Endogenous n-3 Polyunsaturated Fatty Acids Delay Progression of Pancreatic Ductal Adenocarcinoma in Fat-1\textsuperscript{p48Cre/++}– LSL-Kras\textsuperscript{G12D/+} Mice\textsuperscript{1,2}


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Abstract
Preclinical studies suggest that diets rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs) may be beneficial for prevention of pancreatic cancer. Nutritional intervention studies are often complex, and there is no clear evidence, without potential confounding factors, on whether conversion of n-6 PUFAs to n-3 PUFAs in pancreatic tissues would provide protection. Experiments were designed using n-3 fatty acid desaturase (Fat-1) transgenic mice, which can convert n-6 PUFA to n-3 FAs endogenously, to determine the impact of n-3 PUFAs on pancreatic intraepithelial neoplasms (PanINs) and their progression to pancreatic ductal adenocarcinoma (PDAC). Six-week-old female p48\textsuperscript{Cre/++}–LSL-Kras\textsuperscript{G12D/+} and compound Fat-1–p48\textsuperscript{Cre/++}–LSL-Kras\textsuperscript{G12D/+} mice were fed (AIN-76A) diets containing 10% safflower oil for 35 weeks. Pancreata were evaluated histopathologically for PanINs and PDAC. Results showed a dramatic reduction in incidence of PDAC (84%; \textit{P} < .02) in Fat-1–p48\textsuperscript{Cre/++}–LSL-Kras\textsuperscript{G12D/+} mice compared to p48\textsuperscript{Cre/++}–LSL-Kras\textsuperscript{G12D/+} mice. Importantly, significant reductions of pancreatic ducts with carcinoma (90%; \textit{P} < .0001) and PanIN 3 (\textasciitilde 50%; \textit{P} < .001) lesions were observed in the compound transgenic mice. The levels of n-3 PUFA were much higher (>85%; \textit{P} < .05–0.01) in pancreas of compound transgenic mice than in those of p48\textsuperscript{Cre/++}–LSL-Kras\textsuperscript{G12D/+} mice. Molecular analysis of the pancreas showed a significant down-regulation of proliferating cell nuclear antigen, cyclooxygenase-2, 5-lipoxygenase (5-LOX), 5-LOX–activating protein, Bcl-2, and cyclin D1 expression levels in Fat-1–p48\textsuperscript{Cre/++}–LSL-Kras\textsuperscript{G12D/+} mice compared to p48\textsuperscript{Cre/++}–LSL-Kras\textsuperscript{G12D/+} mice. These data highlight the promise of dietary n-3 FAs for chemoprevention of pancreatic cancer in high-risk individuals.

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Introduction
Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive cancer with a dismal 5-year survival rate (<5%) and usually is diagnosed in an advanced stage with no curative therapies. Developing novel strategies to prevent or delay the progression of PDAC is of utmost importance. In 2012, more than 43,920 Americans will be diagnosed with pancreatic cancer and there will be about 37,390 deaths from
this disease [1]. The high mortality rate is due, in part, to difficulties in establishing an early and accurate diagnosis and, in part, to the lack of effective prevention strategies [2,3]. Current therapies for managing pancreatic cancer lack efficacy, and an urgent need remains for the development of novel strategies for prevention and treatment. Activating K-ras mutations, mostly at codon 12, occur in more than 95% of the patients with PDAC [2–4]. It is well known that the initiation of the disease begins with genetic alteration in the K-ras gene in the normal pancreatic ductal cell followed by inactivation of tumor suppressor genes (CDKN2A and p53) and activation of growth signaling pathways (epidermal growth factor receptor and insulin-like growth factor) [5,6]. Mice harboring a conditional K-ras mutant allele (LSL-Kras<sup>G12D<sup>+</sup>) in combination with a pancreas-specific Cre recombinase transgene (p<sup>48Cre<sup>+</sup>) develop a full range of premalignant pancreatic intraepithelial neoplasm (PanIN) lesions in the pancreas before succumbing to invasive PDAC and other tumors at late ages [7,8].

From a dietary perspective, published data indicate that bioactive lipids containing omega-3 polyunsaturated fatty acids (n-3 PUFAs) modulate a wide array of chronic diseases, including tumor development and its progression [9,10]. PUFAs are constituents of membrane phospholipids that serve as precursors for signaling eicosanoids including prostaglandins (PGs), thromboxanes, and leukotrienes (LTs) [11]. The principal PUFA, arachidonic acid (AA; C20:4n-6), is the source of PGE<sub>2</sub>, thromboxane, and leukotriene (LT) [12]. The principal PUFA, arachidonic acid (AA; C20:4n-6), is the source of PGE<sub>2</sub>, thromboxane, and leukotriene (LT) [12].

It has been estimated that the Western diet is deficient in n-3 PUFAs with a ratio of ~15:1 n-6 to n-3 PUFAs and this high n-6/n-3 ratio is a risk factor for many cancers [19]. The health benefits of n-3 PUFAs are thought to stem mainly from the EPA and DHA metabolites, which are the main precursors of the eicosanoids that contribute to anti-inflammatory processes [20,21]. Most of the preclinical studies using mouse models demonstrate that dietary intake of n-3 PUFAs (e.g., in the form of diets with a low n-6/n-3 PUFA ratio) reduces the incidence and growth of various cancers, including pancreatic cancer [22–25]. In chemically induced pancreatic carcinogenesis models, fish oils were capable of reducing the incidence of pancreatic cancers and hepatic metastases [25,26].

