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Original Article

Retinoic acid receptor-β deletion in a model of early pancreatic ductal adenocarcinoma (PDAC) tumorigenesis

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Abstract: Vitamin A (VA, retinol) and its metabolites, including retinoic acid (RA), play a major role in the maintenance of cell populations in the adult pancreas. Pancreatic ductal adenocarcinomas (PDACs) contain lower amounts of VA and express lower levels of retinoic acid receptors (RARs) compared to normal human pancreatic tissues. Our goal was to determine if VA signaling directly impacts molecular events underlying pancreatic carcinogenesis using cell-type specific genetic approaches in mice. We knocked out retinoic acid receptor beta (RAR-β) selectively in pancreatic cells by tamoxifen treatment after crossing these adult RAR-$\beta^{fl/fl}$ mice with Pdx1/CreER (PC^{er}) and lox-stop-lox $KRas^{G12D}$ transgenic mice. Our data show that the rounds of tamoxifen we used were able to induce the knockout of the RAR-β gene in pancreatic cells in this PC^{er};KRas;RAR-$\beta^{fl/fl}$ transgenic model. We detected increases in proteins involved in RA metabolism (CYP26A1, RBP1, and ALDH1A2) in the PC^{er};RAR-$\beta^{D/wt}$ pancreata, but the levels of RBP1 and ALDH1A2 were decreased in PC^{er};RAR-β^{D} (both RAR-β alleles deleted) compared to PC^{er};KRas;RAR-β^{D} and wild-type pancreata. Ki67 and vimentin proteins exhibited lower levels in the PC^{er};KRas;RAR-β^{D} and PC^{er};RAR-β^{D} pancreata compared to wild-type, indicating that deletion of RAR-β reduced cell proliferation in acinar cells. Expression of SOX9, a key protein required for formation and maintenance of PDAC, was higher in PC^{er};RAR-$\beta^{D/wt}$ and PC^{er};RAR-β^{D} pancreata compared to wild-type, indicating that deletion of RAR-β increases SOX9 levels even without the KRas activating mutation. In summary, lack of RAR-β in pancreatic acinar cells reduced cell proliferation and increased SOX9 protein levels in this transgenic model.

Keywords: Pancreatic cancer, vitamin A, retinoic acid receptors, cellular proliferation

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer [1]. PDAC originates in the pancreatic ducts, which traverse the central region of the organ and are responsible for its primary exocrine functions [2]. Exocrine cell tumors, particularly PDAC, account for approximately 95% of pancreatic cancers, whereas endocrine pancreatic cancers tend to exhibit a more indolent course and have a generally more favorable prognosis [3]. PDAC is the deadliest form of pancreatic cancer [4]. In 2024, 66,440 people are expected to be newly diagnosed and 51,750 deaths are estimated from pancreatic cancer in the United States, recently eclipsing breast cancer as the third leading cause of over-all cancer deaths [5]. PDAC generally arises from indolent, pre-invasive pancreatic intraepithelial neoplasia (PanINs) that frequently go undetected and persist for many years [6, 7].

PDAC commonly presents with a distinctive desmoplastic stroma characterized by the presence of various cell types, including fibroblasts, immune cells, endothelial cells, and pancreatic stellate cells (PSCs). This stroma is rich in extracellular matrix proteins and polysaccharides such as collagen, laminin, hyaluronic acid (HA), and fibronectin [8-10]. This can create a hypoxic environment, limiting the effectiveness of some chemotherapies [11] and promoting tumor development [12].

Vitamin A (retinol, VA) is essential for regulating cell proliferation and differentiation of epithelial tissues. VA is primarily obtained through dietary sources that contain retinol, retinyl esters, or beta-carotene [13]. Common dietary sources of

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vitamin A include animal liver, fish oils, dairy products, and fortified cereals, particularly in the United States [14]. The absorption and the metabolism of VA to retinoic acid (RA, also known as all-trans retinoic acid) are complex processes and differ by cell type [15, 16]. The release of VA from storage forms and the synthesis of biologically active VA metabolites are crucial processes for the biological functions of retinoids. However, the regulation of these steps remains incompletely understood [15].

RA, an active metabolite of retinol, plays a pivotal role in regulating various biological processes within the pancreas [17]. Its pleiotropic effects on cellular growth and differentiation primarily stem from its action as an endogenous ligand for nuclear receptors known as retinoic acid receptors (RARs), which form heterodimers with retinoid X receptors (RXRs) [18]. There are 3 RARs: RAR-alpha, RAR-beta, and RAR-gamma, as well as three RXRs: RXR-alpha, RXR-beta, and RXR-gamma, encoded, respectively, by their corresponding genes [18]. Approximately one-third of all pancreatic tumors completely lose expression of RAR-β, when compared with non-transformed pancreas, and the other two-thirds of tumors express significantly lower levels of RAR-β mRNA compared to adjacent, normal pancreatic ductal cells [19, 20]. RAR-β is a tumor suppressor, an attractive target in cancer research [21], and may have a significant role in mitigating the malignant phenotype of PDAC [13, 22]. Interestingly, RAR-β expression is also lost or reduced in a variety of carcinomas, including lung [23] and liver [19, 24]. The disruption of RARβ expression frequently occurs during the initial phases of numerous cancers and may coincide with the onset of cancer progression [13]. Consistently, previous studies have noted a correlation between diminished RARβ expression and advancing tumor stages, with a notable decline observed from stage IA to IIA in pancreatic tumors [25].

There is a tight correlation between the loss of RAR-β expression and the degree of cellular dedifferentiation [26]. These data suggested that loss or decreased expression of RAR-β could either be an epiphenomenon associated with malignant transformation or could indeed play a central role in the malignant phenotype in human pancreatic adenocarcinoma [27].

RA is currently under investigation as a potential treatment for PDAC in murine models and clinical trials [28, 29]. RA has been shown to impede the migration of pancreatic carcinoma cells and inhibit the epithelial-mesenchymal transition of tumor cells by downregulating IL-6 expression in cancer-associated fibroblasts [30]. Additionally, RA can diminish the stem-like characteristics of PDAC cells [31]. These findings collectively suggest that activating RA signaling pathways could lead to the development of drugs for preventing recurrence and/or metastasis in pancreatic cancer [14].

