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Endogenous n-3 Polyunsaturated Fatty Acids Delay Progression of Pancreatic Ductal Adenocarcinoma in Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ Mice[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$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and compound Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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%; $P < .02$) in Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice compared to p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice. Importantly, significant reductions of pancreatic ducts with carcinoma (90%; $P < .0001$) and PanIN 3 (~50%; $P < .001$) lesions were observed in the compound transgenic mice. The levels of n-3 PUFA were much higher (>85%; $P < .05$–0.01) in pancreas of compound transgenic mice than in those of p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice compared to p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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

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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 (*CDNK2A* 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-$Kras^{G12D/+}$) in combination with a pancreas-specific Cre recombinase transgene ($p48^{Cre/+}$) 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_2 and LTB_4, which have been shown to promote tumor growth and metastasis, whereas n-3 PUFAs, eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA)–derived eicosanoids, show tumor-suppressive effects [12–14]. Evidence suggests that the n-6:n-3 FA ratio, rather than the absolute levels of these two classes of PUFAs, is the primary factor that influences the tumorigenesis [15–19].

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, type 3 series eicosanoids (e.g., PGE_3) compared to AA metabolites of proinflammatory type 2 series eicosanoids (e.g., PGE_2) [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^{G12D}–activated pancreatic cancers would be inhibited by increased n-3 FA without any experimental confounding factors. The recently 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 $p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ pancreatic cancer transgenic mice were bred with Fat-1 mice to generate compound Fat-1–$p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ 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 $p48^{Cre/+}$–$Kras^{G12D/+}$ [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-$Kras^{G12D/+}$, and $p48^{Cre/+}$ mice were maintained in a C57BL/6 heterozygous genetic background. Fat-1, LSL-$Kras^{G12D/+}$, and $p48^{cre/+}$ mice were bred, and the offspring of female activated $p48^{Cre/+}$–LSL-$Kras^{G12D/+}$, Fat-1–$p48^{Cre/+}$–LSL-$Kras^{G12D/+}$, 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′-CCTTTACAAGCGCACGCAGAG-3′ (sense) and 5′-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3′ (antisense) and Cre, 5′-ACCGTCAGTACGTGAGATATCTT-3′ (sense) and 5′-ACCTGAAGATGTTCGCGATTATCT-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′-CTGCACCACGCCTTCACCAACC-3′ (sense) and 5′-ACACAGCAGATTCCAGAGATT-3′ (antisense). PCR products were separated on a 2% agarose gel. Successful recombination yields are 550-, 210-, 251-bp products for *Kras*, *Cre*, and *Fat-1* genes, respectively. The genotype of each pup was confirmed by tail DNA extraction and PCR as described elsewhere (Figure 1*A*) [3,27,28].

Bioassay Study

Genotyped female $p48^{Cre/+}$–LSL-$Kras^{G12D/+}$, Fat-1–$p48^{Cre/+}$–LSL-$Kras^{G12D/+}$, Fat-1 transgenic, and WT mice were used in the bioassay study. The experimental protocol is summarized in Figure 1*B*. Five-week-old mice were selected and randomized so that average body