An important nutritional question as to whether the ratio of n-6: n-3 FAs plays a role in pancreatic tumorigenesis remains to be clarified in well-characterized experimental models of pancreatic cancer. Because 95% of human pancreatic cancers are associated with K-ras mutations, it is important to understand whether K-ras<sup>G12D</sup>-activated pancreatic cancers would be inhibited by increased n-3 FA without any experimental confounding factors. The recent engineered n-3 fatty acid desaturase (Fat-1) mice, which can convert n-6 to n-3 FAs and have a balanced ratio of n-6 to n-3 FAs in their tissues, allow carefully controlled studies to be performed in the absence of potential confounding dietary factors. The Fat-1 gene encodes an n-3 FA desaturase that catalyzes conversion of n-6 to n-3 FAs. This allows production of two different FA profiles (high vs. low n-6/n-3 ratios) in the animals without or with the Fat-1 gene by using just a single diet, thus eliminating the potential diet variations. Hence, the Fat-1 transgenic mouse is a valuable in vivo system for elucidating the role of n-3 FAs and the n-6/n-3 ratio in carcinogenesis. The p<sup>48Cre<sup>+</sup>-LSL-Kras<sup>G12D<sup>+</sup></sup> pancreatic cancer transgenic mice were bred with Fat-1 mice to generate compound Fat-1-p<sup>48Cre<sup>+</sup>-LSL-Kras<sup>G12D<sup>+</sup></sup> transgenic mice. In this study, we fed compound transgenic mice a high n-6-containing diet with 10% safflower oil to test whether endogenous n-3 PUFAs affect the incidence of PanINs and their progression to PDAC. We analyzed amounts of n-3 PUFAs, n-6/n-3 ratio, and various signaling molecules involved in the PDAC development.

**Materials and Methods**

**Animals, Diets, and Care**

All animal experiments were done in accordance with the institutional guidelines of the American Council of Animal Care. All the mice are in the C57BL/6 background. Required quantities of activated p<sup>48Cre<sup>+</sup>-Kras<sup>G12D<sup>+</sup></sup> [3,27,28], Fat-1, and Fat-1–Kras compound mice were generated as described. Animals were housed in ventilated cages under standardized conditions (21°C, 60% humidity, 12-hour light/12-hour dark cycle, 20 air changes/hour) in the University of Oklahoma Health Sciences Center Rodent Barrier Facility. Semipurified modified AIN-76A diet ingredients and safflower oil were purchased from Bioserv, Inc (Frenchtown, NJ). The 10% safflower oil replacing corn oil in the modified AIN-76A diet was blended using a Hobart mixer. Mice were allowed ad libitum access to the respective diets and to automated tap water purified by reverse osmosis.

**Breeding and Genotyping Analysis**

Fat-1, LSL-Kras<sup>G12D<sup>+</sup></sup>, and p<sup>48Cre<sup>+</sup></sup> mice were maintained in a C57BL/6 heterozygous genetic background. Fat-1, LSL-Kras<sup>G12D<sup>+</sup></sup>, and p<sup>48Cre<sup>+</sup></sup> mice were bred, and the offspring of female activated p<sup>48Cre<sup>+</sup>-LSL-Kras<sup>G12D<sup>+</sup></sup>. Fat-1-p<sup>48Cre<sup>+</sup>-LSL-Kras<sup>G12D<sup>+</sup></sup>, Fat-1, and C57BL/6 wild-type (WT) mice were generated at required quantities. Briefly, genomic DNA was extracted from snap-frozen tail tissue samples using the Miniprep Kit (Invitrogen, Carlsbad CA). Polymerase chain reaction (PCR) was performed for K-ras and Cre genes using the following conditions: denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. Oligonucleotide primer sequences used were given as follows: K-ras, 5′-CACCTGACACCCCGCACCCAGAG-3′ (sense) and 5′-AGCTGACACCACCATGGCTTGAGTAAGTCTGCA-3′ (antisense); Cre, 5′-ACCCGTAGCTGATGATCTTT-3′ (sense) and 5′-ACCTGAAGATGTTCCGAGATTCT-3′ (antisense). PCR was performed for Fat-1 gene using the following conditions: denaturation at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 62°C for 20 seconds, and 72°C for 2 minutes. Oligonucleotide primer sequences used for Fat-1 were given as follows: 5′-CTGCACCAACGATCCAGAGATGAG-3′ (sense) and 5′-ACAGACGATTCCAGATTGATGATCTTT-3′ (antisense). PCR products were separated on a 2% agarose gel. Successful recombination yields are 550-, 210-, 251-bp products for K-ras, Cre, and Fat-1 genes, respectively. The genotype of each pup was confirmed by tail DNA extraction and PCR as described elsewhere (Figure 1A) [3,27,28].

**Bioassay Study**

Genotyped female p<sup>48Cre<sup>+</sup>-LSL-Kras<sup>G12D<sup>+</sup></sup>, Fat-1-p<sup>48Cre<sup>+</sup>-LSL-Kras<sup>G12D<sup>+</sup></sup>, Fat-1 transgenic, and WT mice were used in the bioassay study. The experimental protocol is summarized in Figure 1B. Five-week-old mice were selected and randomized so that average body...
weights in each group were equal \((n = 12 \text{ p48Cre/+, LSL-Kras}^{G12D/+} \text{ mice/group, } n = 13 \text{ Fat-1.p48Cre/+, LSL-Kras}^{G12D/+} \text{ mice/group, and } n = 12 \text{ C57BL/6 WT mice/group})\), and mice were fed AIN-76A diet for 1 week. At 6 weeks of age, mice were fed experimental diets containing 10% safflower oil in the modified AIN-76A diet until termination of the study. The used 10% safflower oil diet has an n-6/n-3 FA ratio of 274, with an FA composition having 72 molar percent 18:2n-6 and no significant n-3 FAs. Previously, it was determined and subsequently confirmed in further studies that feeding a 10% safflower oil diet to Fat-1 mice effectively and efficiently alters the n-6/n-3 FA ratio of lipid compositions of measured tissues. This diet has reproducibly provided the desired effect on the FA composition of tissues of interest. Hence, we continued to use the 10% safflower oil diet for these Fat-1 studies [29–31]. Mice were checked routinely for signs of weight loss or any signs of toxicity or abnormalities. Food intake and body weight of each animal were measured once weekly for the first 6 weeks and then once a month until termination (Figure 1C). After 35 weeks (~9 months) on experimental diets, all mice were killed by CO2 asphyxiation and necropsied; pancreata were collected from all groups, weighed, and snap frozen in liquid nitrogen for further analysis.

