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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

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

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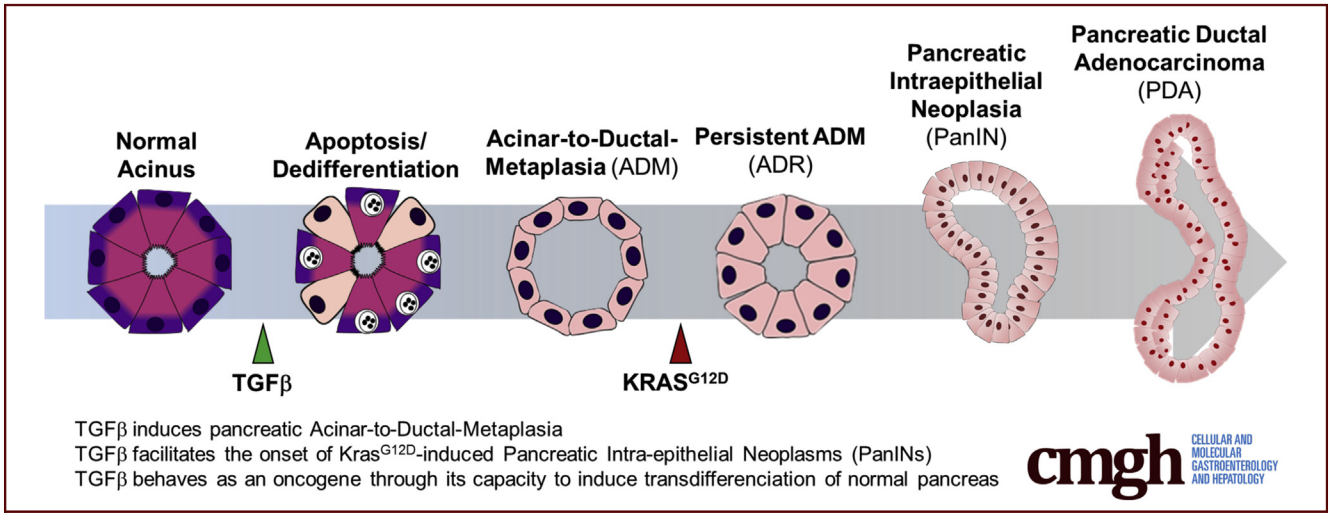

SUMMARY

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 TGFβ, 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 TGFβ in PDA.

BACKGROUND & AIMS: Transforming growth factor beta (TGFβ) acts either as a tumor suppressor or as an oncogene, depending on the cellular context and time of activation. TGFβ activates the canonical SMAD pathway through its interaction with the serine/threonine kinase type I and II heterotetrameric receptors. Previous studies investigating TGFβ-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 (TβRI^{CA}) in the pancreatic acinar compartment.

RESULTS: We observed that *TβRI^{CA}* expression induced acinar-to-ductal metaplasia (ADM) reprogramming, eventually facilitating the onset of KRAS^{G12D}-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 KRAS^{G12D}-dependent carcinogenesis. Such findings are highly relevant for the development of early detection markers and of potentially novel treatments for pancreatic cancer patients. *(Cell Mol Gastroenterol Hepatol 2017;4:263–282; http://dx.doi.org/10.1016/j.jcmgh.2017.05.005)*

Keywords: Pancreas; Cancer; TGFβ; Acinar-to-Ductal Metaplasia; KRAS^{G12D}.

In mammals, the pancreas is a bifunctional organ.[1] The exocrine pancreas, composed of acinar cells and ducts, accounts for more than 90% of the organ's mass. Acinar cells secrete digestive enzymes that are further collected in a network of ducts that discharge pancreatic juices into the duodenum. The endocrine tissue, represented by islets of Langerhans embedded in the exocrine pancreas, controls blood glucose levels by secreting hormones such as insulin and glucagon. Embryonically, all pancreatic lineages arise from a multipotent progenitor present at day E9 in the mouse endoderm.[2] The fate of the pancreas is dictated by the expression of progenitor markers such as SOX9 and CK19 for ductal cells, MIST1, PTF1A, CPA1, and ELA1 for acinar cells, and NGN3 for endocrine cells.[2–4] Note that PTF1A is required for the specification of the pancreatic multipotent progenitor cells and is later expressed only in the adult acinar compartment.[5]

The adult pancreas is highly plastic[6] to ensure organ integrity in response to internal (intracellular activation of digestive enzymes, obstruction of main ducts potentially as a result of gallstones) or external stresses (alcohol, trauma). Repair and regeneration of the injured organ are orchestrated by many cell types including acinar cells, centroacinar cells, ductal cells, immune cells, and stellate cells.[7,8] The existence of a resident stem cell population in the organ remains controversial.[9,10] Many studies[1,11,12] have focused on post-injury pancreatic regeneration and demonstrated the crucial role of acinar cells during this process. Indeed, under severe stress conditions such as pancreatitis, acinar cells undergo acinar-to-ductal metaplasia (ADM), a morphologic and transcriptional conversion into duct-like cells with embryonic progenitor cell properties.[5,7,13] These metaplastic cells are then capable of proliferating to replenish the damaged organ. In the case of a sustained stress signal or concomitant oncogenic activation such as KRAS activating mutations (eg, KRAS^{G12D}), the metaplastic cells cannot revert to a differentiated state, as generally observed in the case of an acute stress. This pancreatic erosion process constitutes a favorable setting for the onset of low-grade pancreatic intraepithelial neoplasia (PanIN).[14] Progression toward PanINs of higher grade and eventually pancreatic ductal adenocarcinoma (PDA) is associated with recurrent mutations or genetic/epigenetic alterations in tumor suppressor genes (*INK4/ARF*, *TP53*).[15,16] Importantly, *SMAD4/DPC4*, a core component of the transforming growth factor beta (TGFβ) signaling pathway, is deleted in 50% of PDAs.[16–18] Members of the TGFβ superfamily of transforming growth factors are involved in embryonic development, regulation of homeostasis, and the pathogenesis of a variety of diseases.[19,20] TGFβ signaling occurs through a heterotetrameric receptor complex composed of 2 subunits, the type I and type II TGFβ receptors (TβRI and TβRII, respectively). On binding to its receptors, TGFβ enables TβRII to transphosphorylate TβRI, which in turn activates the canonical SMAD pathway (phosphorylation of SMAD2 and SMAD3 that further interact with SMAD4 to accumulate inside the nucleus) and other signaling pathways (MAPK, RHOA, and PI3K/AKT).[21] In cancer, TGFβ behaves as either a tumor suppressor or a tumor promoter. Indeed, TGFβ is generally considered to be a tumor suppressor early in tumor development (tumor initiation) by restricting epithelial cell growth (through cytostasis and apoptosis).[19,22,23] However, in later stages of tumorigenesis (tumor progression), TGFβ has oncogenic properties through its capacity to regulate cellular plasticity by stimulating biological processes, including extracellular matrix deposition, immune evasion, epithelial-to-mesenchymal transition (EMT), and stemness.[22,24–27]

Unlike the majority of previously published studies in which the TGFβ signaling pathway was abrogated, our present work addresses the consequences of TGFβ cell-autonomous activation in the pancreatic epithelial lineage. To this end, we generated a unique mouse model enabling us to express a constitutively activated TβRI receptor (TβRI^{CA}) exclusively in pancreatic acinar cells, starting either in the embryo or after birth. Hence, we demonstrate that the specific activation of TGFβ signaling in pancreatic acinar cells induced ADM reprogramming and eventually facilitated the onset of KRAS^{G12D}-induced PanINs and PDA progression.

[a]Authors share co-first authorship.

Abbreviations used in this paper: **ADM, acinar-to-ductal metaplasia; AFI, acinar fatty infiltration; EMT, epithelial-to-mesenchymal transition; PanIN, pancreatic intraepithelial neoplasia; PBS, phosphate-buffered saline; PDA, pancreatic ductal adenocarcinoma; RT-qPCR, reverse transcription quantitative polymerase chain reaction; TGFβ, transforming growth factor beta; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.**

Most current article

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Materials and Methods

Mouse Strains and Handling

The *LSL-TβRI^{CA}* strain was previously generated in the laboratory of Dr Bartholin[28,29] (TβRI^{CA} is tagged with the hemagglutinin epitope at the C-terminus). We and others have functionally validated the *TβRI^{CA}* 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^{CA} 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-Kras^{G12D}*,[39] *Pdx1-Cre*,[40] *Ptf1a-Cre^{ERT2}*,[5] and *Rosa-Cre^{ERT2}*[41] alleles have been described previously. *E2A-Cre*[42] allele has been described previously; [*E2A-Cre$^{+/+}$*] or [*E2A-Cre$^{+/-}$*] mice were crossed with [*LSL-TβRI^{CA}*] (R) mice.