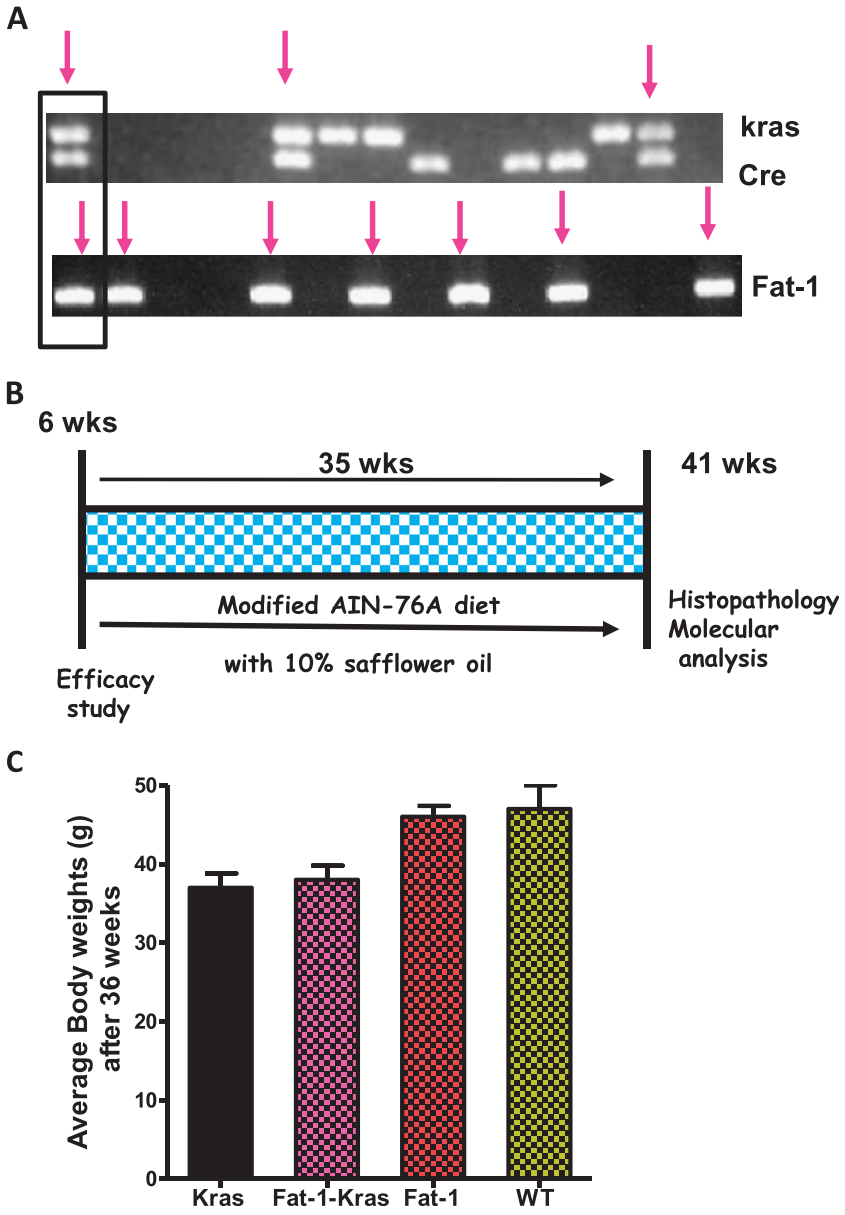

Figure 1. (A) Genotyping of offspring of p48$^{Cre/+}$, LSL-Kras$^{G12D/+}$, and Fat-1 mice by PCR (arrows on top indicate mice with activated Kras and arrows on the bottom indicates mice with *Fat-1* gene expression). (B) Experimental design for preventive efficacy evaluation of endogenous n-3 FAs in female p48$^{Cre/+}$–LSL-K-Ras$^{G12D/+}$ mice. At 6 weeks of age, groups (12 activated p48$^{Cre/+}$.LSL-Kras$^{G12D/+}$ or 13 Fat-1.p48$^{Cre/+}$.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–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and p48$^{Cre/+}$–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.

weights in each group were equal (n = 12 p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice/group, n = 13 Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice/group, and n = 12 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 1*C*). After 35 weeks (~9 months) on experimental diets, all mice were killed by CO_2 asphyxiation and necropsied; pancreata were collected from all groups, weighed, and snap frozen in liquid nitrogen for further analysis.

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 separated from other lipids (primarily cholesterol) by TLC on Silica Gel 60 plates using a solvent system of 80:20 hexane/ether. 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,