Figure 1. (A) Genotyping of offspring of p48Cre/+, LSL-Kras\(^{G12D/+}\), and Fat-1 mice by PCR (arrows on top indicate mice with activated Kras and arrows on the bottom indicates mice with Fat-1 gene expression). (B) Experimental design for preventive efficacy evaluation of endogenous n-3 FAs in female p48Cre/+, LSL-Kras\(^{G12D/+}\) mice. At 6 weeks of age, groups (12 activated p48Cre/+, LSL-Kras\(^{G12D/+}\) or 13 Fat-1.p48Cre/+, LSL-Kras\(^{G12D/+}\)/group and 12 WT/group) of mice were fed AIN-76A diets containing 10% safflower oil continuously for 35 weeks and each pancreas was evaluated histopathologically for various marker expressions as described in the text. (C) Effect of n-3 FAs on body weight (means ± SE, \(N = 13\) or 12) at the termination of the experiment. No significant difference was observed between Fat-1–p48Cre/+, LSL-Kras\(^{G12D/+}\) and p48Cre/+, LSL-Kras\(^{G12D/+}\) mice or between WT and Fat-1 mice. However non-Kras–activated mice showed an increased body weight gain compared with Kras-activated mice.
Pancreata (head to tail) required for histopathologic and immunohistochemistry (IHC) evaluations, identification of PanIN lesions and PDAC, and evaluation of various molecular markers were fixed in 10% neutral-buffered formalin.

**Histologic Evaluation**

Formalin-fixed, paraffin-embedded tissues were sectioned (4 μm) and stained with hematoxylin and eosin (Figure 2, A–D). Sections of each pancreas were evaluated histologically by a pathologist blinded to the experimental groups as previously described [7,27,28]. PanIN lesions and carcinoma were classified according to histopathologic criteria as recommended elsewhere and our previous studies [7,27,28]. To quantify the progression of PanIN lesions, we determined the total number of ductal lesions and their grade.

**FA Analysis**

FA profiles were determined for pancreas. Total lipids were extracted following the method of Folch et al. [32] with modifications [33]. To each lipid extract, we added 15:0, 17:0, 23:0, and 30:3n-6 as internal standards. The lipid extracts were subjected to acid hydrolysis/methanolysis to generate fatty acid methyl esters (FAMEs) [33]. FAMEs were quantified using an Agilent Technologies 6890N gas chromatograph with flame ionization detector [34].

**Immunohistochemistry**

The effects of n-3 PUFAs on expression of proliferating cell nuclear antigen (PCNA), β-catenin, cyclooxygenase-2 (COX-2), Bcl-2, p21, p53, and caspase-3 were evaluated by immunohistochemistry as previously described [7,27,28]. The significance of the differences between groups was assessed by ANOVA with Bonferroni’s correction and the data were expressed as means ± SEM.

**Figure 2.** (A–D) Typical histologic appearance of the pancreas with/without PanIN/PDAC in Fat-1 (A), WT (B), Fat-1–p48Cre/+–LSL-KrasG12D/+ (C), and p48Cre/+–LSL-KrasG12D/+ (D) mice. No evidence of PanINs or PDAC was seen in the pancreas of Fat-1 or WT mice. (E) Pancreata of activated KrasG12D/+ (top) and WT (bottom) mice at 41 weeks of age. (F) Pancreata from p48Cre/+–LSL-KrasG12D/+ (top) or Fat-1–p48Cre/+–LSL-KrasG12D/+ (bottom) mice at 41 weeks of age. As shown in E and F (top), the pancreata from p48Cre/+–LSL-KrasG12D/+ mice were increased in size, weight and thickness compared with those from normal (E, bottom) and Fat-1–p48Cre/+–LSL-KrasG12D/+ mice (F, bottom). Expression of Fat-1 with Kras (Fat-1–p48Cre/+–LSL-KrasG12D/++) significantly decreased the size of the pancreas compared to Kras alone (p48Cre/+–LSL-KrasG12D/+). (G) Pancreas weights (means ± SE, N = 13 or 12) at the termination of the experiment. The significant differences between Fat-1–p48Cre/+–LSL-KrasG12D/+ and p48Cre/+–LSL-KrasG12D/++ mice or WT and Fat-1 mice were analyzed by unpaired t test with Welch’s correction. (H) Effect of Fat-1 gene expression on the incidence (percentage of mice with carcinomas) of PDAC. (I) Percentage of carcinoma spread per pancreas. (J) Effect of Fat-1 expression on the PanIN multiplicity (means ± SE). The data in H to J were analyzed by unpaired t test with Welch’s correction; values are considered statistically significant at P < .05.
and 5-lipoxygenase (5-LOX) were evaluated by IHC as described elsewhere. Briefly, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in phosphate-buffered saline (PBS). Antigen retrieval was carried out by heating sections in 0.01 M citrate buffer (pH 6) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H2O2 in PBS for 5 minutes. Nonspecific binding sites were blocked using Protein Block for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin–biotin complex reagent (Zymed Laboratories, Cambridge, MA; Cell Signaling Technology, Danvers, MA; Santa Cruz Biotechnology Santa Cruz, CA).