KRas mutations are one of the leading dominant mutations in PDAC [32]. One of the transgenic mouse models that is often used is the KC model (Pdx1/CreER; lox-stop-lox $KRas^{G12D}$). This model shows the spectrum of PDAC progression, from PanINs to invasive and metastatic PDACs at low frequency [33]. The goal of our research is to delineate the impacts of changes in retinoid signaling in acinar cells on the molecular events underlying early PDAC tumorigenesis by using mice with RAR-β knocked out in the Pdx1/CreER;lox-stop-lox KRas transgenic line.

Materials and methods

Transgenic mouse model

These experiments were conducted with the Institutional Animal Care and Use Committee (IACUC) guidelines at Weill Cornell Medical College (WCMC) in accord with all applicable federal, state, and local regulations. All experimental protocols for this specific study were approved by the IACUC, including animal care, tumor size, housing, and sanitization (WCMC Animal Welfare Assurance Number: D16-00186); WCMC Institutional Biosafety Committee (IBC) Laboratory Registration #: IBC-18783. We confirm that the maximal tumor size/burden was not exceeded.

We mated homozygous Pdx-CreER double-positive transgenic mice (Cat #024968) and lsl-$KRas^{G12D}$ (Cat #008179) from Jackson Lab (Bar Harbor, ME), and RAR-$\beta^{fl/fl}$ double-positive transgenic mice (gift from Dr. Pierre Chambon) [34] to generate Pdx1-CreER;lox-stop-lox $KRas^{G12D}$;RAR-$\beta^{fl/wt}$ and Pdx1-CreER;RAR-$\beta^{fl/wt}$; and Pdx1-CreER;lox-stop-lox $KRas^{G12D}$;RAR-$\beta^{fl/fl}$ and Pdx1-CreER;RAR-$\beta^{fl/fl}$ mice (**Figure 1A**). The

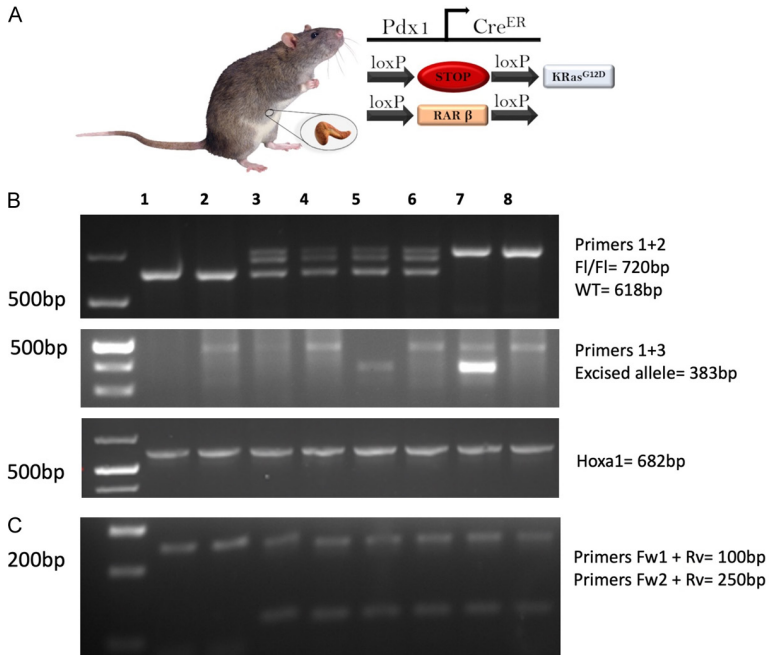

Figure 1. Generation of the Pdx-creER (PC^{ER}); Isl-$KRas^{G12D/+}$; $RAR\beta^{fl/fl}$ mouse line. A. Scheme of the mouse line generation. B. Genotyping PCR confirmation of excised alleles from RARβ specifically in the pancreas. 1 = pancreas from Wild-type; 2 = liver from Wild-type; 3 = pancreas from PC^{er};KRas;$RAR\beta^{fl/wt}$ (No tam); 4 = liver from PC^{er};KRas;$RAR\beta^{fl/wt}$ (No tam); 5 = pancreas from PC^{er};KRas;$RAR\beta^{D/wt-}$ (1 round of tam); 6 = liver PC^{er};KRas;$RAR\beta^{D/wt-}$ (1 round of tam); 7 = pancreas from PC^{er};KRas;$RAR\beta^{D}$ (3 rounds of tam); 8 = liver from PC^{er};KRas;$RAR\beta^{D}$ (3 rounds of tam). C. Genotyping PCR confirmation of mutant KRas in the DNA of all tissues of transgenic lines. 1 = pancreas from Wild-type; 2 = liver from wild-type; 3 = pancreas from PC^{er};KRas;RAR $\beta^{fl/wt}$ (No tam); 4 = liver from PC^{er};KRas;$RAR\beta^{fl/wt}$ (No tam); 5 = pancreas from PC^{er};KRas;$RAR\beta^{D/wt-}$ (1 round of tam); 6 = liver PC^{er};KRas;$RAR\beta^{D/wt-}$ (1 round of tam); 7 = pancreas from PC^{er};KRas;$RAR\beta^{D}$ (3 rounds of tam); 8 = liver from PC^{er};KRas;$RAR\beta^{D}$ (3 rounds of tam).

transgenic mice were genotyped using the primers for the different genes studied (**Table 1**). According to Jackson Lab (jax.org/strain/024968), we used the wild-type mouse line C57BL/6 as a control for Pdx1-CreER.