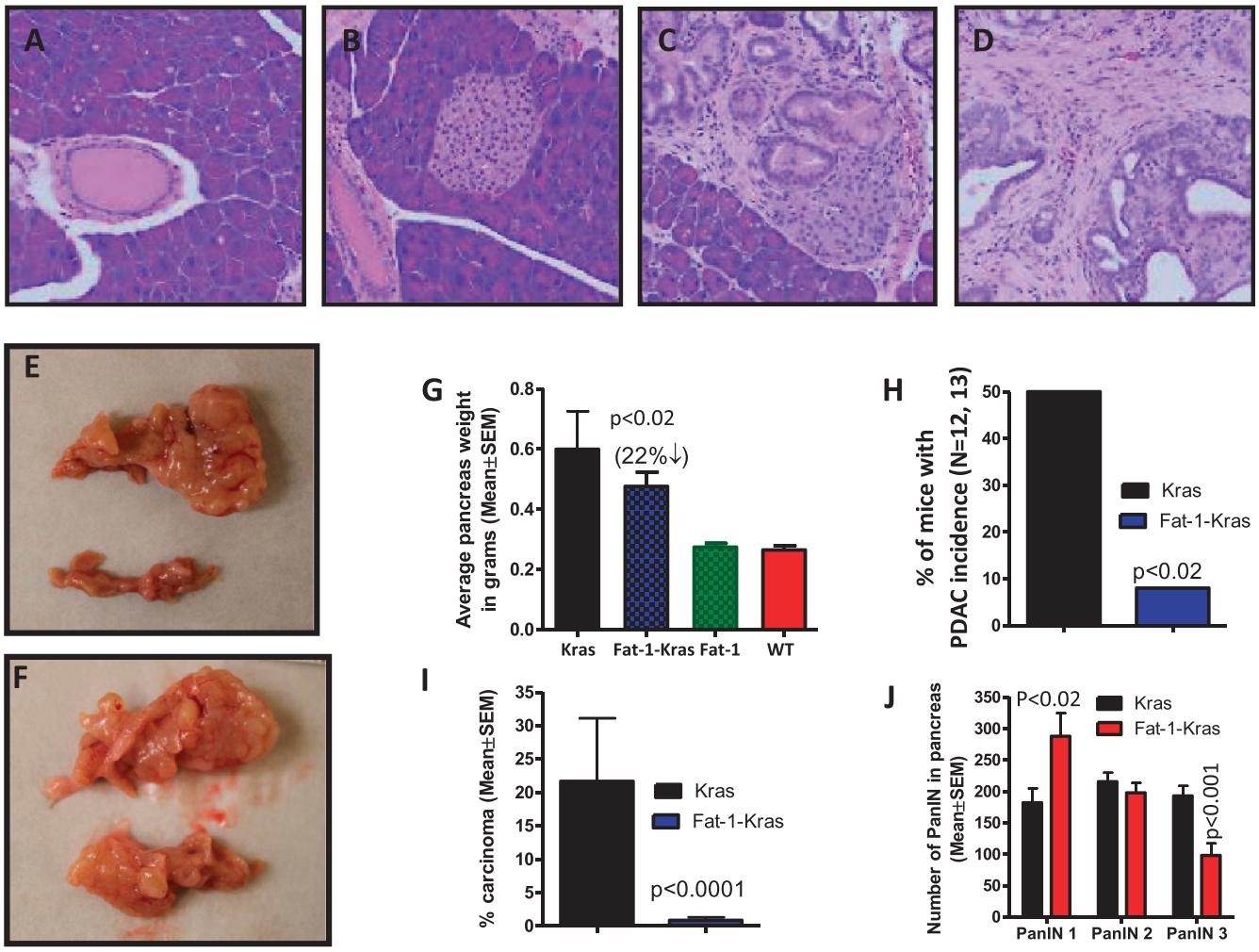

Figure 2. (A–D) Typical histologic appearance of the pancreas with/without PanIN/PDAC in Fat-1 (A), WT (B), Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ (C), and p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ (D) mice. No evidence of PanINs or PDAC was seen in the pancreas of Fat-1 or WT mice. (E) Pancreata of activated Kras$^{G12D/+}$ (top) and WT (bottom) mice at 41 weeks of age. (F) Pancreata of p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ (top) or Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ (bottom) mice at 41 weeks of age. As shown in E and F (top), the pancreata from p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice were increased in size, weight and thickness compared with those from normal (E, bottom) and Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice (F, bottom). Expression of Fat-1 with Kras (Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$) significantly decreased the size of the pancreas compared to Kras alone (p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$). (G) Pancreas weights (means ± SE, $N = 13$ or 12) at the termination of the experiment. The significant differences between Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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% H_2O_2 in PBS for 5 minutes. Nonspecific binding sites were blocked using Protein Block for 20 minutes. Sections then were incubated overnight at 4°C with 1:300 dilutions of monoclonal antibodies against PCNA, β-catenin, COX-2, Bcl-2, p21, and pAKT (AbCam, 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, Camarillo, CA). After rinsing with PBS, the slides were incubated with the chromogen 3,3′-diaminobenzidine for 3 minutes, then rinsed and counterstained with hematoxylin. Nonimmune rabbit immunoglobulins were substituted for primary antibodies as negative controls. Slides were observed under an Olympus microscope 1X701, and digital computer images were recorded with an Olympus DP70 camera.

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 12,000*g* 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× RT2 SYBR master mix (SABiosciences, Frederick, MD), and nuclease-free water were incubated for 40 cycles on a Bio-Rad IQ-5 real-time PCR System (Bio-Rad). Oligonucleotide primer sequences used for COX-2 were given as follows: 5′-AGAAACGGCTACCACATCCAA-3′ (sense) and 5′-GGGTCGGGAGTGGGTAATTT-3′ (antisense). Oligonucleotide primer sequences used for 5-LOX were given as follows: 5′-GGACCTCAGCATGTGGTATG-3′ (sense) and 5′-GCTGGGTCAGGGGTACTTTA-3′ (antisense). Oligonucleotide primer sequences used for mPGES-1 were given as follows: 5′-GGAACGACATGGAGACCATCTAC-3′ (sense) and 5′-TCCAGGCGACAAAAGGGTTA-3′ (antisense). Oligonucleotide primer sequences used for FLAP were given as follows: 5′-GCCGGACTGATGTACCTGTT-3′ (sense) and 5′-GGTGAGCGTCCTTCTCTGTC-3′ (antisense). Oligonucleotide primer sequences used for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were given as follows: 5′-CCTCGTCCCGTAGACAAAATG-3′ (sense) and 5′-TGAAGGGGTCGTTGATGGC-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′-ATGGAACACCAGCTCCTGTG-3′ (sense) and 5′-ACCTCCAGCATCCAGGTGGC-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 used for p21 were given as follows: 5′-TCCTGGTGATGTCCGACCTG-3′ (sense) and 5′-TCCGTTTTCGGCCCTGAG-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

given as follows: 5′-CCAAGAATGCAAAGCACATCC-3′ (sense) and 5′-CCCAGCCTCCGTTATCCTG-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 p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and the Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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 p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and the Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice (Figure 1*C*). 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 p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$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, p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, and Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice (Figure 2, *E* and *F*). Pancreas 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 2*G*). However, the pancreas from p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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 p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice. Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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 2*H* summarizes the effectiveness of endogenous n-3 PUFAs on PDAC incidence by comparison of Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice. Over an observation period of 41 weeks of age, the p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice had an incidence of 50% (percentage of mice with PDAC), whereas Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice showed an incidence of 8% PDAC ($P < .02$), as determined with histologic analysis ($P < .02$). The p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice showed about 22% of the pancreas involved with invasive ductal carcinoma, whereas Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice showed only about 1% of the pancreas involved with invasive ductal carcinoma (Figure 2*I*). In addition, p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice developed, on the average, about 182 PanIN-1, 215 PanIN-2, and 192 PanIN-3 lesions, whereas Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice had a significant increase in PanIN-1 lesions (288) and significant inhibition of PanIN-3 (98) lesions (Figure 2*J*).

FA Profiles of Pancreatic Tissues of Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, Fat-1, and C57BL/6 Mice

Analysis of the total lipids extracted from the pancreata showed distinct lipid profiles among Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, 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 [docosapentaenoic acid (DPA)], and 22:6n-3 [DHA]} in the pancreas derived from WT and p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice than in the pancreata from Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and Fat-1 mice. The ratios of the $C \geq 20$ 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, p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, and Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ transgenic mice, respectively (Table 1). Notably, we observed that p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$–induced pancreatic tumors showed significant blockage of chain elongation steps > C:20 FAs (Table 1). In addition, p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mouse pancreas showed significantly higher levels of AA (C20:4) compared with Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mouse pancreas (Figure W1).