Apoptosis Assay

Paraffin sections of 5-μm thickness mounted on slides were rehydrated and stained using the Fragment End Labeling ( FragEL) DNA Fragmentation Detection Kit with the terminal deoxynucleotidyl transferase dUTP nick end labeling ( TUNEL) method following the manufacturer’s instructions ( Millipore, Billerica, MA). This kit allows the recognition of apoptotic nuclei in paraffin-embedded tissue sections fixed on slide by FragEL of DNA. The terminal deoxynucleotidyl transferase binds to exposed ends of DNA fragments generated in response to apoptotic signals and catalyzes the template-dependent addition of biotin-labeled and biotin-unlabeled deoxynucleotides. Biotinylated nucleotides are detected using streptavidin–HRP conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble colored product at the site of DNA fragmentation. Counterstaining with methyl green aids in the morphologic evaluation and characterization of normal and apoptotic cells. Stained apoptotic epithelial cells (a minimum of 10 microscopic fields per section) were counted manually in a single-blind fashion.

Western Blot Analysis of Protein Expression

Pancreata harvested from mice were homogenized and lysed in ice-cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT, and 1× protease inhibitor cocktail ( Sigma, St Louis, MO)]. After a brief vortexing, the lysates were separated by centrifugation at 12,000g for 15 minutes at 4°C, and protein concentrations were measured with the Bio-Rad Protein Assay reagent ( Bio-Rad, Hercules, CA). An aliquot (50 μg protein/lane) of the total protein was separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with 5% milk powder, membranes were probed for expression of COX-2, 5-LOX, β-catenin, Bcl-2, caspase-3, PCNA, and β-actin in hybridizing solution (1:500, in TBS–Tween 20 solution) using respective primary antibodies ( Cell Signaling Technology, AbCam, and Santa Cruz Biotechnology) and then probed with their respective HRP-conjugated secondary antibodies. Detection was performed using the SuperSignal West Pico Chemiluminescence procedure ( Pierce, Rockford, IL). The bands were captured on Ewen Parker Blue sensitive X-ray films and analyzed by densitometry.

Real-time Quantitative PCR for COX-2, 5-LOX, mPGES-1, and 5-LOX–activating Protein mRNA Expression

Total RNA from pancreas samples was extracted using the TRizol RNA Kit ( Invitrogen) as per the manufacturer’s instructions. Equal quantities of DNA-free RNA were used in reverse transcription ( RT) reactions for making cDNA using SuperScript Reverse Transcriptase ( Invitrogen). Quantitative PCR was performed for COX-2, 5-LOX, microsomal prostaglandin E synthase-1 ( mPGES-1), and 5-LOX–activating protein ( FLAP). The quantitative real-time PCR reaction mixtures (primers and cDNA), 2× RT SYBR master mix ( SABiosciences, Frederick, MD), and nuclease-free water were incubated for 40 cycles on a Bio-Rad IQ5 real-time PCR System ( Bio-Rad). Oligonucleotide primer sequences used for COX-2 were given as follows: 5’-AGAAGGGCTACCACACATCCAA-3’ (sense) and 5’-GGGTCGGGAGTGGGTATT-3’ (antisense). Oligonucleotide primer sequences used for 5-LOX were given as follows: 5’-GGACCTTCAGCATGGTGGTATG-3’ (sense) and 5’-GCTGGGCAGGGCTTACTTATTA-3’ (antisense). Oligonucleotide primer sequences used for mPGES-1 were given as follows: 5’-GGAAGCACTGAGACACCTAC-3’ (sense) and 5’-TCCAGGGCAGAAAAGGTTA-3’ (antisense). Oligonucleotide primer sequences used for FLAP were given as follows: 5’-GCGGACGTGATGTAACTGTT-3’ (sense) and 5’-GGTGACGTCTCTTCTCCTGC-3’ (antisense). Oligonucleotide primer sequences used for glyceraldehyde 3-phosphate dehydrogenase ( GAPDH) were given as follows: 5’-CCTCGTCTCCGATAGCAAAATG-3’ (sense) and 5’-TGAAGGGCTGTGGTTATGGCC-3’ (antisense). The cDNA samples were amplified at 95°C for 3 minutes, 95°C for 30 seconds (5-LOX, mPGES-1, FLAP) and 1 minute (COX-2), 60°C for 25 seconds (5-LOX, mPGES-1, FLAP), and 55°C for 35 seconds (COX-2) for a total of 40 cycles. The comparative C_t method was used to compute relative levels of target mRNAs by subtracting the C_t values of the endogenous control (GAPDH). Given that the relative value of the calibrator is 1.0, the other samples were n-fold relative to the calibrator.

RT-PCR for p21, Cyclin D1, and Bcl-2 mRNA Expression

Total RNA from pancreas samples was extracted using the TRizol RNA Kit ( Invitrogen) as per the manufacturer’s instructions. Equal quantities of DNA-free RNA were used in RT reactions for making cDNA using SuperScript Reverse Transcriptase ( Invitrogen). RT-PCR reactions were performed for p21, cyclin D1, and Bcl-2 using the Taq polymerase, 10 mM deoxyribonucleotide triphosphates ( dNTP), respective primers [3,28], and buffers from Invitrogen. For cyclin D1, denaturation at 94°C for 3 minutes was followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. Oligonucleotide primer sequences used for the cyclin D1 gene were given as follows: 5’-ATGGAAACCACTCTCTTGTC-3’ (sense) and 5’-ACC-TCCAGCATCCAGTGGCG-3’ (antisense). For p21, denaturation at 94°C for 3 minutes was followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. Oligonucleotide primer sequences for the p21 gene were given as follows: 5’-TCCGCACTCGAGGTGGC-3’ (sense) and 5’-TCGCTTTTTTCGCGCTAG-3’ (antisense). For Bcl-2, denaturation at 94°C for 2 minutes was followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. Oligonucleotide primer sequences used for Bcl-2 were

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given as follows: 5′-CCAAGATGCAAAGCACATCC-3′ (sense) and 5′-CCCCGCTCGTTATCTCCTG-3′ (antisense). The PCR products were visualized and photographed under UV illumination and analyzed by densitometry.