E19.5 embryos were removed from [*LSL-TβRI^{CA}*] 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-Cre^{ERT2}* allele along with *LSL-TβRI^{CA}* and/or *LSL-Kras^{G12D}* transgenes were injected with tamoxifen (Sigma-Aldrich #T5648; St Louis, MO; 3 mg per injection) to induce recombination of the *LSL-TβRI^{CA}* and *LSL-Kras^{G12D}* 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, 2-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-Cre^{ERT2}* 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 (CECCAPP protocol #CLB-2014-008).

Histology and Immunohistochemistry/Immunofluorescence

Histologic (H&E staining) and immunohistochemical experiments were performed as described previously.[43,44] 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.

RNAscope

TβRI^{CA} 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 KRAS^{G12D}-expressing vector (*pZIP-Neo-KRAS^{G12D}*; 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–KRAS^{G12D} 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 #S4317), 10 μmol/L MEK inhibitor (U0126 monoethanolate, 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-EI286-0001). 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 dodecylsulfate, 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.[29] The primary antibodies used were anti-phospho-SMAD2 (Cell Signaling #3101), anti-SMAD2 (Invitrogen #51-1300; Carlsbad, CA), anti-SMAD2/3 (Cell Signaling #3102), anti-SMAD4 (Epitomics #1676-1; Burlingame, CA), anti-RAS (Santa Cruz Biotechnology), anti-β-actin (Sigma-Aldrich #A5441 Clone AC15), and anti-β-tubulin (mouse monoclonal; Sigma-Aldrich). 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 recombined $T\beta RI^{CA}$ allele was performed as described previously.[28]

AR42J-WT and AR42J-KRAS^{G12D} 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:

Ela1:	Forward: 5′-TGGTCCTGTATGGACACAGT-3′
	Reverse: 5′-CCGTCATTCTGACTCAGGTT-3′
Mist1:	Forward: 5′- TGGCTAAAGCGACGTGTCC-3′
	Reverse: 5′- CTTCCGACTGGGGATCCGA-3′
Cpa1:	Forward: 5 ′- ACTTTGTGGGACACCAGGTT-3′
	Reverse: 5′- ACATCAATGGGGATACCGGC-3′
Sox9:	Forward: 5′- GTGCTGAAGGGCTACGACTGGA-3′
	Reverse: 5′- GTTGTGCAGATGCGGGTACTGG-3′
Hnf1β:	Forward: 5′- TCCCATCTGCAATGGTGGTC-3′
	Reverse: 5′- GCTGTGCACAAAGTGAGTGG-3′
Hes1:	Forward: 5′- GTCCCCGGTGGCTGCTAC-3′
	Reverse: 5′- AACACGCTCGGGTCTGTGCT-3′
Serpine-1:	Forward: 5′- CCCACGGAGATGGTTATAG -3′
	Reverse: 5′- ATCACTTGCCCATGAAGAG -3′
TβRI^{CA}:	Forward: 5′- TTGTGAACAGAAGTTAAGGC -3′
	Reverse: 5′- AGCATAATCAGGAACATCAT -3′
Actb:	Forward: 5′- GCAGGAGTACGATGAGTCCG-3′
	Reverse: 5′- ACGCAGCTCAGTAACAGTCC-3′
Bmf:	Forward: 5′- AGGTACAGATCGCCAGAAAGC-3′
	Reverse: 5′- CTCGGTTCTGCTGGTGTTGTTG-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-KRAS^{G12D} 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-KRAS^{G12D} cells were seeded in duplicate and treated with 10 ng/mL TGFβ for 48 hours (PeproTech). 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-KRAS^{G12D} (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 buffer (30 mmol/L Tris, 150 mmol/L sodium cacodylate, pH7.5), 1 mmol/L CoCl2, Biotin16-dUTP (Sigma-Aldrich #11093070910), 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 1*A*). 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 1*B*). 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 1*C*). 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 1*D*). 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 1*B*, *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 2*A*), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Figure 2*B*), and annexin-V (Figure 2*C*) 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 2*D*). 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 3*A*). 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*, *Cpa1,* 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 3*B*). 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 3*B*. TGFβ signaling is mediated by both the canonical SMAD pathway and non-canonical pathways (MAPK, PI3K, RHO-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 3*C*).

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^{CA}*] 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

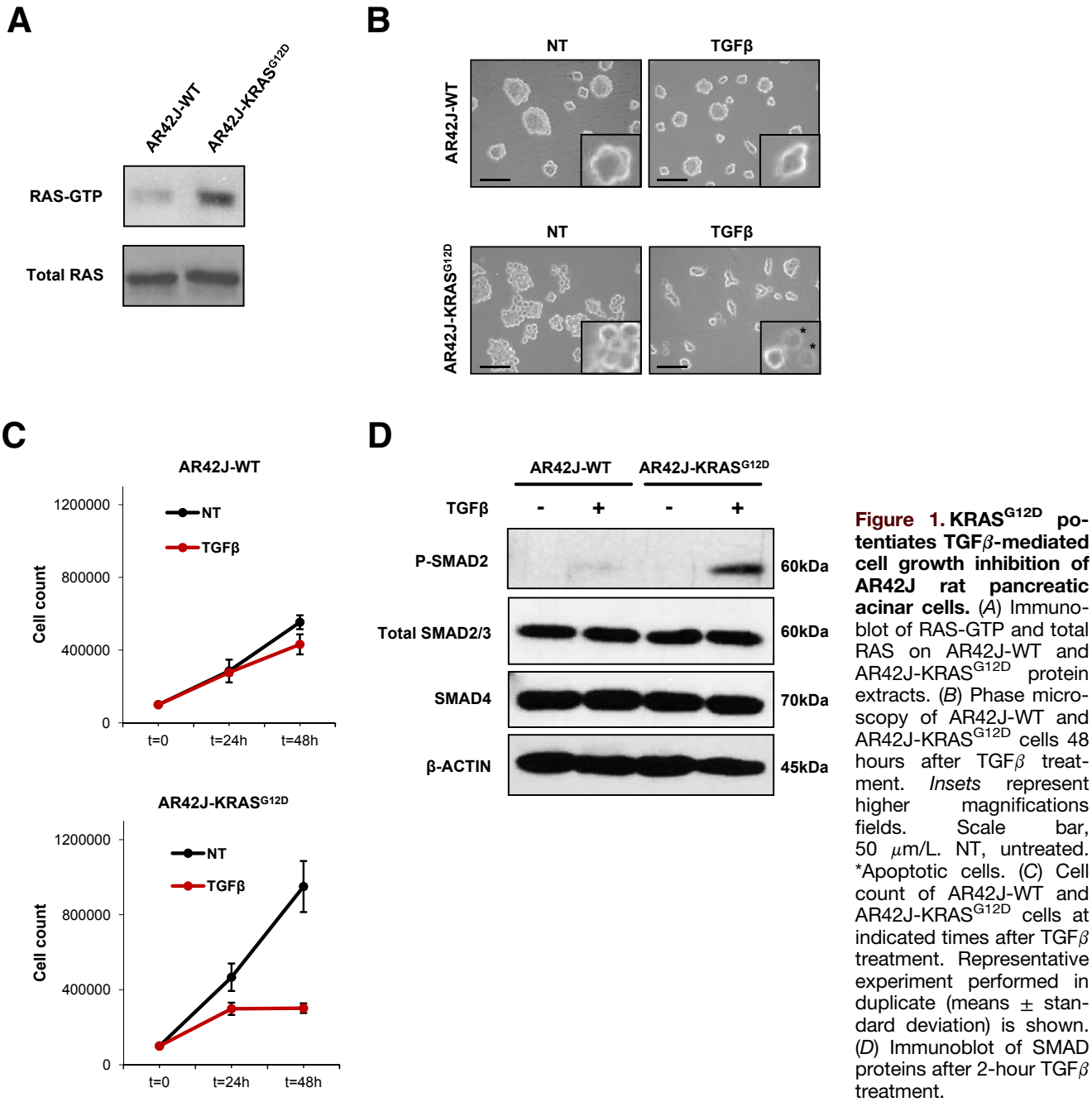


Figure 1. KRAS^{G12D} potentiates TGFβ-mediated cell growth inhibition of AR42J rat pancreatic acinar cells. (*A*) Immunoblot of RAS-GTP and total RAS on AR42J-WT and AR42J-KRAS^{G12D} protein extracts. (*B*) Phase microscopy of AR42J-WT and AR42J-KRAS^{G12D} cells 48 hours after TGFβ treatment. *Insets* represent higher magnifications fields. Scale bar, 50 μm/L. NT, untreated. *Apoptotic cells. (*C*) Cell count of AR42J-WT and AR42J-KRAS^{G12D} cells at indicated times after TGFβ treatment. Representative experiment performed in duplicate (means ± standard deviation) is shown. (*D*) Immunoblot of SMAD proteins after 2-hour TGFβ treatment.