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

PUFAs	WT	Kras	Fat-1	Fat-1–Kras
14:0	1.04 ± 0.16	1.71 ± 0.15	1.31 ± 0.18	1.92 ± 0.22
16:0	23.21 ± 0.10	22.50 ± 2.58	19.63 ± 1.44	21.35 ± 1.95
16:1	5.11 ± 1.29	8.54 ± 0.42	7.48 ± 1.62	9.05 ± 1.96
18:0	8.36 ± 0.74	3.40 ± 1.05	3.20 ± 0.51	2.87 ± 0.38
18:1	22.73 ± 2.72	24.05 ± 2.96	30.35 ± 1.68	25.88 ± 3.70
18:2n-6	26.19 ± 3.17	32.70 ± 2.98	32.62 ± 3.79	34.97 ± 4.01
18:3n-6	0.11 ± 0.02	0.14 ± 0.04	0.09 ± 0.02	0.12 ± 0.02
18:3n-3	0.02 ± 0.00	0.03 ± 0.02	1.95 ± 0.82	0.33 ± 0.21
20:5n-3	0.01 ± 0.01	0.00 ± 0.00	1.33 ± 0.55***	0.45 ± 0.38***
20:3n-6	0.27 ± 0.03	0.30 ± 0.06	0.13 ± 0.04	0.19 ± 0.04
20:4n-6	10.52 ± 1.47	4.35 ± 1.69	0.46 ± 0.15**	1.21 ± 0.60**
22:4n-6	0.55 ± 0.07	0.76 ± 0.32	0.13 ± 0.04	0.25 ± 0.09
22:5n-6	0.71 ± 0.12	0.60 ± 0.24	0.05 ± 0.02	0.12 ± 0.03
22:5n-3	0.03 ± 0.00	0.02 ± 0.01	0.21 ± 0.07*	0.28 ± 0.17*
22:6n-3	0.20 ± 0.03	0.14 ± 0.07	0.41 ± 0.18	0.45 ± 0.21*
n-3 ≥ C20	0.23 ± 0.04	0.16 ± 0.07	2.01 ± 0.77*	1.21 ± 0.80*
n-6 ≥ C20	12.16 ± 1.62	6.08 ± 2.28	0.85 ± 0.23**	1.82 ± 0.75**
n-6/n-3	113.98	219.64	9.95***	34.75***
AA/EPA + DPA + DHA	43.8	27	0.23*	1.02*

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 separate measurements ± SD (N = 3–6). The significant differences of important FAs between p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and Fat-1 or Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice were analyzed by unpaired *t* test with Welch's correction. Values are means ± SE (N = 3–6). *$P \leq 0.05$ to 0.01, **$P \leq 0.01$ to 0.001, and ***$P < 0.001$ to 0.0001.