Statistical Analysis

The data are presented as means ± SE. Differences in body weights were analyzed by analysis of variance. Statistical differences between control and treated groups were evaluated using Fisher’s exact test for PDAC incidence, and unpaired *t* test with Welch’s correction was used for PanIN and PDAC lesions. Differences between groups are considered significant at *P* < .05.

Results

General Observations

The p48Cre+/−–LSL-KrasG12D/+ and the Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice fed the 10% safflower oil experimental diets had similar body weight gains at the end of the experiment. However, WT and Fat-1 mice had slightly higher body weight gains (*P* < .05) in comparison with the p48Cre+/−–LSL-KrasG12D/+ and the Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice (Figure 1C). None of the animals fed the experimental diets exhibited any observable toxicity or any gross changes attributable to liver, kidney, or lung toxicity despite notable difference in the pancreatic weights as described below.

Expression of Fat-1 Gene Delays the Progression of PanIN Lesions to PDAC

To test whether increasing the pancreatic tissue levels of n-3 FAs in Fat-1 transgenic mice may be associated with a lower risk for tumorigenesis than in p48Cre+/−–LSL-KrasG12D/+ mice, we evaluated tumorigenesis and analyzed PUFA in the pancreas. C57BL/6 WT and Fat-1 mice fed AIN-76A diet containing 10% safflower oil showed no evidence of PanIN lesions or carcinoma (Figure 2, A and B); however, Kras-activated mice showed 100% penetrance with PanIN lesions and carcinoma (Figure 2, C and D). Significant difference in the size and texture of the pancreata was noticed among C57BL/6 WT, Fat-1, p48Cre+/−–LSL-KrasG12D/+, and Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice (Figure 2, E and F). Pancreases from C57BL/6 WT and Fat-1 mice fed AIN-76A diet containing 10% safflower oil weighed about 0.17 to 0.3 g (Figure 2G). However, the pancreas from p48Cre+/−–LSL-KrasG12D/+ mice weighed 0.6 g, almost two-fold more than the pancreas from WT mice. A significant decrease in pancreas weights (−22%; *P* < .02) was observed in Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice (Figure 2, F and G). To assess effect of Fat-1 gene on K-ras–activated pancreatic tumorigenesis, we measured the PDAC incidence and the extent of pancreas invaded by carcinoma. As shown in Figure 2, H and I, there is a marked difference in the incidence of PDAC formation and degree of invasion between p48Cre+/−–LSL-KrasG12D/+ and Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice. Fat-1–p48Cre+/−–LSL-KrasG12D/+ and p48Cre+/−–LSL-KrasG12D/+ mice spontaneously develop pancreatic cancer arising from progression of PanINs through low-grade PanINs (1A and 1B) to high-grade PanINs (PanIN-2 and PanIN-3) (Figure 2, C and D). Figure 2H summarizes the effectiveness of endogenous n-3 PUFAs on PDAC incidence by comparison of Fat-1–p48Cre+/−–LSL-KrasG12D/+ and p48Cre+/−–LSL-KrasG12D/+ mice.

Over an observation period of 41 weeks of age, the p48Cre+/−–LSL-KrasG12D/+ mice had an incidence of 50% (percentage of mice with PDAC), whereas Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice showed an incidence of 8% PDAC (*P* < .02), as determined with histologic analysis (*P* < .02). The p48Cre+/−–LSL-KrasG12D/+ mice showed about 22% of the pancreata involved with invasive ductal carcinoma, whereas Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice showed only about 1% of the pancreata involved with invasive ductal carcinoma (Figure 2I). In addition, p48Cre+/−–LSL-KrasG12D/+ mice developed, on the average, about 182 PanIN-1, 215 PanIN-2, and 192 PanIN-3 lesions, whereas Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice had a significant increase in PanIN-1 lesions (288) and significant inhibition of PanIN-3 (98) lesions (Figure 2J).

FA Profiles of Pancreatic Tissues of Fat-1–p48Cre+/−–LSL-KrasG12D/+, p48Cre+/−–LSL-KrasG12D/+, Fat-1, and C57BL/6 Mice

Analysis of the total lipids extracted from the pancreata showed distinct lipid profiles among Fat-1–p48Cre+/−–LSL-KrasG12D/+, p48Cre+/−–LSL-KrasG12D/+, Fat-1, and WT mice (Table 1). There are significantly higher levels of n-6 FAs (20:4n-6 (AA) and 22:5n-6) and much lower concentrations of n-3 FAs (18:3n-3, 20:5n-3 [EPA], 22:5n-3 [DPA], and 22:6n-3 [DHA]) in the pancreas derived from WT and p48Cre+/−–LSL-KrasG12D/+ mice than in the pancreata from Fat-1–p48Cre+/−–LSL-KrasG12D/+ and Fat-1 mice. The ratios of the C ≥ 20 n-6 FA, AA (20:4n-6), to the n-3 FAs (EPA, DPA, and DHA) were 43.8, 0.23, 27, and 1.02 in WT, Fat-1, p48Cre+/−–LSL-KrasG12D/+, and Fat-1–p48Cre+/−–LSL-KrasG12D/+ transgenic mice, respectively (Table 1). Notably, we observed that p48Cre+/−–LSL-KrasG12D/+–induced pancreatic tumors showed significant blockage of chain elongation steps > C:20 FAs (Table 1).

Table 1. Profiles of Polyunsaturated n-6 and n-3 FAs in Pancreas from WT, Fat-1, Kras, and Fat-1–Kras Transgenic Mice.