obtained [*E2A-Cre*; *LSL-TβRI^{CA}*] mice (Figure 4), strongly suggesting that the expression of *TβRI^{CA}* at an early stage of development was embryonically or prenatally lethal. To restrict TGFβ gain-of-function to the whole organism of mice *postpartum*, we generated [*Rosa-Cre^{ERT2}*; *LSL-TβRI^{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 5*A*). All [*Rosa-Cre^{ERT2}*; *LSL-TβRI^{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 5*B*). Histologic examination of the internal organs revealed that the pancreas was the most impaired organ, with many apoptotic figures (Figure 5*C*), clearly indicating a deleterious effect of TβRI activation in the pancreas, which could explain the severe phenotype of these animals. To test this hypothesis, we expressed *TβRI^{CA}* in all of the pancreatic epithelial lineages by crossing [*LSL-TβRI^{CA}*] (R) and [*Pdx1-Cre*] (C) mice,[40] which drives Cre recombinase expression in embryonic progenitors (at E8.5) of all pancreatic epithelial

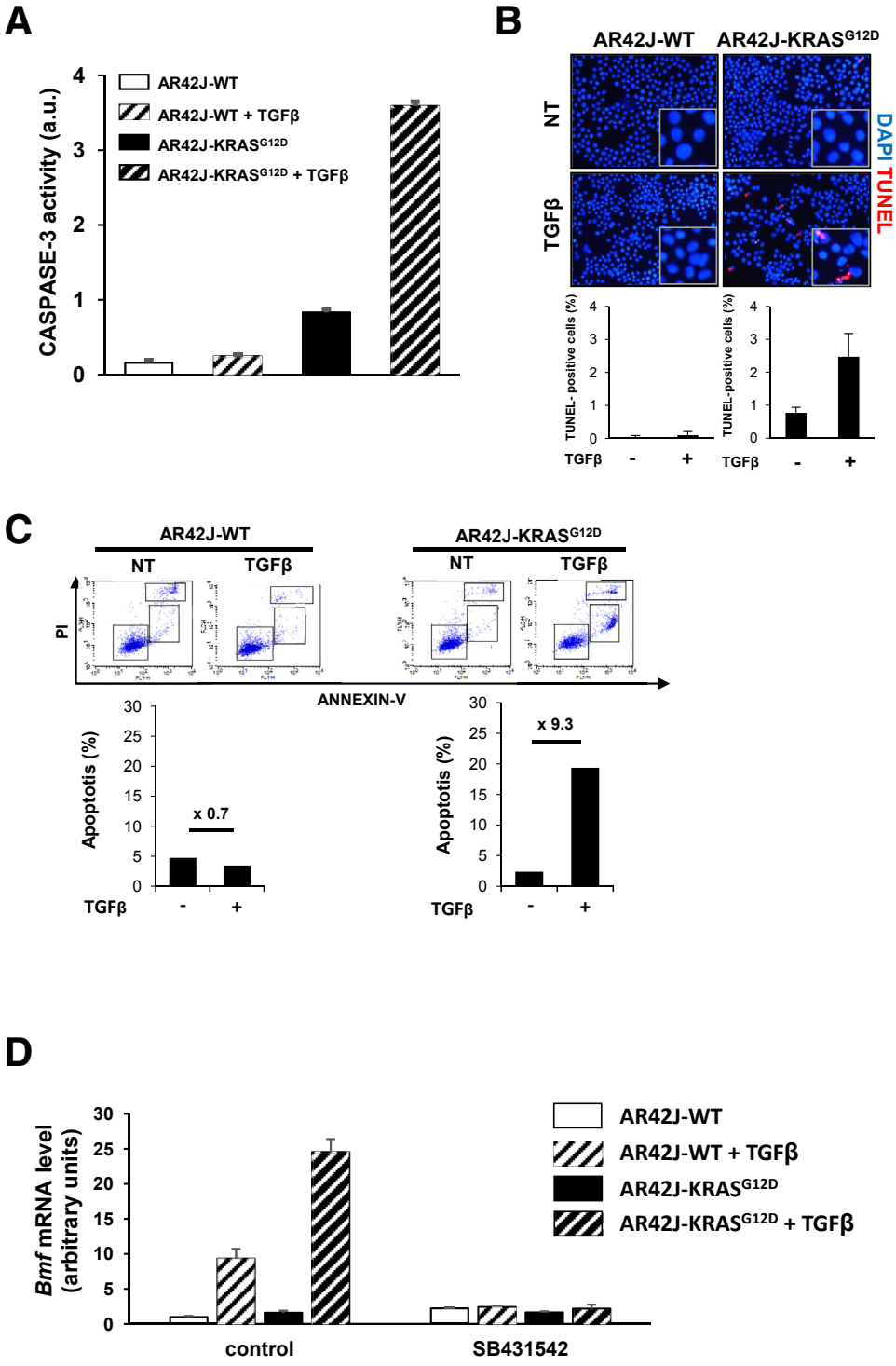


Figure 2. KRAS^{G12D} and TGFβ cooperate to activate apoptosis of AR42J rat acinar cells. (*A*) Caspase-3 assay after 12-hour TGFβ treatment. (*B*) TUNEL assay after 24-hour TGFβ treatment. *Upper panel*, fluorescence microscopy. *Lower panel*, graphs showing percentage of TUNEL-positive cells under fluorescence microscopy and counted in each condition (>1000 cells). (*C*) Annexin-V/propidium iodide (PI) assay after 48-hour TGFβ treatment. *Upper panel*, raw FACS data. *Upper right box* drawn on each plot corresponds to apoptotic cell population (cells positive for both annexin-V and PI). *Lower panel*, quantification of apoptotic cells in upper right boxes drawn on plots above. For (*A–C*), representative experiment performed in triplicate (means ± standard deviation) is shown. (*D*) RT-qPCR of proapoptotic *Bmf* (BCL2-modifying factor) marker. Cells were treated (or not) with TGFβ ± TβRI inhibitor (SB431542) for 48 hours. For each condition, mRNA level is represented as mean ± standard deviation of 1 representative experiment performed in triplicate.

lineages (Figure 5*D*). Histologic analysis revealed that [*Pdx1-Cre*; *LSL-TβRI^{CA}*] 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 5*E*). No obvious defect in the endocrine compartment was observed (using INSULIN staining as a marker) (Figure 5*E*). 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.