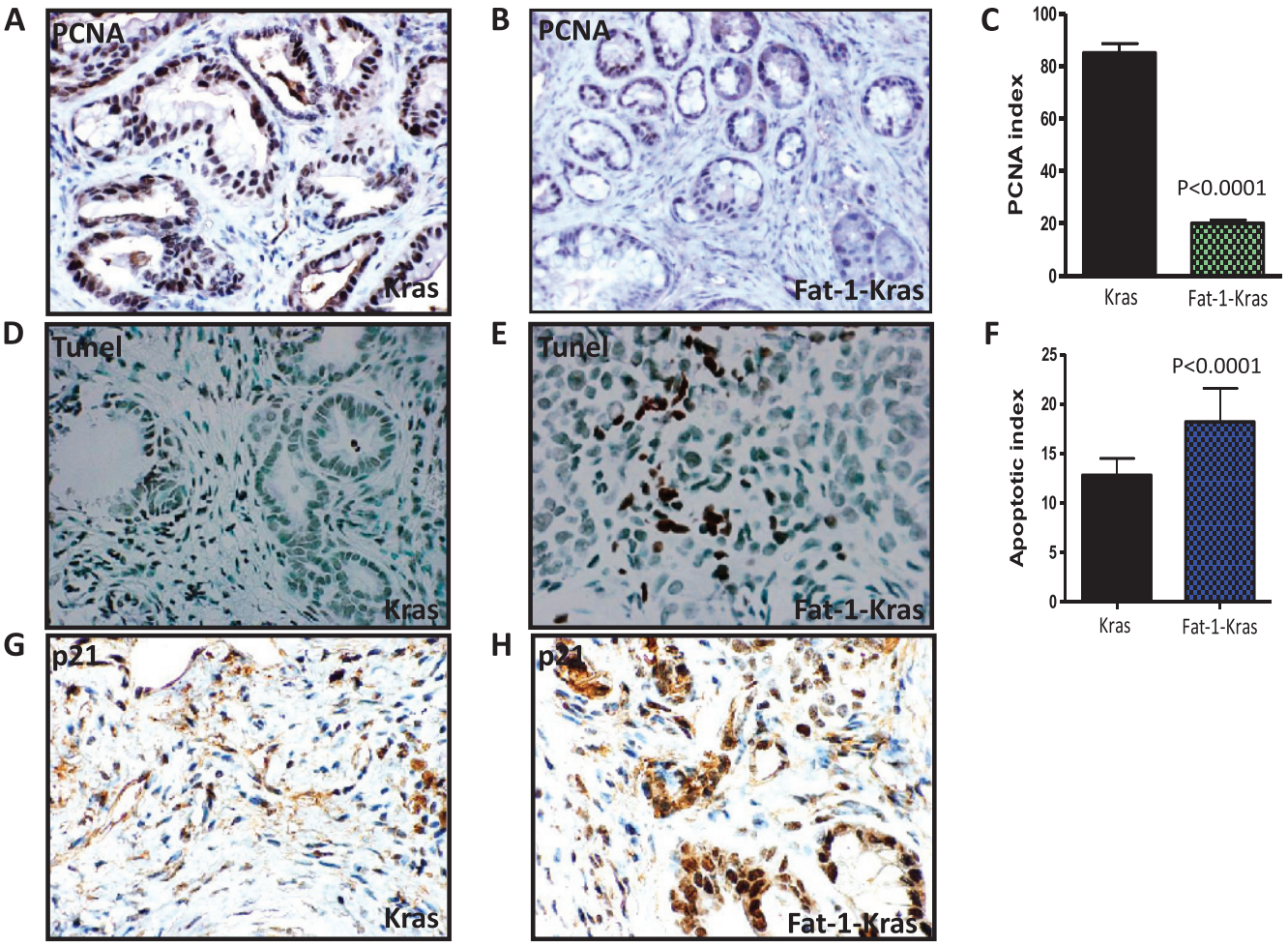

Figure 3. Effect of Fat-1 expression on cell proliferation (A–C), apoptosis (D–F), and p21 (G and H) in pancreatic tumors. Immunohistochemical analysis was performed with paraffin-embedded and microsectioned pancreatic tissues as described in Materials and Methods section. A, D, and G represent pancreas from $p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ mice, and B, E, and H represent pancreas from Fat-1–$p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ mice. (A and B) Immunohistochemical analysis of PCNA expression in PanIN lesions and in ductal cells. (C) Significant differences in proliferative indices were observed between the groups. (D–F) Effect of Fat-1 expression on apoptosis in pancreatic tissues by TUNEL assay. A significant increase in TUNEL-positive cells was observed in pancreatic lesions and PDAC because of Fat-1 expression in mice. (F) Significant differences were observed in apoptotic indices between PanIN and PDAC of Fat-1–$p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ and $p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ mice. (G and H) Immunohistochemical analysis of 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.

Inhibition of Proliferation and Induction of Apoptosis by Endogenous n-3 PUFAs in PanINs and Carcinoma of the Pancreas

Figure 3 (*A–C*) 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-$Kras^{G12D/+}$. 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% ($P < .0001$) (Figure 3*C*). 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% ($P < .0001$) (Figure 3*F*). 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 the expression of p21 was seen in the pancreatic tissues of Fat-1–$p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ mice (Figure 3, *G* and *H*).

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–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice exhibited significantly reduced protein expressions of COX-2, 5-LOX, PCNA, Bcl-2, and β-catenin compared with pancreatic tissues from p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice compared with p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice (Figures 3, *G* and *H*, and 5*A*).

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–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ (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–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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*-nitroso*bis*(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–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ transgenic mice. These results clearly support the notion that