All recombinant mice were treated with tamoxifen at 4 weeks of age. A minimum of three mice in each group were used, each group receiving 1 or 3 rounds of 5 intraperitoneal injections of 200 mg tamoxifen/kg body weight each (Cayman Chemical Company, #13258, MI) in cottonseed oil (0.1 mL - Sigma, #C7767). Each round consisted of once daily injections on alternating days (days 1, 3, 5, 7 and 9), and we waited one month between each round. The mice were sacrificed 4 weeks after the last injection; thus, the mice treated with 3 rounds of tamoxifen were 4 months old when sacrificed. The PC^{er};$RAR\beta^{D/wt}$ and PC^{er};KRas;$RAR\beta^{D/wt}$ heterozygotes were generated from Pdx1-CreER;$RAR\beta^{fl/wt}$ and Pdx-CreER;lox-stop-lox $KRas^{G12D}$;$RAR\beta^{D/wt}$ treated with one round of tamoxifen, respectively; the PC^{er};$RAR\beta^{D}$ and PC^{er};KRas;$RAR\beta^{D}$ homozygous knockout mice were generated from Pdx1-CreER;$RAR\beta^{fl/fl}$ and Pdx1-CreER;lox-stop-lox$KRas^{G12D}$;$RAR\beta^{fl/fl}$ treated with 3 rounds of tamoxifen, respectively. We sacrificed the mice by cervical dislocation, strictly following the guidelines approved by the IACUC. The tamoxifen treatments caused both activation of the KRas mutant and the knockout of RAR-β selectively in pancreatic cells.

PCR conditions

The polymerase chain reaction for Cre was performed with an initial denaturation step at 95°C for 10 min, followed by 34 cycles consisting of denaturation at 95°C for 1 min, annealing at 60°C for 2 min, elongation at 72°C for 1 min, and a final extension at 72°C for 10 min. For the mutant KRas gene, PCR was performed with an initial denaturation step at 94°C for 3 min, followed by 9 cycles of 65°C for 30 sec, decreasing 0.5°C per cycle, and 72°C for 30 sec; this was followed by 27 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min. For the RAR-β floxed (fl/fl) gene, the primers P1 and P2 were used (**Table 1**); the PCR was performed with an initial denaturation step at 95°C for 5 min, followed by 39 cycles consisting of denaturation at 95°C for 30 sec, annealing at 57°C for 45 sec, elongation at 72°C for 1 min, and a final extension at 72°C for 5 min. To assess the knockout induced by tamoxifen, the primers P1 and P3, which yielded the excised genomic DNA product, were used. Primers for the amplification of Hoxa1 DNA, a transcription factor regulated by RA and RAR-β, were used as positive control for DNA quality and the PCR was performed at 94°C for 10 min, followed by

Table 1. Sequence of primers used to genotype of mice models

Gene	Sequence Primers	Size of fragment (bp)
PdxCreEr	5'GCAGTGGAGAACTGTCAAAGC3' 5'CTAGGCCACAGAATTGAAAGATCT3'	410
KRas	Fw1 5'TGTCTTTCCCCAGCACAGT3' Fw2 5'GCAGGTCGAGGGACCTAATA3' Rv 5'CTGCATAGTACGCTATACCCTGT3'	Mutant 100 Wild-type 250
RAR-β	P1 5'TGTACCCAGAGTCAACAAA3' P2 5'GGCCATCAGAGAAAGTCAT3' P3 5'CAACCCAGTCCAGCACCAG3'	Primer P1 + Primer P2 = 720 RAR-β floxed (fl/fl) band Primer P1 + primer P3 = 383 Excised RAR-β band by Cre
HOXA1	Fw 5'TAACTCCTTATCCCCTCTCCAC3' Rv 5'ACCCACGTAGCCGTACTCTCCA3'	682

39 cycles consisting of 94°C for 1 min, 60°C for 2 min, and 72°C for 1 min, with a final extension at 72°C for 10 min (**Table 1**).

Immunostaining conditions

We fixed pancreas samples in 4% paraformaldehyde buffer (pH 7.4) and embedded them in paraffin blocks. Next, we stained 5 µm thick sections. The slides were de-paraffinized with xylene for 10 min twice, placed in 100% ethanol for 10 min, 95% ethanol for 5 min, 70% ethanol for 5 min, and rinsed with dH_2O twice for 5 min for each rinse. The slides were incubated in 15 mL antigen unmasking solution (Vector antigen Unmasking solution Citrate-base, pH 6, Cat H3300 or Tris-Based, pH 9, cat H3301) in 1.6 L of dH_2O for 3 min at 176°C in a pressure cooker. The slides were then incubated with 3% hydrogen peroxide in methanol for 1 hour to quench endogenous peroxidase activity, followed by incubation for 10 min in PBS-T and 10 min in PBS. We blocked the slides with 2% bovine serum albumin (BSA) in PBS/Triton X-100 (0.1%) and normal goat serum (Vector Laboratories #S-1000), for 1 hour at room temperature (RT).

After optimization of the staining protocols, we incubated the slides for 1 hour at RT with Ki67 (rabbit monoclonal, 1:100, #9129, Cell Signaling, MA), SOX9 (rabbit monoclonal, 1:100, #14366, Cell Signaling, MA), RBP1 (rabbit monoclonal, 1:75, Ab543900101, AbClonal), ALDH1A2 (rabbit polyclonal, 1:100, Custom Made), Vimentin (rabbit monoclonal, 1:100, #5741, Cell Signaling, MA), Muc1 (rabbit monoclonal, 1:100, #MA5-14077, Thermo Fisher), and CYP26a1 (rabbit monoclonal 1:50, A5982, AbClonal), followed by an incubation overnight at 4°C. The antibodies were diluted in the same blocking buffer. To assess non-specific staining, we included a negative control slide incubated without primary antibodies. After rinsing in PBS, we incubated the slides with 1× goat anti-rabbit IgG secondary antibody, poly HRP conjugate (Invitrogen, #B40962, OR) for 1 hour at 22°C. After washes with PBS, the slides were incubated with 3,3'-diaminobenzidine substrate (DAB) (Vector Laboratories), according to the manufacturer's instructions, counterstained with hematoxylin (Poly Scientific R&D, Bay Shore, NY), and mounted for image acquisition with a Nikon TE2000 inverted fluorescence microscope. All slides for one antibody were stained at the same time.

Statistical analysis

Images were acquired from 5 random fields per tissue section per mouse from a total of 3 mice per group and the areas of staining were quantified with Fiji (Image J) software (v1.48, NIH) according to previously published methods [35]. We quantified the level of staining as the percentage positive area and intensity per field. The data were then represented as the means ± standard deviation (SD). The statistical significance among groups was determined by one-way ANOVA and Bonferroni multiple comparison post-hoc analysis (black stars) using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA), and p values of < 0.05 were considered statistically significant.