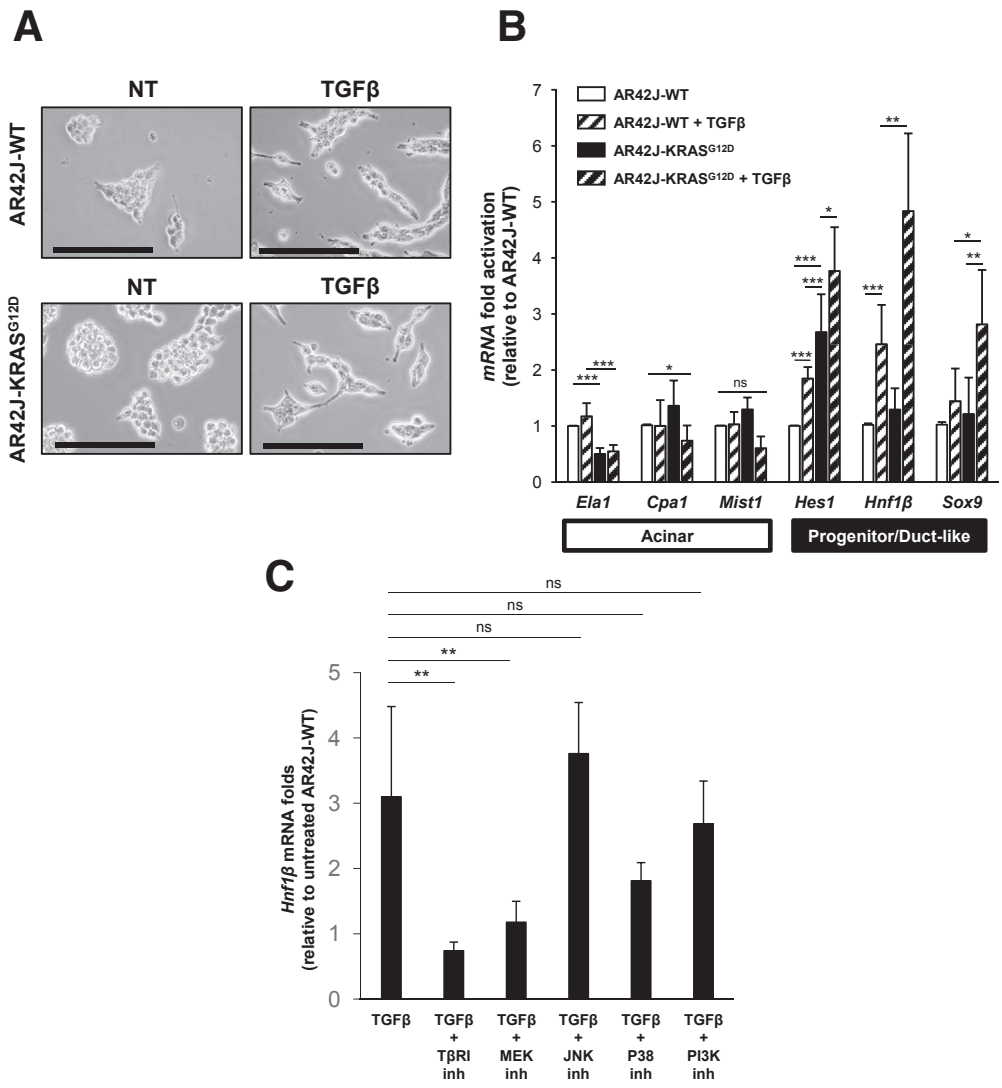


Figure 3. KRAS^{G12D} and TGFβ cooperate to activate dedifferentiation of AR42J rat acinar cells. (*A*) High-magnification microscopy of rat acinar cells (AR42J). Phase-contrast microscopy of AR42J-WT and AR42J-KRAS^{G12D} cells treated or not with TGFβ for 48 hours. NT, untreated. Scale bars, 100 μm. (*B*) RT-qPCR of acinar (*Ela-1*, *Cpa1*, *Mist1*), progenitor (*Hnf1*β, *Hes1*), and ductal (*Sox9*) markers after 48-hour TGFβ treatment. For each condition, expression is represented as mean $\pm$ standard deviation of at least 3 independent experiments. Statistical analyses were performed by using Mann-Whitney test: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Non-significant (ns) if $P > .05$. (*C*) Signaling pathways involved in TGFβ-induced *Hnf1*β mRNA activation. AR42J-WT cells were cultured in presence of TGFβ along with different kinase inhibitors (inh) of canonical and non-canonical TGFβ pathways: TβRI inh, SB431542; MEK inh, U0126 monoethanolate; JNK inh, JNK-IN-8; P38 inh, SB203580; PI3K inh, LY294002. *Hnf1*β mRNA was detected by RT-qPCR after 48-hour treatment. For each condition, folds are represented as mean $\pm$ standard deviation of at least 3 independent experiments. Statistical analyses were performed by using Mann-Whitney *U* test: $^{**}P < .01$. Nonsignificant (ns) if $P > .05$.

Inducible TβRI^{CA} Expression in Pancreatic Acinar Cells After Birth Results in SMAD Pathway Activation

To circumvent the afore-mentioned embryonic developmental defects, we investigated the effects of *TβRI^{CA}* expression starting after birth. To that end, we used the tamoxifen-inducible Pft1a-Cre^{ERT2} mouse allele (C^{ER}),[5] which limits the expression of Cre recombinase to adult acinar cells (Figure 6*A*). Mice were injected with tamoxifen at 5 weeks of age. Excision of the floxed transcriptional Stop in the pancreas of C^{ER}R(+TAM) mice was validated by PCR on genomic DNA (Figure 6*B*). RT-qPCR experiments revealed a significant enrichment in *TβRI^{CA}* mRNA in C^{ER}R(+TAM) pancreata compared with R pancreata, which was correlated with upregulation of the TGFβ-target gene *Serpine-1* (Figure 6*C*). Immunoprecipitation assays and Western blot analyses revealed a marked accumulation of P-SMAD2 in the pancreata of C^{ER}R(+TAM) compared with wild-type mice (Figure 6*D*) (as expected the level of total SMAD2 in the lysates or in the immunoprecipitates did not differ between wild-type and C^{ER}R(+TAM) mice). Next, we ascertained that the expression of the *TβRI^{CA}* transgene was restricted to the pancreatic acinar compartment by performing

	# litters	# pups	# pups/litter	Genotype			
				WT	[*E2a-Cre*]	[*LSL-TβRI^{CA}*]	[*E2a-Cre; LSL-TβRI^{CA}*]
[*E2a-Cre$^{+/+}$*] x [*LSL-TβRI^{CA}*]	4	18	4.5		18		0
[*E2a-Cre$^{+/-}$*] x [*LSL-TβRI^{CA}*]	5	29	5.8	10	7	12	0
			Fisher's exact test	ns	ns	ns	*

Figure 4. Early activation of TβRI^{CA} during mouse development is embryonically lethal. [*E2A-Cre$^{+/+}$*] or [*E2A-Cre$^{+/-}$*] mice were crossed with [*LSL-TβRI^{CA}*] (R) mice. Total number of litters, pups, and offspring genotype distribution are presented. Fisher's exact test was performed to statistically confirm absence of [*E2A-Cre$^{+/-}$; LSL-TβRI^{CA}*] mice. Nonsignificant (ns), $P > .05$; *$P < .05$.

RNAscope (Figure 6*E*). 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 *TβRI^{CA}*. These results clearly indicate that the conditional *TβRI^{CA}* 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 TβRI^{CA} Expression

Activating mutations of KRAS (eg, KRAS^{G12D}) are found in >90% of human PDAs and are sufficient to trigger carcinogenesis when targeted in mice.[49] We generated mice expressing *TβRI^{CA}* (R) and *Kras^{G12D}* (K) transgenes either alone or in combination under the control of Pft1a-Cre^{ERT2} (C^{ER}), 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 7*A*), C^{ER}R(+TAM) and C^{ER}KR(+TAM) pancreata presented a severe reduction in the acinar compartment associated with increased apoptosis (Figure 7*B*; H&E, *black arrowheads*; TUNEL). C^{ER}K(+TAM) were normal and C^{ER}KR(+TAM) undistinguishable from C^{ER}R(+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 that C^{ER}R(+TAM) and C^{ER}KR(+TAM) pancreata displayed double-positive CK19^{+}/amylase^{+} or SOX9^{+}/amylase^{+} acinar cells (Figure 7*B*, 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^{+}/amylase^{+}, SOX9^{+}/amylase^{+}) most likely correspond to a regenerative process accompanying acinar cell loss. Three weeks after tamoxifen treatment (Figure 8), C^{ER}K(+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 (amylase+), concomitantly with a large increase in the number of duct-like structures (CK19+), when TβRI^{CA} was expressed (C^{ER}R(+TAM)), independently of oncogenic KRAS activation (eg, KRAS^{G12D}).

Tumorigenesis Induced by KRAS^{G12D} Is Enhanced by TβRI^{CA}

Two and 6 months after tamoxifen treatment (Figure 9*A* and *B*), we observed in C^{ER}R(+TAM) pancreata a massive loss of acinar tissue replaced by adipose tissue, a phenomenon known as acinar fatty infiltration (AFI). In C^{ER}K(+TAM) pancreata, PanINs and ADM lesions were clearly distinguishable without clear evidence of AFI. Interestingly, C^{ER}KR(+TAM) double mutant pancreata presented both AFI and PanINs. Precise quantitative and qualitative analysis of epithelial lesions (Figure 9*C*) revealed at 2 months a 3-fold increase in the number of PanIN1 in C^{ER}KR(+TAM) compared with C^{ER}K(+TAM) and the absence of PanIN2 in C^{ER}K(+TAM), whereas these lesions were detectable in C^{ER}KR(+TAM). At 6 months the analysis showed the presence of PanIN3/PDA only in C^{ER}KR(+TAM) (absence in C^{ER}K(+TAM)), with three-fourths of C^{ER}KR(+TAM) mice developing PDA compared with 0 of 5 C^{ER}K(+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 *TβRI^{CA}* expression in C^{ER}R(+TAM) mice does not further affect endocrine islets compared with C^{ER}K(+TAM), which we have previously shown to present disorganized islets architecture[51] (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