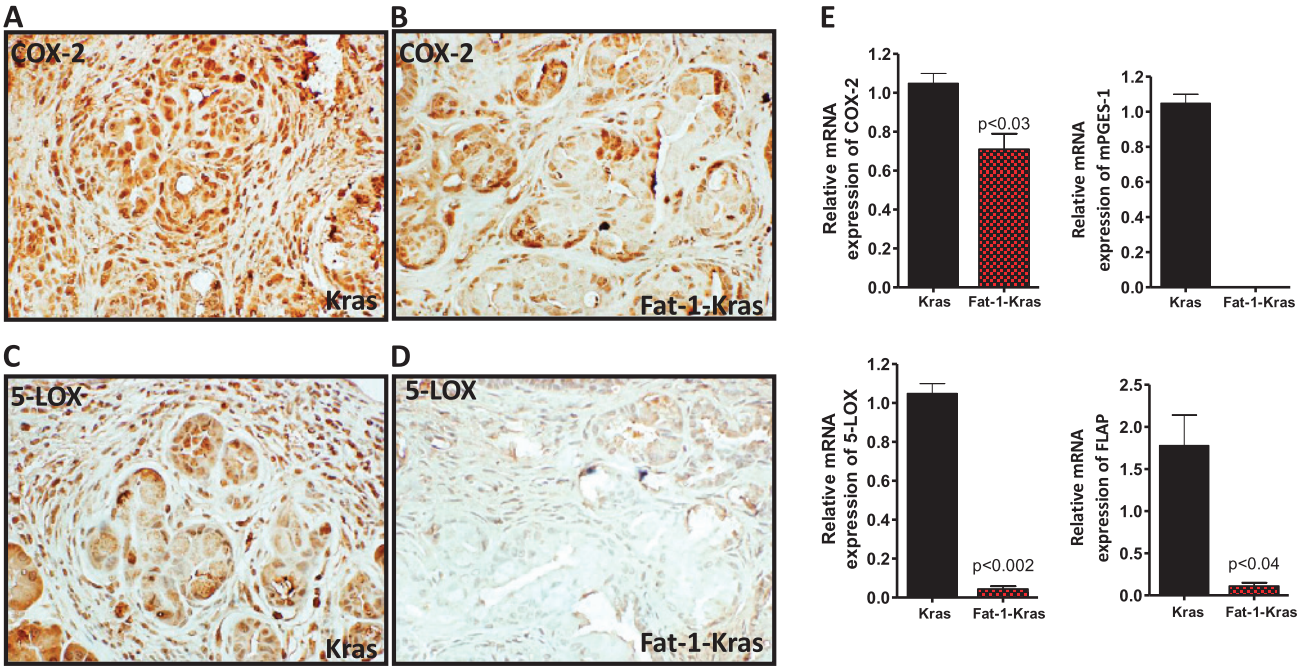

Figure 4. Immunohistochemical analysis of the effect of Fat-1 expression on AA metabolism in pancreatic tumors. Immunohistochemical analysis was performed with paraffin-embedded and microsectioned pancreatic tissues as described in Materials and Methods section. Immunohistochemical analysis of COX-2 (A and B) and 5-LOX (C and D) expressions in PanIN lesions/PDAC. A and C represent pancreas from p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice, and B and D represents pancreas from Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice. A significant decrease in the expression of COX-2 and 5-LOX was seen in the pancreatic tissues of Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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 p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ and Fat-1–Kras represents Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$). A significant decrease in the expression of COX-2, mPGES-1, 5-LOX, and FLAP was seen in the pancreatic tissues of Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice.

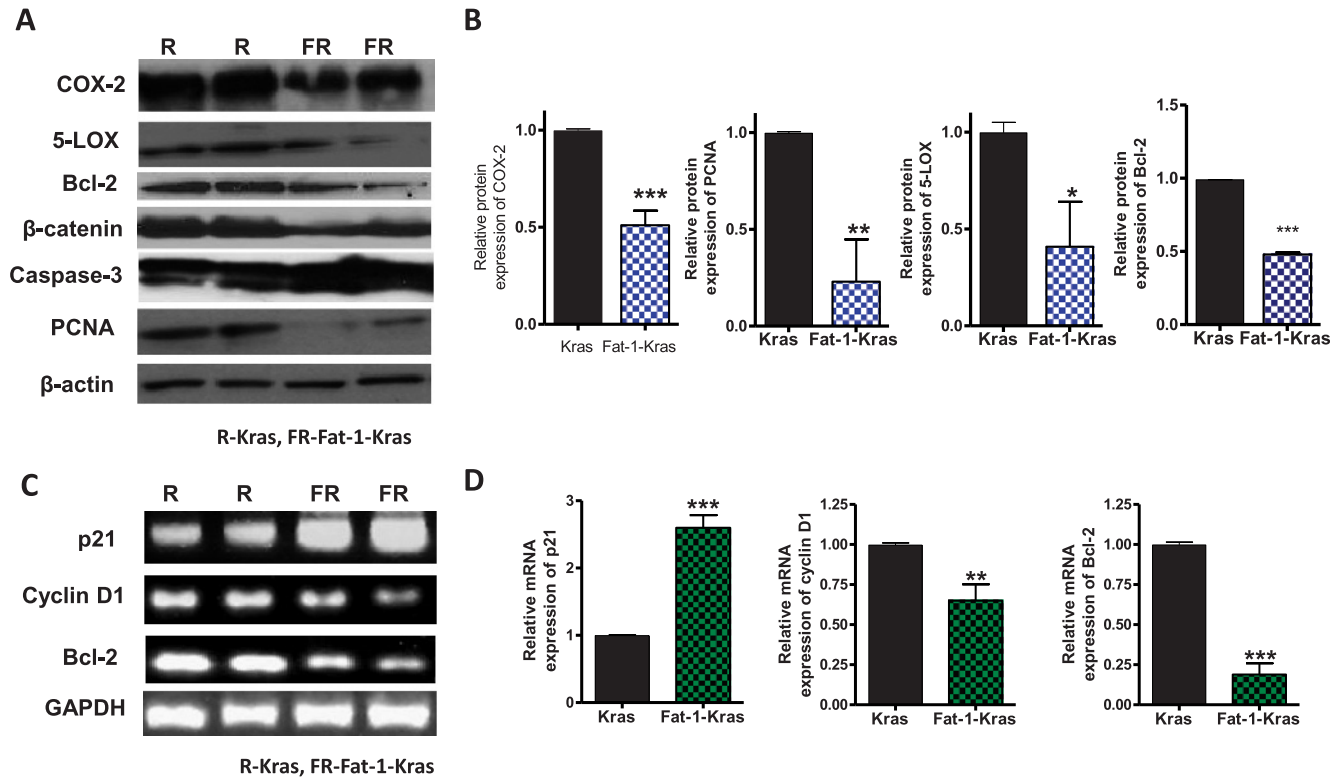

Figure 5. (A and B) Western blot analysis (A) and densitometry (B) quantification of the effect of Fat-1 expression on expression of COX-2, 5-LOX, β-catenin, caspase-3, and PCNA in PDAC (R represents p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, FR represents Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, and $*P = .05$, $**P < .05$–0.01, $***P < .001$–0.0001, respectively). (C and D) RT-PCR (C) and densitometry (D) quantification of the effect of Fat-1 expression on the mRNA expression levels of p21, cyclin D1, and Bcl-2 in the pancreatic tumor tissue (R represents p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, FR represents Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$, and $*P < .05$, $**P < .05$–0.01, $***P < .001$–0.0001, respectively).

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–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ 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–$p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ 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 protumorigenic 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 resolvin 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

[1] American Cancer Society (2012). *Cancer Facts and Figures (2012)*. American Cancer Society, Atlanta, GA.