<table>
<thead>
<tr>
<th>PUFAs</th>
<th>WT</th>
<th>Kras</th>
<th>Fat-1</th>
<th>Fat-1–Kras</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.04 ± 0.16</td>
<td>1.71 ± 0.15</td>
<td>1.31 ± 0.18</td>
<td>1.92 ± 0.22</td>
</tr>
<tr>
<td>16:0</td>
<td>23.21 ± 0.10</td>
<td>22.50 ± 2.58</td>
<td>19.63 ± 1.44</td>
<td>21.35 ± 1.95</td>
</tr>
<tr>
<td>18:1</td>
<td>5.11 ± 1.29</td>
<td>8.54 ± 0.42</td>
<td>7.48 ± 1.62</td>
<td>9.05 ± 1.96</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>23.63 ± 0.87</td>
<td>3.40 ± 1.05</td>
<td>3.20 ± 0.51</td>
<td>2.87 ± 0.38</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>22.73 ± 2.72</td>
<td>24.05 ± 2.96</td>
<td>30.35 ± 1.68</td>
<td>25.88 ± 3.70</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>26.19 ± 3.17</td>
<td>32.70 ± 2.98</td>
<td>32.62 ± 3.79</td>
<td>34.97 ± 4.01</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.11 ± 0.02</td>
<td>0.14 ± 0.04</td>
<td>0.09 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.02 ± 0.00</td>
<td>0.03 ± 0.02</td>
<td>0.00 ± 0.00</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.01 ± 0.01</td>
<td>0.00 ± 0.00</td>
<td>0.33 ± 0.55***</td>
<td>0.45 ± 0.13***</td>
</tr>
<tr>
<td>20:5n-6</td>
<td>0.27 ± 0.03</td>
<td>0.30 ± 0.06</td>
<td>0.13 ± 0.04</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>10.52 ± 1.47</td>
<td>4.35 ± 1.69</td>
<td>0.46 ± 0.15**</td>
<td>1.21 ± 0.60**</td>
</tr>
<tr>
<td>20:5n-6</td>
<td>0.55 ± 0.07</td>
<td>0.76 ± 0.32</td>
<td>0.13 ± 0.04</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.71 ± 0.12</td>
<td>0.60 ± 0.24</td>
<td>0.05 ± 0.02</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.03 ± 0.00</td>
<td>0.02 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.28 ± 0.17**</td>
</tr>
<tr>
<td>AA/EPA + DPA + DHA</td>
<td>43.8</td>
<td>27</td>
<td>0.23*</td>
<td>1.02*</td>
</tr>
</tbody>
</table>

Total lipids of the pancreas were extracted and FAMEs were subjected to analysis by gas chromatography. The values (molar percent of total FAs) are means of six measurements ± SD (*N* = 3–6). The significant differences of important FAs between p48Cre+/−–LSL-KrasG12D/+ and Fat-1 or Fat-1–p48Cre+/−–LSL-KrasG12D/+ mice were analyzed by unpaired *t* test with Welch’s correction. Values are means ± SE (*N* = 3–6). *P* ≤ 0.05 to 0.01, **P* ≤ 0.01 to 0.001, and ***P* ≤ 0.001 to 0.0001.
Inhibition of Proliferation and Induction of Apoptosis by Endogenous n-3 PUFAs in PanINs and Carcinoma of the Pancreas

Figure 3 summarizes the effects of n-3 PUFAs on tumor cell proliferation as measured by fraction of PCNA-positive cells (labeling index). PCNA labeling indices were significantly lowered in the pancreas of Fat-1–p48 CRE/LSL-Kras G12D/+ animals (Figure 3, B and C) compared with pancreas from p48 CRE/LSL-Kras G12D/+ mice. Quantification of the PCNA staining showed 85 ± 3.6 (mean ± SEM) PCNA labeling index in p48 CRE/LSL-Kras G12D/+ PDAC compared with 20 ± 1.4 PCNA labeling index in Fat-1–p48 CRE/LSL-Kras G12D/+ PDAC, accounting for a decrease in the proliferation index by ~75% (P < 0.0001) (Figure 3C). Figure 3, D to F, summarizes the effects of n-3 PUFAs on tumor cell apoptosis. Qualitative microscopic examination of TUNEL-stained sections showed a substantial increase in TUNEL-positive cells in the pancreatic tissue of Fat-1–p48 CRE/LSL-Kras G12D/+ mice compared with that of p48 CRE/LSL-Kras G12D/+ mice. The quantification of apoptotic staining showed 12.8 ± 1.7 (mean ± SEM) TUNEL-positive cells (apoptotic index) in PDAC from p48 CRE/LSL-Kras G12D/+ mice compared with 18.20 ± 3.4 (mean ± SEM) TUNEL-positive cells in PDAC from Fat-1–p48 CRE/LSL-Kras G12D/+ mice, accounting for an increase in the apoptotic index of ~29% (P < 0.0001) (Figure 3F). Figure 3, G and H, summarizes the effects of n-3 PUFAs on p21 expression in PanIN lesions and in ductal cells. A significant increase in expression of p21 was seen in the pancreatic tissues of Fat-1–p48 CRE/LSL-Kras G12D/+ mice.

Differential Expression and/or Modulation of COX-2, 5-LOX, β-catenin, Bcl-2, Caspase-3, PCNA, p21, Cyclin D1, Bcl-2, mPGES-1, and FLAP in the Pancreata of Transgenic Mice

Because COX-2 and 5-LOX play critical roles in AA metabolism and Wnt/β-catenin pathways are active in pancreatic tumor development [2,3,5,6], we examined the expression of these pathways in Fat-1–expressing mice. To determine whether Fat-1 expression decreases
COX-2 and 5-LOX, thereby modulating several related signaling molecules and exerting a role in anticancer effects, we analyzed protein and mRNA expression profiles by IHC, Western immunoblot analysis, and RT and/or real-time PCR (Figures 4, 5, and W2). Pancreatic tissues from Fat-1–p48Cre/+–LSL-KrasG12D/+ mice exhibited significantly reduced protein expressions of COX-2, 5-LOX, PCNA, Bcl-2, and β-catenin compared with pancreatic tissues from p48Cre/+–LSL-KrasG12D/+ mice (Figures 4, A–D, 5, A and B, and W2). In addition, caspase-3 and p21 expressions were slightly higher in the pancreata of Fat-1–p48Cre/+–LSL-KrasG12D/+ mice compared with p48Cre/+–LSL-KrasG12D/+ mice (Figures 3, G and H, and W2).