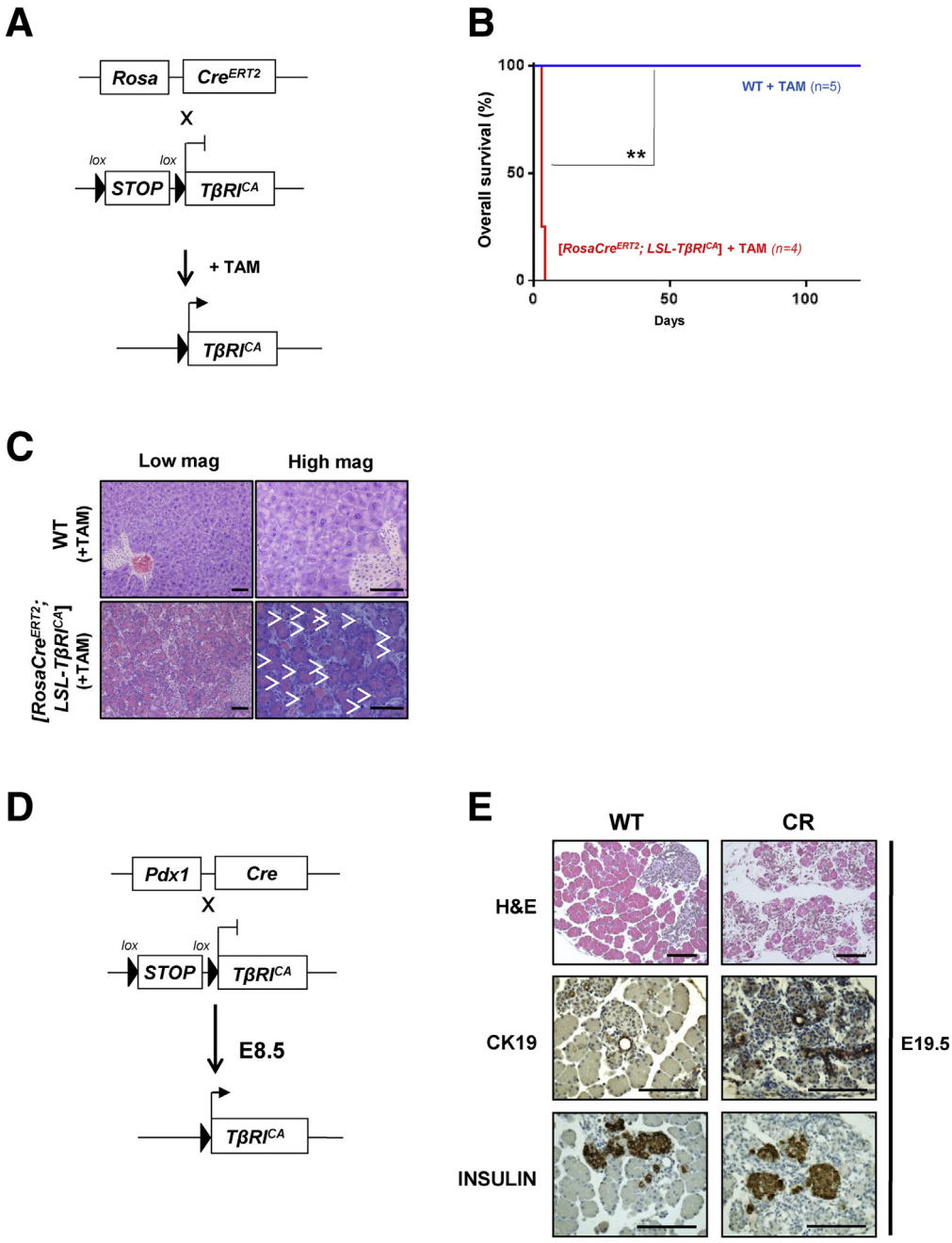


Figure 5. Expression of $T\beta RI^{CA}$ in mouse embryo compromises development of acinar compartment. (*A*) Breeding strategy to target $T\beta RI^{CA}$ expression in whole adult body by using tamoxifen-inducible *Rosa26-Cre*^{ERT2} allele. (*B*) Overall survival (Kaplan-Meier analysis) of wild-type (WT) and [*Rosa26-Cre*^{ERT2}*; LSL-*$T\beta RI^{CA}$] mice after tamoxifen injection. Log-rank (Mantel-Cox) test. $^{**}P = .0047$. (*C*) Histology of pancreata prepared from WT and [*Rosa26-Cre*^{ERT2}*; LSL-*$T\beta RI^{CA}$] mice 5 days after tamoxifen injection. *White arrows*, apoptotic cells. Scale bars, 200 μm. (*D*) Breeding strategy to target $T\beta RI^{CA}$ 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-$T\beta RI^{CA}$] (CR) E19.5 embryos. Scale bars, 200 μm. mag, magnification; TAM, tamoxifen.

(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 I TGFβ receptor ($T\beta RI^{CA}$) in the pancreas. We demonstrated that cell-autonomous expression of $T\beta RI^{CA}$ 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

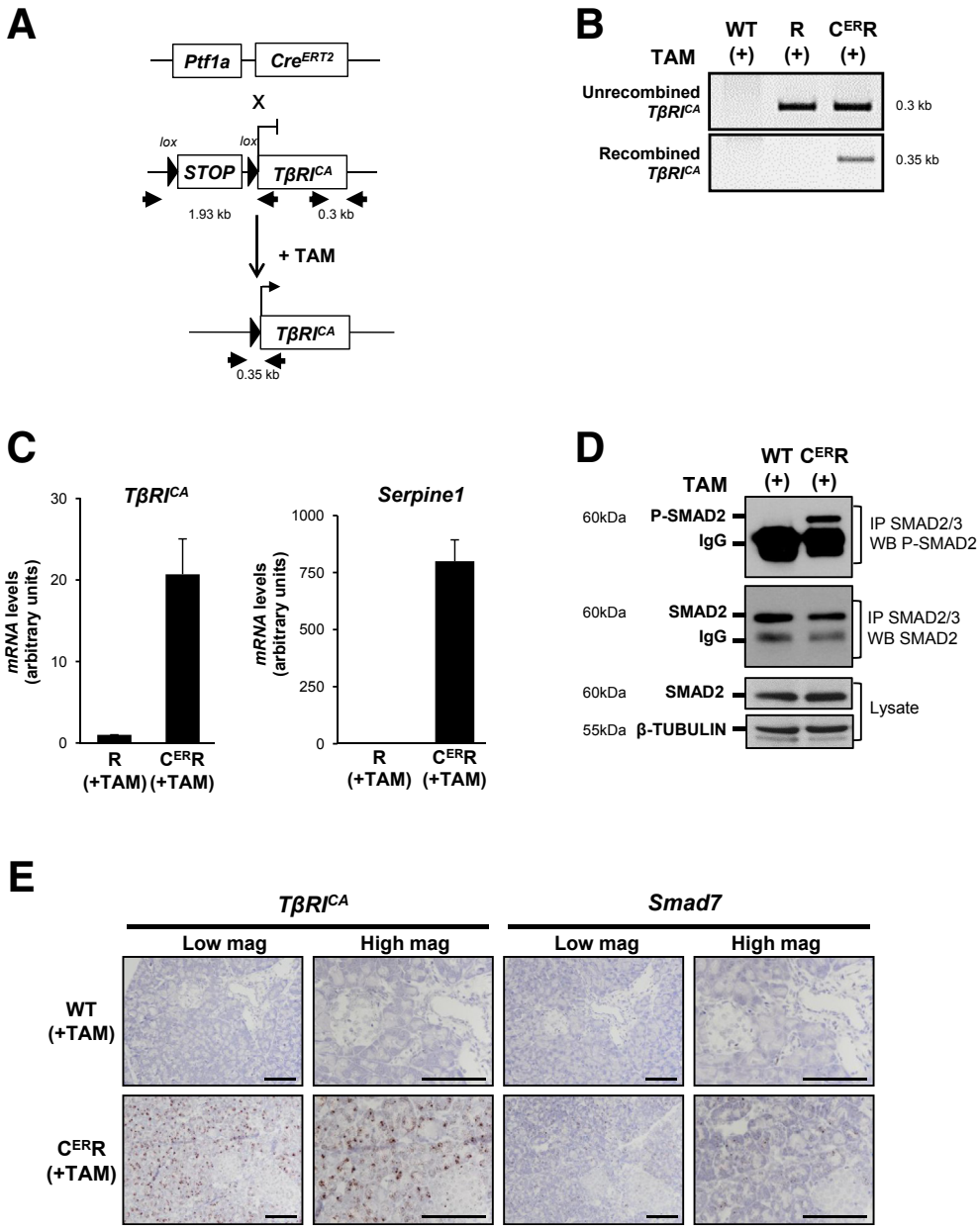


Figure 6. Inducible *TβRI*^{CA} expression in pancreatic acinar cells after birth results in SMAD pathway activation *in vivo*. (*A*) Breeding strategy to express *TβRI*^{CA} after birth in pancreatic acinar cells by using tamoxifen-inducible *Pft1a-Cre*^{ERT2} allele. *Black arrows* represent genotyping primers. Sizes of expected fragments are shown. (*B*) PCR on genomic DNA to assess excision of STOP signal in C^{ER}R pancreas after tamoxifen injection. (*C*) RT-qPCR of *TβRI*^{CA} and *Serpine-1* on total RNA from pancreata prepared from mice treated with tamoxifen. Expression level in R mice was arbitrarily set at 1 (mean ± standard deviation; 2 independent experiments). (*D*) Immunoprecipitation of SMAD2/3 and Western blot analysis of P-SMAD2 (phospho-SMAD2) and total SMAD2. Total lysate was assessed for β-tubulin and SMAD2. (*E*) RNAscope detection of *TβRI*^{CA} and *Smad7* mRNA on pancreatic sections from WT and C^{ER}R mice. Scale bars, 100 μm. (*A–E*): WT, wild-type; R, [*LSL-TβRI*^{CA}]; C^{ER}R, [*Pft1a-Cre*^{ERT2}; *LSL-TβRI*^{CA}]. mag, magnification; TAM, tamoxifen.