Results

Generation of the RAR-β knockout specifically pancreatic cells

We used PC^{er};KRas;$RAR\beta^{fl/wt}$ (heterozygous for RARβ deletion) and PC^{er};KRas;$RAR\beta^{fl/fl}$ (homozy-

gous for RARβ deletion) lines as models in our study to compare loss of one versus both copies of the RARβ gene (**Figure 1A**). Genotyping results confirm tamoxifen induction of the knockout of the RARβ gene in the pancreas, but not in the liver (**Figure 1B**). Here we show the band of 383 bp that represents the knockout of RARβ in the pancreas after the tamoxifen treatments. We did not observe RARβ knockout in all pancreatic cells because the floxed RAR-β allele is still present, in addition to the band for the knockout, after tamoxifen treatment (**Figure 1B**). This result is expected since a 100% tamoxifen-induced knockout is not guaranteed due to various factors, including the cell type, the location of the target gene, and the doses of tamoxifen. According to **Figure 1B**, we observed that the PCR product of the PC^{er} RAR β^{D} mice shows a greater intensity of the excised allele when mice were treated with 3 rounds of injections comparing to the band from the mouse (PdxCreEr RAR-$\beta^{D/wt}$) treated with one round of injection, using HOXA1 as a loading control. Also, we obtained a band of 100 bp that represents the presence of the mutant KRas in the pancreata of the appropriate mice (**Figure 1C**).

Effects of the RAR-β knockout on the protein levels of retinoic acid metabolism enzymes and intracellular retinol binding protein (RBP1)

We first determined if the knockout of RARβ affects CYP26A1 protein levels in mouse pancreata. We detected higher CYP26A1 protein levels in the pancreata of PC^{er};RAR-$\beta^{D/wt}$ compared to wild-type (**Figure 2A**). These results suggest that a reduction in RAR-β expression in pancreatic cells could lead to increases in RA metabolism in the pancreas. This result was extended when we evaluated CYP26A1 protein levels in the pancreata from PC^{er};RAR-β^{D} compared to wild-type and PC^{er};KRas;RAR-β^{D} mice (**Figure 3A**). However, the addition of the activated KRas oncogene did not further increase CYP26A1 levels and in fact, decreased these levels relative to those in the PC^{er};RAR-β^{D} pancreata (**Figure 3A**).

We detected greater RBP1 staining in PC^{er};RARβ$^{D/wt}$ compared to wild-type and PC^{er};KRas;RAR-$\beta^{D/wt}$ pancreata (**Figure 2B**). In contrast, we detected decreased expression of RBP1 protein in the PC^{ER};RAR-β^{D} and PC^{er};KRas;RAR-β^{D} compared to wild type pancreata (**Figure 3B**). Thus, the homozygous knockout of RAR-β in pancreata is associated with reduced RBP1 levels and this reduction is not dependent on the presence of the activated KRas oncogene. We conclude that the degree of RAR-β knockout in pancreatic cells does not correlate with RBP1 protein levels.

ALDH1A2 protein levels in PC^{er};RAR-$\beta^{D/wt}$ were higher compared to wild-type and PC^{er};KRas;RAR-$\beta^{D/wt}$ pancreata (**Figure 2C**). ALDH1A2 levels in the PC^{er};RAR-β^{D} and PC^{er};KRas;RAR-β^{D} pancreata were lower compared to wild-type (**Figure 3C**). Thus, the homozygous knockout of RAR-β in pancreata reduces ALDH1A2 levels and this reduction is not dependent on the presence of the activated KRas oncogene.

Cellular proliferation in RAR-β knockout acinar cells

We detected different degrees of cell proliferation in the mice with knockouts in RARβ (**Figure 4A**). We saw increases in Ki67 staining in pancreatic acinar cells in PC^{er};RAR-$\beta^{D/wt}$ and PC^{er};KRas;RAR-$\beta^{D/wt}$ compared to wild-type. This result suggests that RARβ limits pancreatic cell proliferation. However, we detected lower Ki67 levels in both PC^{er};RAR-β^{D} and PC^{er};KRas;RAR-β^{D} than in wild-type (**Figure 5A**), indicating that Ki67 levels did not correlate with the degree of deletion (heterozygous vs. homozygous deletion) of RAR-β in the pancreata.

We measured lower vimentin expression in both wild-type and PC^{er};RAR-$\beta^{D/wt}$ than in the PC^{er};KRas;RAR-$\beta^{D/wt}$ pancreata (**Figure 4B**). When we next evaluated the staining in the PC^{er};RAR-β^{D} and PC^{er};KRas;RAR-β^{D} pancreata, vimentin expression was greatly reduced relative to that in wild-type pancreata (**Figure 5B**). These results suggest that RAR-β knockout leads to a decrease in pancreatic vimentin expression. Addition of the KRas mutation showed a trend (p value = 0.09) toward a greater reduction in vimentin expression upon RAR-β deletion (**Figure 5B**).

Immunohistochemical detection of a ductal lineage marker

The transcription factor SOX9 plays a major role in the development and differentiation of