[2] Eckel F, Schneider G, and Schmid RM (2006). Pancreatic cancer: a review of recent advances. *Expert Opin Investig Drugs* **15**, 1395–1410.

[3] Mohammed A, Janakiram NB, Lightfoot S, Gali H, Vibhudutta A, and Rao CV (2012). Early detection and prevention of pancreatic cancer: use of genetically engineered mouse models and advanced imaging technologies. *Current Med Chem* **19**(22), 3701–3713.

[4] Ren YX, Xu GM, Li ZS, and Song YG (2004). Detection of point mutation in K-ras oncogene at codon 12 in pancreatic diseases. *World J Gastroenterol* **10**, 881–884.

[5] Hruban RH, Adsay NV, Albores-Saavedra J, Anver MR, Biankin AV, Boivin GP, Furth EE, Furukawa T, Klein A, Klimstra DS, et al. (2006). Pathology of genetically engineered mouse models of pancreatic exocrine cancer: consensus report and recommendations. *Cancer Res* **66**, 95–106.

[6] Mazur PK and Siveke JT (2011). Genetically engineered mouse models of pancreatic cancer: unravelling tumour biology and progressing translational oncology. *Gut* **61**, 1488–1500.

[7] Mohammed A, Janakiram NB, Li Q, Madka V, Ely M, Lightfoot S, Steele VE, and Rao CV (2010). The epidermal growth factor receptor inhibitor gefitinib prevents the progression of pancreatic lesions to carcinoma in a conditional LSL-$Kras^{G12D/+}$ transgenic mouse model. *Cancer Prev Res* **3**, 1417–1426.

[8] Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, et al. (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* **4**, 437–450.

[9] Clinton SK and Giovannucci E (1998). Diet, nutrition, and prostate cancer. *Annu Rev Nutr* **18**, 413–440.

[10] Kolonel LN (1997). Fat and cancer. The epidemiologic evidence in perspective. *Adv Exp Biol Med* **422**, 1–19.

[11] James MJ, Gibson RA, and Cleland LG (2000). Dietary polyunsaturated fatty acids and inflammatory mediator production. *Am J Clin Nutr* **71**, 343S–348S.

[12] Rose DP and Connolly JM (2000). Regulation of tumor angiogenesis by dietary fatty acids and eicosanoids. *Nutr Cancer* **37**, 119–127.

[13] Ge Y, Chen ZH, Kang ZB, Brown J, Laposata M, and Kang JX (2002). Effects of adenoviral gene transfer of *C. elegans* n-3 fatty acid desaturase on the lipid profile and growth of human breast cancer cells. *Anticancer Res* **22**, 537–543.

[14] Yang P, Chan D, Felix E, Cartwright C, Menter DG, Madden T, Klein RD, Fischer SM, and Newman RA (2004). Formation and antiproliferative effect of prostaglandin E_3 from eicosapentaenoic acid in human lung cancer cells. *J Lipid Res* **45**, 1030–1039.

[15] Gago-Dominguez M, Yuan JM, Sun CL, Lee HP, and Yu MC (2003). Opposing effects of dietary n-3 and n-6 fatty acids on mammary carcinogenesis: the Singapore Chinese Health Study. *Br J Cancer* **89**, 1686–1692.

[16] Maillard V, Bougnoux P, Ferrari P, Jourdan ML, Pinault M, Lavillonniere F, Body G, Le Floch O, and Chajès V (2002). N-3 and N-6 fatty acids in breast adipose tissue and relative risk of breast cancer in a case-control study in Tours, France. *Int J Cancer* **98**, 78–83.

[17] Xia S, Wang JD, and Kang JX (2005). Decreased n-6/n-3 fatty acid ratio reduces the invasive potential of human lung cancer cells by downregulation of cell adhesion/invasion-related genes. *Carcinogenesis* **26**, 779–784.

[18] Granados S, Quiles JL, Gil A, and Ramirez-Tortosa MC (2006). Dietary lipids and cancer. *Nutr Hosp* **21**(2), 42–54.

[19] Simopoulos AP (2006). Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomed Pharmacother* **60**, 502–507.

[20] Larsson SC, Kumlin M, Ingelman-Sundberg M, and Wolk A (2004). Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am J Clin Nutr* **79**, 935–945.

[21] Smith WL (2005). Cyclooxygenases, peroxide tone and the allure of fish oil. *Curr Opin Cell Biol* **17**, 174–182.

[22] Kelavkar UP, Hutzley J, Dhir R, Kim P, Allen KG, and McHugh K (2006). Prostate tumor growth and recurrence can be modulated by the omega-6:omega-3 ratio in diet: athymic mouse xenograft model simulating radical prostatectomy. *Neoplasia* **8**, 112–124.

[23] Kobayashi N, Barnard RJ, Henning SM, Elashoff D, Reddy ST, Cohen P, Leung P, Hong-Gonzalez J, Freedland SJ, Said J, et al. (2006). Effect of altering dietary omega-6/omega-3 fatty acid ratios on prostate cancer membrane composition, cyclooxygenase-2, and prostaglandin E2. *Clin Cancer Res* **12**, 4662–4670.

[24] Swamy MV, Citineni B, Patlolla JM, Mohammed A, Zhang Y, and Rao CV (2008). Prevention and treatment of pancreatic cancer by curcumin in combination with omega-3 fatty acids. *Nutr Cancer* **60**(1), 81–89.

[25] Simopoulos AP (2000). Human requirement for N-3 polyunsaturated fatty acids. *Poultry Sci* **79**, 961–970.