programmed form of necrosis death) in acinar cell death was reported in severe experimental mouse pancreatitis.[52] We also demonstrated that in the presence of mutated KRAS^{G12D}, TGFβ-induced ADM reprogramming facilitates the onset of PanINs. This work represents a demonstration that the activation of cell-autonomous TGFβ signaling compromises pancreatic acinar cell identity and eventually potentiates KRAS^{G12D}-driven tumor initiation.

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

Three days after TβRI^{CA} 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 TGFβ on the fate of acinar cells. Indeed, it was demonstrated that TGFβ could inhibit the formation of acinar tissue *ex vivo* in cultures of pancreatic embryonic buds.[53] *In vivo*, by using a transgenic mouse model expressing a type II TGFβ dominant-negative mutant receptor under the regulation of the metallothionein 1 (*Mt1*) promoter, TGFβ was shown to be essential for the maintenance of the acinar compartment homeostasis.[54] In the present study, we observed that targeted activation of TGFβ signaling in acinar cells *in vivo* efficiently compromised acinar identity. At the microscopic level, we observed that the loss of acinar tissue was associated with massive

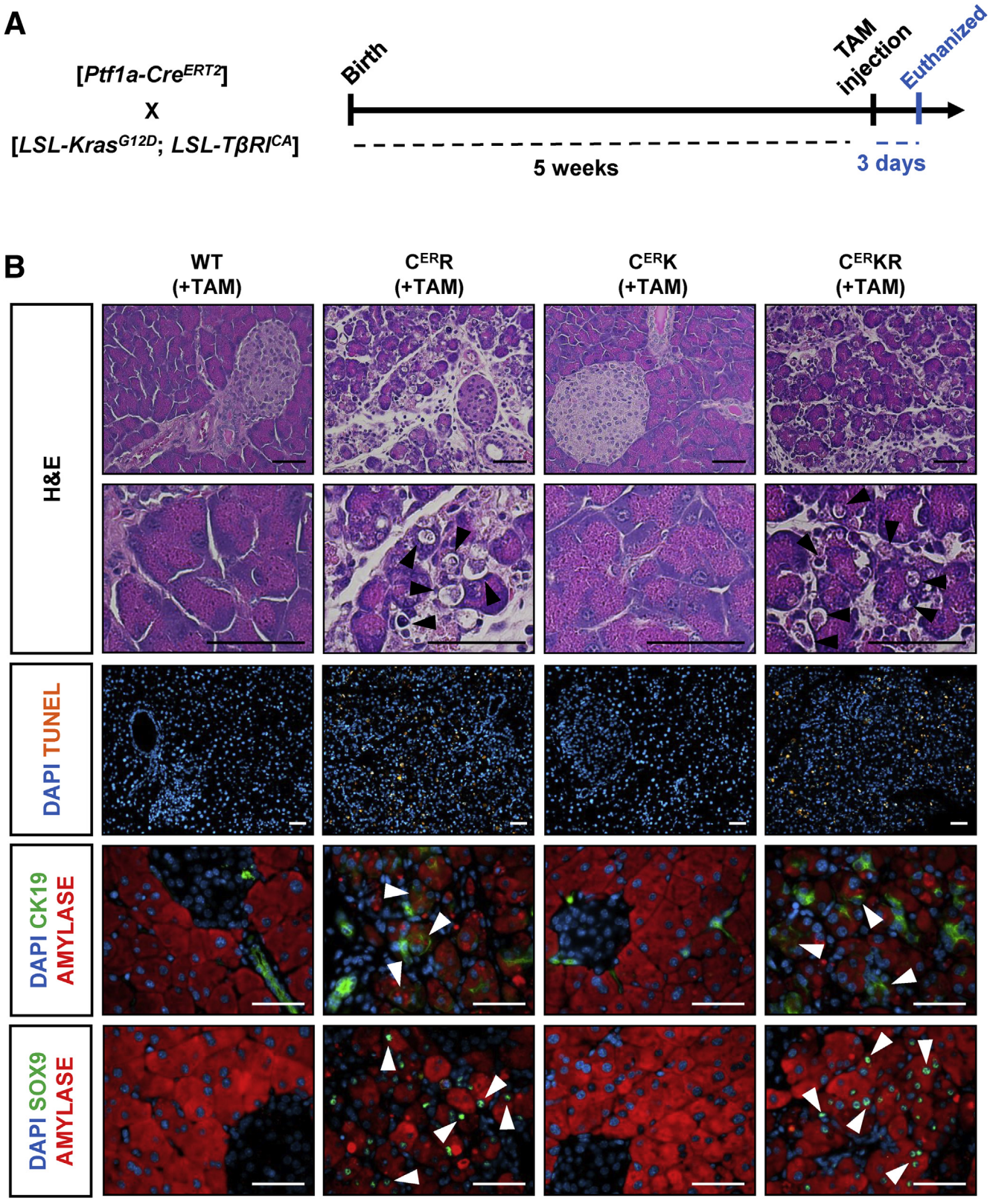


Figure 7. $T\beta RI^{CA}$ expression in acinar cells induces apoptosis and ductal-like differentiation 3 days after induction. (*A*) Diagram of experimental design for 5-week-old mice injected with tamoxifen and euthanized 3 days later. (*B*) H&E staining, TUNEL assay, and immunofluorescence of amylase, CK19, and SOX9. *Black arrowheads*, apoptotic cells; *white arrowheads*, CK19/amylase and SOX9/amylase double-positive cells; WT, wild-type; [*Ptf1a-Cre*$^{ERT-}$; *LSL-*$T\beta RI^{CA}$], $C^{ER}R$; [*Ptf1a-Cre*^{ERT2}; *LSL-Kras*^{G12D}], $C^{ER}K$; [*Ptf1a-Cre*^{ERT2}; *LSL-*$T\beta RI^{CA}$; *LSL-Kras*^{G12D}], $C^{ER}KR$. TAM, tamoxifen. Scale bars, 50 μm.