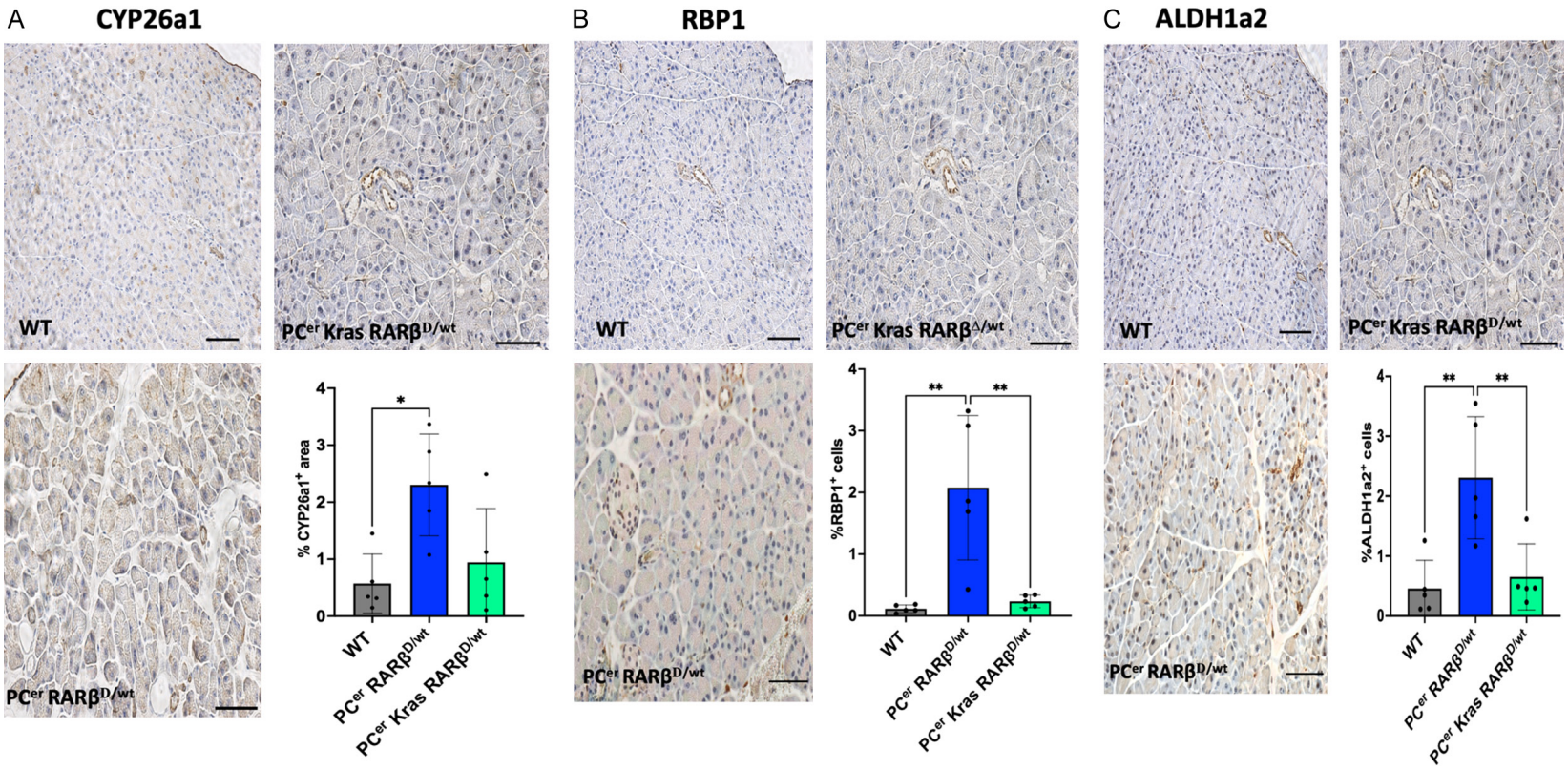

Figure 2. Representative images showing the staining of CYP26a1 (A), RBP1 (B) and Aldh1A2 (C) protein in pancreatic tissues in mice treated with one round of tamoxifen. (A) Representative images of pancreata from Wt, PC^{er};RARβ$^{D/wt}$ and PC^{er};KRas;RARβ$^{D/wt}$ mice stained with CYP26a1 antibody, (B) RBP1 antibody stained the pancreata from mice in (A), and (C) ALDH1A2 antibody stained the pancreata from mice in (A) (300×; scale bar: 100 μm; *N* = 3 mice/group, 5 fields/mouse; representative fields are shown).