The real-time PCR results showed a significant decrease in COX-2, mPGES-1, 5-LOX, and FLAP mRNA expressions in the pancreatic tumor tissues of Fat-1–p48Cre/+–LSL-KrasG12D/+ (Figure 4, E), further confirming the results observed by IHC and Western immunoblot analysis. RT-PCR analysis also demonstrated that pancreatic tissues from Fat-1–p48Cre/+–LSL-KrasG12D/+ mice exhibited significant decreases in cyclin D1 and Bcl-2 with an increase in p21 expressions (Figure 5, C and D). These results support the immunohistologic and/or Western immunoblot analysis observations.

Discussion
There is increasing evidence that dietary intake of n-3 FAs suppresses progression of breast, prostate, and colon cancers, and a number of epidemiological and preclinical studies implicate mostly a protective role of n-3 PUFAs against pancreatic cancer [18–20,35–43]. However, variable diets were a confounder in the earlier studies, and there is limited understanding of how dietary FAs may affect the process of pancreatic carcinogenesis particularly Kras-activated tumor progression. In an attempt to eliminate dietary variables, we compared the incidence and characteristics of pancreatic tumors in WT and K-ras transgenic mice with and without co-expression of Fat-1, which increases the percentage of endogenous n-3 PUFAs. All animals were maintained on identical diets. Previously, decreased tumor growth rates in N-nitrosobis(2-oxopropyl)amine–induced pancreatic cancer were observed in hamsters fed a high n-3 fat diet [27]. To date, several of the in vivo studies regarding the effect of different PUFA-rich diets during pancreatic carcinogenesis have used carcinogen-induced cancers with limited ductal tumor cell origin and not much associated with K-ras mutations [26,27,44]. The, relevance of these chemically induced pancreatic models to human pancreatic cancer, which is predominately ductal cell origin and >95% associated with K-ras mutations, is less. In that context, transgenic models are highly relevant to human pancreatic cancer. Recently, Strouch et al. have shown that a high n-3 fat diet mitigates pancreatic precancer by inhibition of cellular proliferation through induction of cell cycle arrest and apoptosis in the EL-Kras mice and human pancreatic cancer cell lines [35]. To our knowledge, this is the first study to evaluate the effects of endogenous n-3 PUFA derived from n-6 PUFA on transgenic mice that develop a distinct PanIN to PDAC paradigm. The most striking finding is that there is a dramatic inhibition of PDAC incidence and a decreased frequency of PanIN-3 formation and their progression to PDAC in Fat-1–p48Cre/+–LSL-KrasG12D/+ transgenic mice. These results clearly support the notion that

Figure 4. Immunohistochemical analysis of the effect of Fat-1 expression on AA metabolism in pancreatic tumors. Immunohistochemical analysis was performed with paraffin-embedded and microsectioned pancreatic tissues as described in Materials and Methods section. Immunohistochemical analysis of COX-2 (A and B) and 5-LOX (C and D) expressions in PanIN lesions/PDAC. A and C represent pancreas from p48Cre/+–LSL-KrasG12D/+ mice, and B and D represents pancreas from Fat-1–p48Cre/+–LSL-KrasG12D/+ mice. A significant decrease in the expression of COX-2 and 5-LOX was seen in the pancreatic tissues of Fat-1–p48Cre/+–LSL-KrasG12D/+ mice. (E) Effect of Fat-1 expression on the mRNA expression levels of COX-2, mPGES-1, 5-LOX, and FLAP as determined by real-time PCR (Kras represents p48Cre/+–LSL-KrasG12D/+ and Fat-1–Kras represents Fat-1–p48Cre/+–LSL-KrasG12D/+). A significant decrease in the expression of COX-2, mPGES-1, 5-LOX, and FLAP was seen in the pancreatic tissues of Fat-1–p48Cre/+–LSL-KrasG12D/+ mice.
increased tissue levels of n-3 FAs can reduce pancreatic tumor progression (Figure 2). Furthermore, our data show that the cellular proliferation rate in PanIN lesions and PDAC was significantly decreased in the Fat-1−p48Cre/+–LSL-KrasG12D/+ transgenic mice (Figures 3 and 5).

The COX-2/5-LOX and Wnt/β-catenin signaling pathways are active in pancreatic cancers [2,3,5,6]. There is constitutively high expression and activation of COX-2 and β-catenin in human PanIN lesions and PDAC. COX-2 activation enhances the production of PGs from AA that subsequently promote pancreatic inflammation and neoplasia. Therefore, we postulate that strategies aimed at simultaneous disruption of the COX-2/5-LOX and Wnt/β-catenin signaling pathways may produce effective chemopreventive and antitumorigenic effects. Our work shows that Fat-1 expression affects cell growth and the production of eicosanoids that are likely to contribute to cancer cell growth by effectively decreasing COX-2/5-LOX and β-catenin signaling pathways. This study supports the possibility that inhibition of these signaling pathways by n-3 PUFAs contributes to the significant decrease in tumor development with Fat-1 expression.