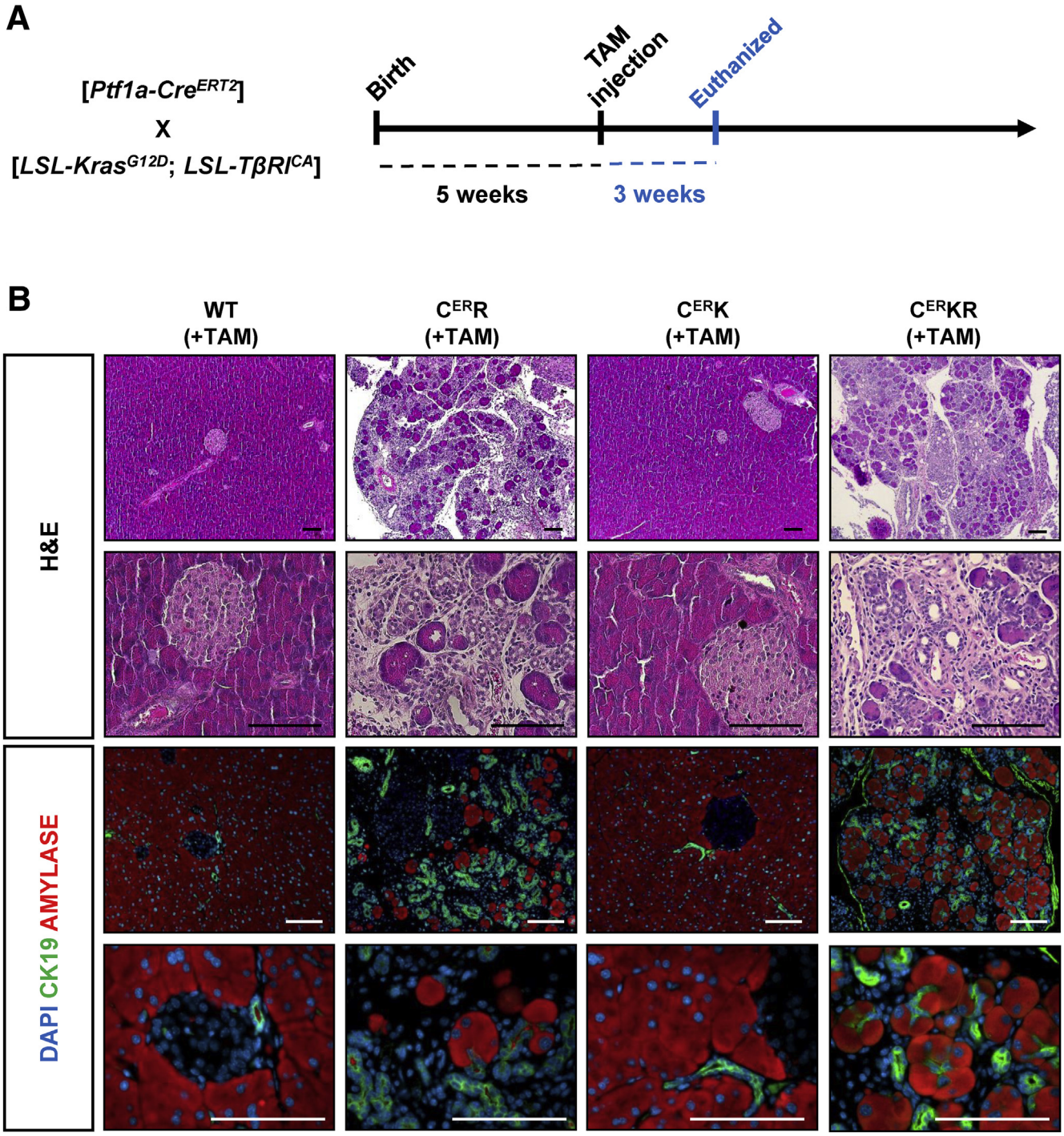
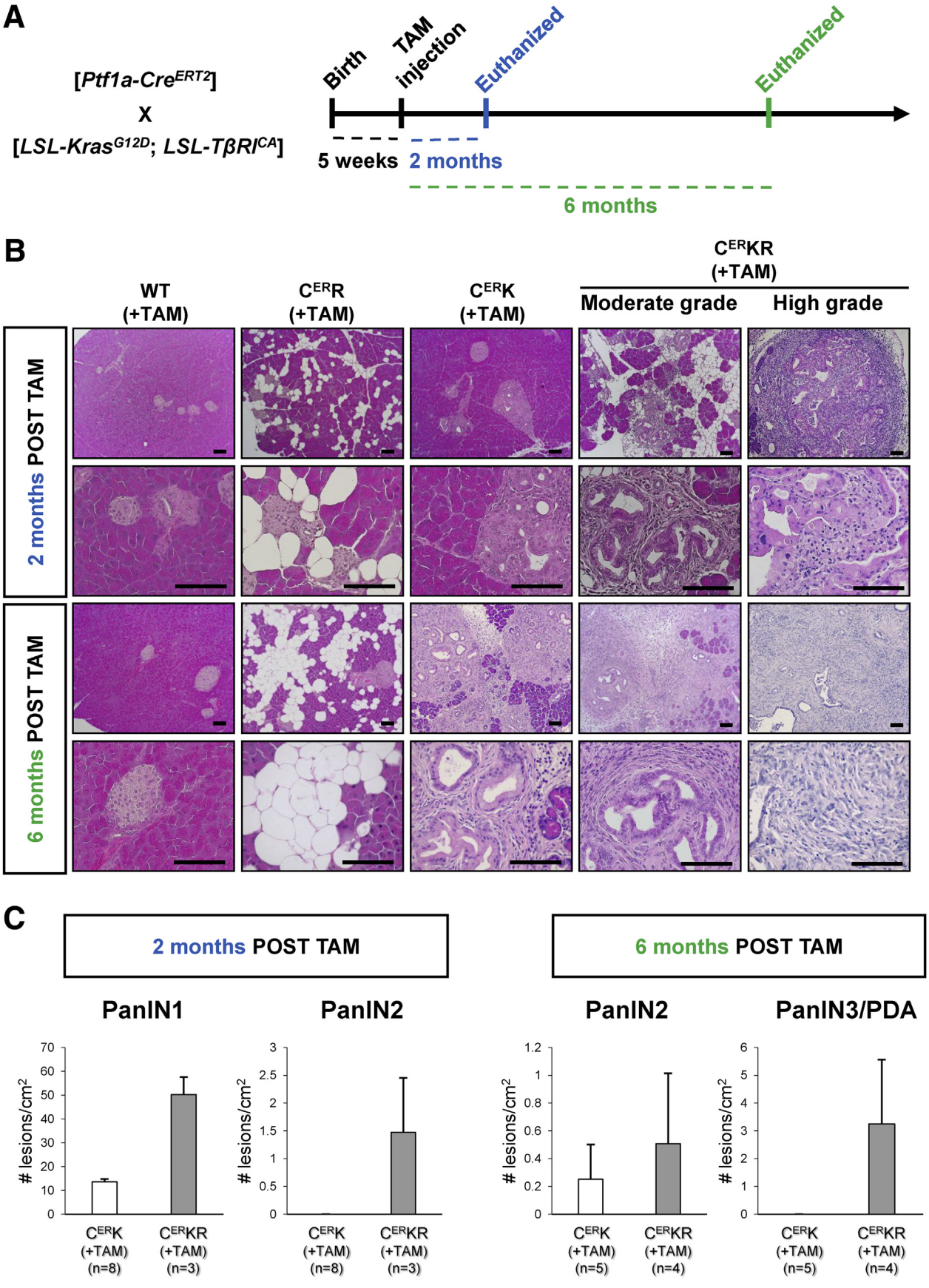


Figure 8. $T\beta RI^{CA}$ expression in acinar cells leads to regenerative ADM 3 weeks after induction. (*A*) Diagram of experimental design for 5-week-old mice injected with tamoxifen and euthanized 3 weeks later. (*B*) H&E staining and immunofluorescence of amylase and CK19. WT, wild-type; [*Pft1a-Cre*$^{ERT-}$; *LSL-TβRI*^{CA}], $C^{ER}R$; [*Pft1a-Cre*^{ERT2}; *LSL-Kras*^{G12D}], $C^{ER}K$; [*Pft1a-Cre*^{ERT2}; *LSL-TβRI*^{CA}; *LSL-Kras*^{G12D}], $C^{ER}KR$. TAM, tamoxifen. Scale bars, 100 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.[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[55] have shown that TGFβ could convert primary human acinar cells to ductal-like cells *in vitro*, which corroborates our current *in vivo* demonstration that TGFβ induces ADM. Although the signals triggering ADM


A
[Ptf1a-CreERT2]
X
[LSL-KrasG12D; LSL-TβRICA]
Birth
TAM injection
Euthanized
Euthanized
5 weeks
2 months
6 months
B
WT (+TAM)
CERR (+TAM)
CERK (+TAM)
CERKR (+TAM)
Moderate grade
High grade
2 months POST TAM
6 months POST TAM
C
2 months POST TAM
6 months POST TAM
PanIN1
PanIN2
PanIN2
PanIN3/PDA
lesions/cm2
CERK (+TAM) (n=8)
CERKR (+TAM) (n=3)
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CERKR (+TAM) (n=4)