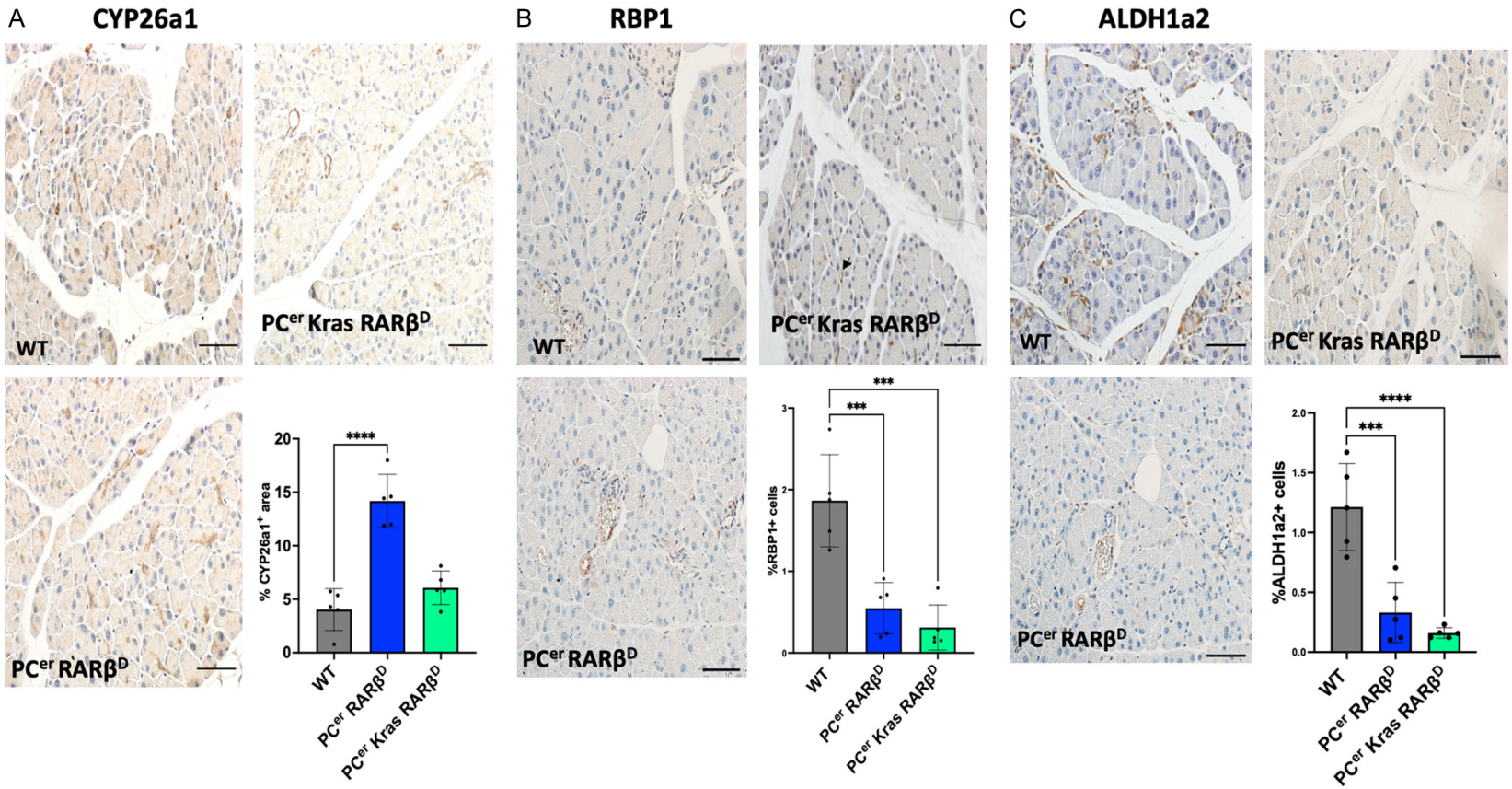

Figure 3. Representative images showing the staining of CYP26a1 (A), RBP1 (B) and Aldh1A2 (C) protein in pancreatic tissues in mice treated with 3 rounds of tamoxifen. (A) Representative images of pancreata from Wt, PC^{er};RARβ^{D} and PC^{er};KRas;RARβ^{D} mice stained with CYP26a1 antibody, (B) RBP1 antibody stained the pancreata from mice in (A), and (C) ALDH1A2 antibody stained the pancreata from mice in (A) (300×; scale bar: 100 μm; *N* = 3 mice/group, 5 fields/mouse; representative fields are shown).

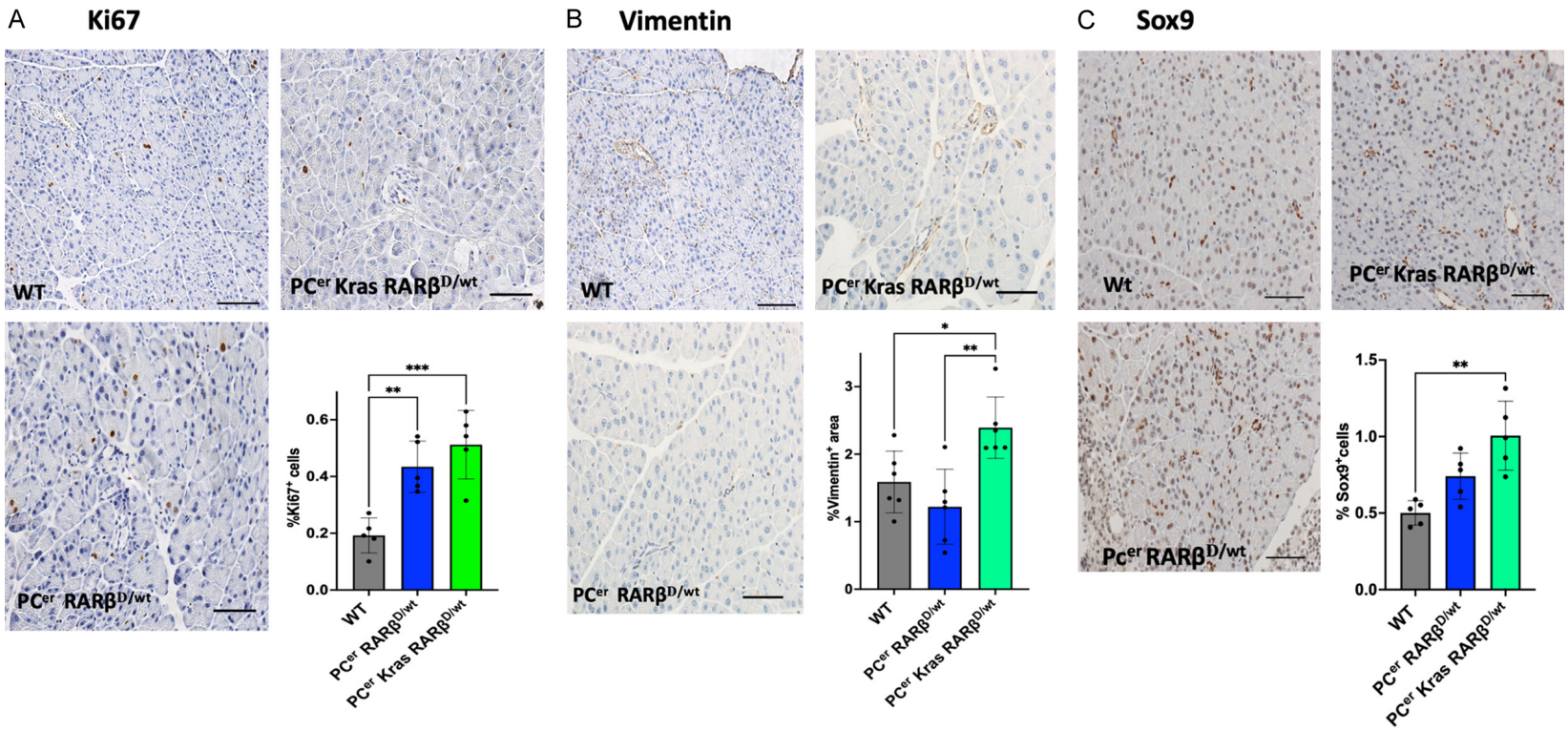

Figure 4. Representative micrographs of the staining of Ki67 (A), vimentin (B) and SOX9 (C) protein in pancreatic tissues in RARβ heterozygotes treated with one round of tamoxifen. (A) Ki67 antibody stained Wt, PC^{er};$RARβ^{D/wt}$ and PC^{er};KRas;$RARβ^{D/wt}$ pancreata and (B) Vimentin antibody stained pancreata from mice in (A); and (C) SOX9 antibody stained pancreata from mice in (A) (300×; scale bar: 100 μm; *N* = 3 mice/group, 5 fields/mouse; representative fields are shown).

