complexity for the dual role of TGFβ as a tumor promoter and suppressor.

TGFβ or components of its signaling pathway are being extensively evaluated as a potential therapeutic target, as attested by the compelling preclinical and encouraging clinical studies. As a consequence of its dual role in cancer, deciphering TGFβ-related functional processes is a prerequisite to develop efficient anti-TGFβ therapies. Understanding the first steps of pancreatic tumorigenesis is necessary to determine how pancreatic tumor cells acquire plasticity. Such an effort may provide new therapeutic strategies aimed at restoring a normal differentiated state. An example of a successful differentiation therapy was observed in acute promyelocytic leukemia, characterized by the accumulation of incompletely differentiated leukemic cells, which could be forced to fully differentiate on treatment with all-trans retinoic acid. More recently, Fitamant et al. provided proof-of-concept for differentiation therapy in solid tumors by deleting Yap1 in hepatocellular carcinoma, which present features of progenitor cells. In this context, unveiling the specific effects of TGFβ at different stages of tumorigenesis may lead to innovative therapeutic strategies aimed at restoring acinar differentiation and, therefore, at decreasing the deleterious onset of cellular plasticity. Defining the molecular mechanisms
underlying the initiation of pancreatic cancer is highly relevant for the development of early detection markers and new therapies. Indeed, inhibition of TGFβ may represent a therapeutic strategy for impeding ductal reprogramming of acinar cells to prevent the initiation of PDA in high-risk patients (hereditary syndrome or chronic pancreatitis).

In conclusion, the present study demonstrates that TGFβ is an ADM inducer, facilitating the development of pancreatic neoplastic lesions in a KRASG12D-dependent context. According to the activated state of KRAS and TGFβ signaling, we propose an integrated and dynamic model for pancreatic cancer initiation; TGFβ activation is not sufficient to induce PanINs, and KRASG12D is poorly efficient at inducing ADM. However, when combined, TGFβ induces ADM and provides a propitious setting for KRASG12D-induced transformation (Figure 11). Defining the molecular mechanisms underlying the initiation of pancreatic cancer is highly relevant for the development of early detection markers and of potentially novel treatments.

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Author Contributions
Study concept and design were performed by NC, DFV, RMP, LBA, and LB. Acquisition of data was performed by NC, DFV, RMP, LBA, and LB. Analysis and interpretation of data were performed by NC, DFV, RMP, LBA, IT, VR, AC, OJS, SS, PD, and LB. Critical revision of the manuscript for important intellectual content was performed by NC, DFV, RMP, VR, AC, JM, Ju.G, OJS, UV, SS, PD, and LB. SS, LB and OJS obtained funding. Technical support was performed by SGL, AC, JV, NG, CC, JV, ES, and IG. LB supervised the study.

Conflicts of interest
The authors disclose no conflicts.

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