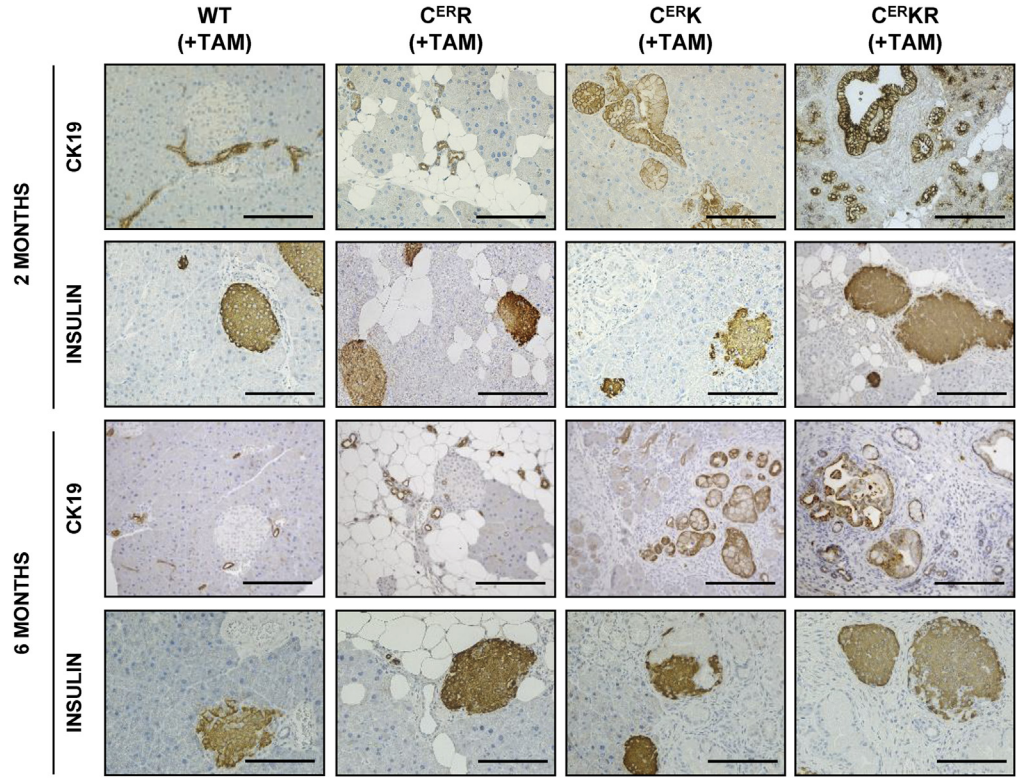

Figure 10. KRAS^{G12D} and TGFβ activation cooperate to accelerate pancreatic tumorigenesis without affecting endocrine compartment. Immunohistochemical detection of CK19 and INSULIN in mice of indicated genotypes 2 and 6 months after tamoxifen induction. [*Pft1a-Cre^{ERT2}*; *LSL-TβRI^{CA}*] (C^{ER}R); [*Pft1a-Cre^{ERT2}*; *LSL-Kras^{G12D}*] (C^{ER}K); [*Pft1a-Cre^{ERT2}*; *LSL-TβRI^{CA}*; *LSL-Kras^{G12D}*] (C^{ER}KR). Scale bars, 200 μm.

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 KRAS^{G12D}-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 *KRAS^{G12D}*.[12] 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 *Kras^{G12D}* transgenes in comparison with age-matched KRAS^{G12D} mice. PanINs were never observed in the presence of *TβRI^{CA}* alone. This observation supports the idea that KRAS^{G12D} generates a persistent ADM (known as for acinar-to-ductal reprogramming[56]), facilitating the subsequent onset of PanINs. Active TGFβ thus appears to be crucial to potentiate KRAS^{G12D} 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 KRAS^{G12D} 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 KRAS^{G12D}-induced PanINs. Hence, cell-autonomous activation of TGFβ

Figure 9. (*See previous page*). TβRI^{CA} expression in acinar cells accelerates KRAS^{G12D}-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; [*Pft1a-Cre^{ERT-}*; *LSL-TβRI^{CA}*], C^{ER}R; [*Pft1a-Cre^{ERT2}*; *LSL-Kras^{G12D}*], C^{ER}K; [*Pft1a-Cre^{ERT2}*; *LSL-TβRI^{CA}*; *LSL-Kras^{G12D}*], C^{ER}KR. Scale bars, 100 μm. (*C*) Quantification of pancreatic epithelial lesions at different grades and observed in C^{ER}K and C^{ER}KR pancreata 2 months and 6 months after TAM injection. TAM, tamoxifen.

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.[7] Importantly, in the tamoxifen-inducible model, 100% of pancreatic epithelial cells expressed the *TβRI^{CA}* transgene *in vivo*, thus indicating that the deleterious phenotype observed in the presence of the *KRAS^{G12D}* transgene resulted from a cell-autonomous functional interaction. Although our observations indicate that TGFβ activation induces direct ADM reprogramming, the mechanisms dictating cell fate, *ie*, 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).[64] David et al[64] 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 KRAS^{G12D} 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,[3] which is in accordance with the well-documented role of TGFβ as a promoter of stemness.[65,66] Indeed, SOX9 and HNF1β are the first transcription factors that commit endoderm cells to pancreatic progenitors during organogenesis.[5] They are markers of the ductal tree, re-expressed during ADM.[3,14] Moreover, the adult SOX9 has been shown to be a master positive regulator of the ADM process.[56] Indeed, the ectopic expression of SOX9 in acinar cells activates the expression of ductal markers and increases KRAS^{G12D}-induced ADM, and conversely, inactivation of *Sox9* in KRAS^{G12D}-expressing adult acinar cells compromises the onset of PanINs, confirming its critical role in the transition toward a ductal phenotype.[56] In the present study, we reported that the *in vivo* co-expression of TβRI^{CA} and KRAS^{G12D} 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 KRAS^{G12D} 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 KRAS^{G12D}-induced lesions.[67–72] 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 KRAS^{G12D}-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 KRAS^{G12D}-induced preexisting PanINs. This observation is consistent with the loss of *SMAD4* at late stages and widespread metastatic human diseases.[73,74] 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.[36] 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.[75] 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,[76,77] 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[78] 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

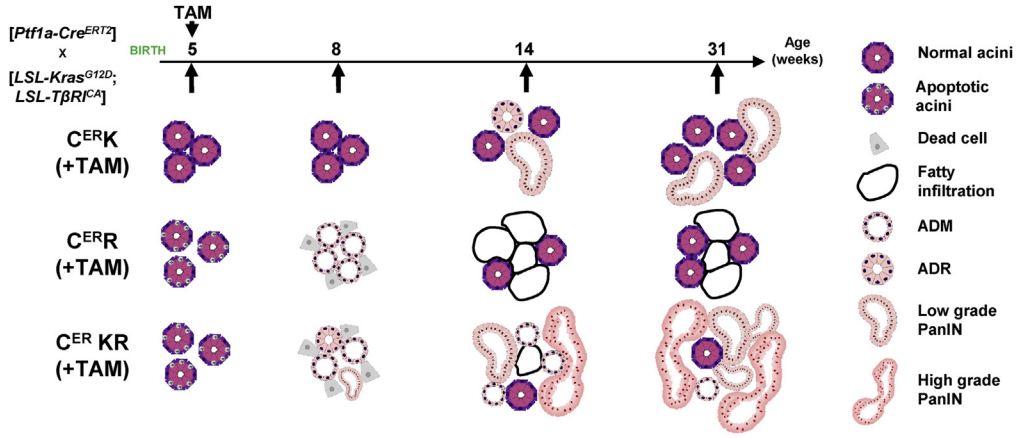

Figure 11. Schematic diagram showing effect of TGFβ signaling activation (alone or in combination with KRAS^{G12D}) on pancreatic acinar compartment. A few days after tamoxifen induction, TβRI^{CA} induces acinar cell apoptosis and subsequent regenerative metaplasia (ADM). In aging mice, ADM is overcome and replaced by AFI. KRAS^{G12D} activation alone induces ADM and PanINs several months after induction. When TβRI^{CA} and KRAS^{G12D} are simultaneously induced, PanINs develop much earlier. In the context of TβRI^{CA}-induced ADM, KRAS^{G12D} induces regenerative cells to undergo the ADM>ADR>PanIN sequence (ADR, acinar-to-ductal reprogramming). Hence, TGFβ signaling activation enhances the properties of KRAS^{G12D} through its capacity to prime a suitable surrounding for transformation. TAM, tamoxifen. [*Pft1a-Cre^{ERT-}*; *LSL-TβRI^{CA}*], C^{ER}R; [*Pft1a-Cre^{ERT2}*; *LSL-Kras^{G12D}*], C^{ER}K; [*Pft1a-Cre^{ERT2}*; *LSL-TβRI^{CA}*; *LSL-Kras^{G12D}*], C^{ER}KR. TAM, tamoxifen. WT, wild-type.

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 KRAS^{G12D}-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 KRAS^{G12D} is poorly efficient at inducing ADM. However, when combined, TGFβ induces ADM and provides a propitious setting for KRAS^{G12D}-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, Jo.G, KY, CC, VC, ER, BK, SM, and CC. Analysis and interpretation of data were performed by NC, DFV, RMP, LBA, IT, VR, AC, OJS, UV, SS, PD, and LB. Drafting of the manuscript was performed by NC, DFV, 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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