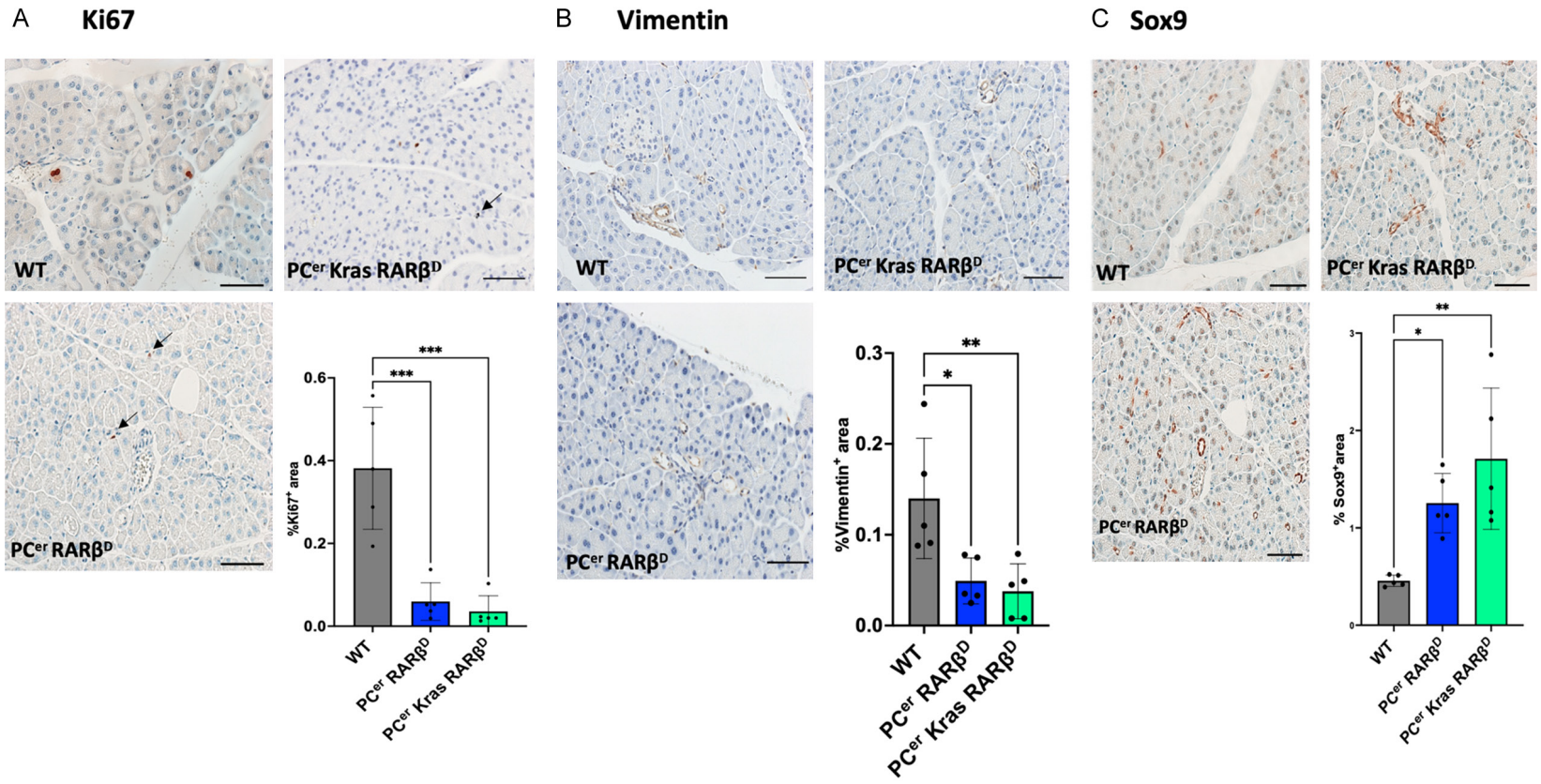

Figure 5. Representative micrographs of the staining of Ki67 (A), vimentin (B) and SOX9 (C) protein in pancreatic tissues in RARβ homozygotes treated with 3 rounds of tamoxifen. (A) Ki67 antibody stained Wt, PC^{er};RARβ^{D} and PC^{er};KRas;RARβ^{D}, treated with 3 rounds of tamoxifen, (B) Vimentin antibody stained pancreata from mice in (A), and (C) SOX9 antibody stained pancreata from mice in (A) (300×; scale bar: 100 μm; *N* = 3 mice/group, 5 fields/mouse; representative fields are shown).

multiple tissues during embryogenesis [36], and SOX9 plays a critical role in pancreatic ductal development. SOX9 expression was higher in both PC^{er};RAR-β^{D} and PC^{er};KRas;RAR-β^{D} compared to wild-type pancreata (**Figure 5C**) and in PC^{er};KRas;RAR-$\beta^{D/wt}$ compared to wild-type pancreata (**Figure 4C**).

Discussion

We used PC^{er};KRas;RAR-$\beta^{fl/wt}$ and PC^{er};KRas;RAR-$\beta^{fl/fl}$ lines as models in our study to compare loss of one versus both copies of the RARβ gene. We detected the band of 383 bp that represents the knockout of RARβ in the pancreas after the tamoxifen treatments. From this result we concluded that the tamoxifen protocol we used is suitable for the experiments and that the knockout of RARβ occurs with both one and 3 rounds of tamoxifen treatments. Also, we obtained a band of 100 bp that represents the presence of the mutant KRas in the pancreata of the mice. We compared pancreata from mice with one allele of RAR-β knocked out in pancreatic cells with mice with both alleles of RAR-β knocked out so that we could assess whether the impact from the RAR-β deletion in pancreatic cells is correlated with the extent of RARβ expression.

CYP26A1 (cytochrome P450 26A1), a member of the cytochrome P450 superfamily of enzymes, metabolizes retinoic acid (RA) to more polar metabolites [37] and shows higher expression in multiple cancers [38-40]. Importantly, The Cancer Genome Atlas (TCGA) database shows that the CYP26A1 mRNA level is associated with worse clinical features and prognosis in pancreatic cancers; higher CYP26A1 is associated with the proliferation, migration, and invasion of pancreatic cancer cells [41]. We detected higher CYP26A1 protein levels in both heterozygous and homozygous RARβ knockout pancreata (PC^{er};RAR-$\beta^{D/wt}$ and PC^{er};RAR-β^{D}), but in pancreata with activated KRas we did not see an increase in CYP26A1 (PC^{er};KRas;RAR-$\beta^{D/wt}$ and PC^{er};KRas;RAR-β^{D}) compared to wild-type. Previous studies show that targeting RA signaling has the potential for prevention/treatment of pancreatic cancer [14]. Our data (**Figures 2A** and **3A**) indicate that the deletion of RAR-β in acinar cells leads to increases in CYP26A1 expression which could facilitate RA metabolism, thereby reducing RA's effects in the treatment of pancreatic cancers.