The endogenous n-3 PUFAs are likely to play a role both in retarding the transformation process, which leads to the initial formation of pancreatic precursors because of K-ras mutation, and then in dampening the proliferative ability of the precancerous lesions once they develop. Previous findings in prostate, mammary, skin, and colon precancerous conditions demonstrated that administration of n-3 PUFA-rich diets suppressed tumor progression [40–43]. Our results extend this idea to pancreatic cancer development in a preclinical setting that recapitulates human disease and support previously identified in vitro and in vivo effects of n-3 PUFAs during pancreatic carcinogenesis. One of the notable changes accompanying the reduction of PDAC formation is a significantly lower n-6/n-3 ratio, a decrease in tumor cell proliferation, and an increase in apoptosis associated with alteration in various signaling molecules as evidenced by RT-PCR, IHC, and/or Western blot analysis.

This genetic approach of modifying FA composition by converting n-6 to n-3 FAs endogenously not only effectively increases the absolute amount of n-3 FAs but also significantly decreases the level of n-6 FAs (Table 1 and Figure W1), leading to a balanced ratio of n-6 to n-3 FAs in the pancreas. The increase in apoptosis (Figure 3) is noteworthy because apoptosis is progressively inhibited during PanIN progression to PDAC development. Therefore, it is possible that the observed protective effect of n-3 PUFA is due, in part, to the enhanced deletion of cells through the activation of targeted apoptosis [45].

More than 80% of PDAC patients show insulin resistance and type 2 diabetes, and epidemiological studies in humans showed that populations traditionally consuming high levels of long-chain n-3 PUFAs display a lower prevalence of type 2 diabetes [46]. Interestingly, data from a clinical study suggest that the positive influence of long-chain n-3 PUFA supplementation on insulin sensitivity is greater in obese populations that display an inflammatory phenotype [47]. These data lend support to our findings, which indicate that the anti-inflammatory actions of n-3 PUFAs in metabolic tissues are a key to the positive impact of long-chain n-3 PUFAs on insulin sensitivity. A clinical study showed that patients with pancreatic cancer given approximately 2 g of EPA and 1 g of DHA per day for 7 weeks
showed significant weight gain and improvement in functional status and appetite [48]. The results presented here show that an increased n-3 PUFA level in the pancreas is associated with diminished inflammatory changes in the organ. Particularly noteworthy in this context is the reduction of COX-2 and 5-LOX, because these contribute to morbidity and prognosis of the disease. However, detailed studies on insulin signaling and glucose metabolism are warranted.

Dietary n-3 PUFAs are well known for both their anti-inflammatory and tumor-suppressing properties. Important n-3 PUFAs involved in human nutrition are α-linolenic acid, EPA, and DHA [36]. Furthermore, the synthesis of longer chain n-3 FAs from linolenic acid is competitively slowed by n-6 analogs. Therefore, the concentration of long-chain n-3 PUFAs in tissues is enhanced when they are directly ingested or when the competing amounts of n-6 FAs are relatively small. The lipid data in Fat-1–p45<sup>Cat</sup>–LSL-Kras<sup>G12D</sup> mice (Table 1 and Figure W1) demonstrate significantly decreased levels of n-6 PUFAs, particularly AA in the pancreas. Activation of Kras signaling leads to blockage of long-chain FA synthesis and, notably, accumulation of C20:4 AA, the most important substrate for COX-2 and 5-LOX–mediated protumorigenetic metabolites. In contrast, in the presence of Fat-1 gene, there are significantly lower levels of AA and higher n-3 PUFA present in pancreatic tissues, even with activated Kras signaling. In addition, EPA is a precursor of resolvins E1 and resolvin E2, the potent anti-inflammatory substances described and characterized recently [49]. Future studies are needed to address the role of resolvins in pancreatic cancer.

Several other signaling pathways also affected by Fat-1 expression may contribute to prevention of tumor development and progression. EPA and DHA also induce cancer cell apoptosis and decrease of Bcl-2 expression [41], and Fat-1 gene expression in tumor cells reduces protein expression of β-catenin and cyclin D1 as well [41]. Our work (Figures 4, 5, and W2), as well as other studies [50], show that n-3 PUFAs block tumor cell growth at least in part through inhibition of Wnt/β-catenin and COX-2 signaling pathways [50]. The higher n-3 PUFA in the pancreatic tissues of Fat-1 gene expressing Kras mice therefore significantly inhibited the progression of PanIN lesions to carcinoma in part through cell cycle arrest and targeted apoptosis in the mutant Kras cells. The potential reasons for this could be the modulatory effects of n-3 PUFA on the accumulated AA because of Kras mutation by inhibiting COX-2 and 5-LOX pathways, thereby reducing tumor cell proliferation and increasing apoptosis.

In summary, this is the first exploration of the function of the Fat-1 gene in pancreatic tumor cells in vivo. We have shown that Fat-1 gene expression inhibits tumor cell proliferation, induces tumor cell apoptosis, and alters AA metabolism. Our results suggest that endogenous n-3 PUFAs delay the progression of PanIN-1 and PanIN-2 to PanIN-3 and PDAC. Elevating n-3 PUFAs may be an important strategy to delay/prevent pancreatic cancer in high-risk patients.

Acknowledgments
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References


Figure W1. Effect of Fat-1 expression on AA. A significant decrease in the AA synthesis was observed in the pancreatic tissues of Fat-1–p48Cre+–LSL-KrasG12D/+ mice.

Figure W2. Immunohistochemical analysis of the effect of Fat-1 expression on expression of Bcl-2 and β-catenin in pancreatic tumors. Immunohistochemical analysis was performed with paraffin-embedded and microsectioned pancreatic tissues as described in Materials and Methods section. Immunohistochemical analysis of Bcl-2 (A and B) and β-catenin (C and D) expressions in PanIN lesions/PDAC. A and C represent pancreas from p48Cre+–LSL-KrasG12D/+ mice, and B, D, and F represent pancreas from Fat-1–p48Cre+–LSL-KrasG12D/+ mice. A significant decrease in the expression of Bcl-2 and β-catenin was seen in the pancreatic tissues of Fat-1–p48Cre+–LSL-KrasG12D/+ mice.