Retinol-binding protein 1 (RBP1 or CRBP1), an intracellular retinol-binding protein, is ubiquitously expressed in various tissues. It serves as a chaperone protein, facilitating the uptake, esterification, and bioavailability of retinol. The essential role of RBP1 in retinoid metabolism has been well-documented [42], suggesting a potential role for RBP1 in inhibiting pancreatic carcinogenesis. Loss or significant downregulation of RBP1 occurs in 70% of human pancreatic cancers and is evident in the very earliest precursor lesions (PanIN-1A) [43]. Our data (**Figures 2B** and **3B**) suggest that the levels of RAR-β in pancreatic cells do not correlate with RBP1 levels. Notably, we detected lower RBP1 when both alleles of RAR-β were knocked out. Since both RAR-β [25] and RBP1 [43] expression are reduced in human pancreatic tumor tissues, the decreased expression of RBP1 in pancreatic cells after homozygous knockout of RAR-β (**Figure 3B**) suggests that the reduction in RBP1 in human pancreatic cancer could result from lower RARβ expression levels in these tumor cells.

We also evaluated the pathway of VA metabolism in pancreatic cells by measuring ALDH1A2, an enzyme responsible for the synthesis of retinoic acid (RA) from retinaldehyde [44]. Prior research has indicated that ALDH1A2 functions as a potential tumor suppressor, and reduced expression of ALDH1A2 is associated with poor prognosis in various cancer types, serving as an unfavorable prognostic biomarker for survival [45]. The reductions in ALDH1A2 protein levels caused by RAR-β deletion in PC^{er};RAR-$\beta^{D/wt}$ and PC^{er};RAR-β^{D} pancreata (**Figures 2C** and **3C**) suggest that less RA is present upon RAR-β deletion.

We also analyzed pancreatic tissues from mice by immunostaining using a Ki67 antibody. Ki67 reflects cell proliferation rates and higher Ki67 levels are correlated with initiation, progression, and metastasis in many types of tumors [46]. We measured increases in Ki67 staining in pancreatic cells in PC^{er};RAR-$\beta^{D/wt}$ and PC^{er};KRas;RAR-$\beta^{D/wt}$ compared to wild-type. This result suggests that RARβ limits pancreatic cell proliferation. However, in contrast we detected lower Ki67 levels in both PC^{er};RAR-β^{D} and PC^{er};KRas;RAR-β^{D} than in wild-type, indicating that Ki67 levels did not correlate with the degree of deletion of RAR-β in the pancreata (**Figures 4A** and **5A**). Why heterozygous dele-

tion of RAR-β in the pancreas results in a different level of Ki67 compared to homozygous deletion of RAR-β is not clear.

Vimentin is considered a marker of mesenchymal differentiation, expressed by normal mesenchymal tissue [47]. There is evidence that carcinomas with markers of mesenchymal differentiation have different biological and clinical behaviors [48, 49]. Vimentin expression patterns in pancreatic cancer have been investigated in a small series [50] and a lack of expression of vimentin in pancreatic cancers was reported [51]. The differential diagnosis between chronic pancreatitis and pancreatic cancer cannot be based on expression of vimentin, however, because vimentin is expressed not only in pancreatic cancer cells, but also in stromal fibroblasts [52]. Our results show that RAR-β knockout leads to a decrease in pancreatic vimentin expression (**Figure 4B**). Addition of the KRas mutation showed a trend (*p* value = 0.09) toward a greater reduction in vimentin expression upon homozygous RAR-β loss (**Figure 4B**).

SOX9 is expressed at a high level in PDAC and in intraductal papillary mucinous neoplasms (IPMNs) [53]. The expression of SOX9 was expected in pancreatic ductal progenitor cells and pancreatic ductal cells [54], and a previous study in genetically engineered mouse models demonstrated that concomitant expression of SOX9 and oncogenic KRas can induce transformation of acinar cells into duct-like cells and subsequent PanIN formation during the initial and developmental stages of PDAC [55]. Our data (**Figures 4B** and **5B**) demonstrate that loss of RAR-β leads to an increase in pancreatic SOX9 protein, which could facilitate PDAC development. We used relatively short time periods for these experiments because we anticipated that the lack of RAR-β would cause neoplastic lesions to develop more rapidly. Although we did not detect pancreatic neoplastic lesions both in cohorts harboring RAR-β and in cohorts treated with 1 round or 3 rounds of tamoxifen in the presence of activated KRas because of the relatively short length of time we used in these experiments, our data, especially the increase in SOX9, suggest that loss of RAR-β could have an impact on accelerating PDAC initiation in this model.

Conclusions

RAR-β expression is reduced in PDAC and reduced RAR-β levels correlate with tumor stage, pointing to RAR-β agonists as a potential treatment for PDAC treatment [25]. Moreover, the micronutrient vitamin A is important for pancreatic health and could reduce pancreatic inflammation from asparaginase-associated pancreatitis [56]. Our data show that the doses of tamoxifen we used caused selective pancreatic acinar cell knockout of the RAR-β gene. We measured a lower level of cellular proliferation by Ki67 staining in the pancreata of the PC^{er};KRas;$RAR\text{-}\beta^{D}$ line compared to wild-type, and the lower expression of vimentin detected shows that the knockout of RARβ may influence the level of mesenchymal differentiation. The deletion of both alleles of RARβ was correlated with higher levels of CYP26A1 and lower levels of RBP1 and ALDH1A2, indicating that deletion of both alleles of RAR-β in pancreatic acinar cells alters RA metabolism. More studies are necessary to compare the effects of deletion of the other RA receptors (RAR-alpha and gamma) in the acinar cells of the pancreas.

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Disclosure of conflict of interest

None.

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