[26] Heukamp I, Gregor JI, Kilian M, Kiewert C, Jacobi CA, Schimke I, Walz MK, Guski H, and Wenger FA (2006). Influence of different dietary fat intake on liver metastasis and hepatic lipid peroxidation in BOP-induced pancreatic cancer in Syrian hamsters. *Pancreatology* **6**, 96–102.

[27] Mohammed A, Qian L, Janakiram NB, Lightfoot S, Steele VE, and Rao CV (2012). Atorvastatin delays progression of pancreatic lesions to carcinoma by regulating PI3/AKT signaling in $p48^{Cre/+}$ LSL-$Kras^{G12D/+}$ mice. *Int J Cancer* **131**, 1951–1962.

[28] Rao CV, Mohammed A, Janakiram NB, Qian L, Ritchie RL, Lightfoot S, Vibhudutta A, and Rao CV (2012). Inhibition of pancreatic intraepithelial neoplasia progression to carcinoma by nitric oxide–releasing aspirin in $p48^{Cre/+}$–LSL-$Kras^{G12D/+}$ mice. *Neoplasia* **14**(9), 778–787.

[29] Tanito M, Brush RS, Elliott MH, Wicker LD, Henry KR, and Anderson RE (2009). High levels of retinal membrane docosahexaenoic acid increase susceptibility to stress-induced degeneration. *J Lipid Res* **50**, 807–819.

[30] Li F, Marchette LD, Brush RS, Elliott MH, Davis KR, Anderson AG, and Anderson RE (2010). High levels of retinal docosahexaenoic acid do not protect photoreceptor degeneration in VPP transgenic mice. *Mol Vis* **16**, 1669–1679.

[31] Tanito M, Brush RS, Elliott MH, Wicker LD, Henry KR, and Anderson RE (2010). Correlation between tissue docosahexaenoic acid levels and susceptibility to light-induced retinal degeneration. *Adv Exp Med Biol* **664**, 567–573.

[32] Folch J, Lees M, and Sloan-Stanley GH (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**, 497–509.

[33] Li F, Marchette LD, Brush RS, Elliott MH, Le YZ, Henry KA, Anderson AG, Zhao C, Sun X, Zhang K, et al. (2009). DHA does not protect ELOVL4 transgenic mice from retinal degeneration. *Mol Vis* **13**(15), 1185–1193.

[34] Ford DA, Monda JK, Brush RS, Anderson RE, Richards MJ, and Fliesler SJ (2008). Lipidomic analysis of the retina in a rat model of Smith–Lemli–Opitz syndrome: alterations in docosahexaenoic acid content of phospholipid molecular species. *J Neurochem* **105**(3), 1032–1047.

[35] Strouch MJ, Ding Y, Salabat MR, Melstrom LG, Adrian K, Quinn C, Pelham C, Rao S, Adrian TE, Bentrem DJ, et al. (2011). A high omega-3 fatty acid diet mitigates murine pancreatic precancer development. *J Surg Res* **165**(1), 75–81.

[36] Arshad A, Al-Leswas D, Stephenson J, Metcalfe M, and Dennison A (2011). Potential applications of fish oils rich in n-3 fatty acids in the palliative treatment of advanced pancreatic cancer. *Br J Nutr* **106**, 795–800.

[37] MacLean CH, Newberry SJ, Mojica WA, Khanna P, Issa AM, Suttorp MJ, Lim YW, Traina SB, Hilton L, Garland R, et al. (2006). Effects of omega-3 fatty acids on cancer risk: a systematic review. *JAMA* **295**, 403–415.

[38] Barber MD, Fearon KC, Tisdale MJ, McMillan DC, and Ross JA (2011). Effect of a fish oil-enriched nutritional supplement on metabolic mediators in patients with pancreatic cancer cachexia. *Nutr Cancer* **40**, 118–124.

[39] Moses AW, Slater C, Preston T, Barber MD, and Fearson KC (2004). Reduced total energy expenditure and physical activity in cachectic patients with pancreatic cancer can be modulated by an energy and protein dense oral supplement enriched with n-3 fatty acids. *Br J Cancer* **90**, 996–1002.

[40] Horia E and Watkins BA (2007). Complementary actions of docosahexaenoic acid and genistein on COX-2, PGE2 and invasiveness in MDA-MB-231 breast cancer cells. *Carcinogenesis* **28**, 809–815.

[41] Lu Y, Nie D, Witt WT, Chen Q, Shen M, Xie H, Lai L, Dai Y, and Zhang J (2008). Expression of the fat-1 gene diminishes prostate cancer growth *in vivo* through enhancing apoptosis and inhibiting GSK-3β phosphorylation. *Mol Cancer Ther* **7**, 3203–3211.

[42] Boudreau MD, Sohn KH, Rhee SH, Lee SW, Hunt JD, and Hwang DH (2001). Suppression of tumor cell growth both in nude mice and in culture by n-3 polyunsaturated fatty acids: mediation through cyclooxygenase-independent pathways. *Cancer Res* **61**, 1386–1391.

[43] Rose DP and Connolly JM (1999). Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol Ther* **83**, 217–244.

[44] O'Connor T, Roebuck B, Peterson F, Lokesh B, Kinsella JE, and Campbell TC (1989). Effect of dietary omega-3 and omega-6 fatty acids on development of azaserine-induced preneoplastic lesions in rat pancreas. *J Natl Cancer Inst* **81**, 858–863.

[45] Park KS, Lim JW, and Kim H (2009). Inhibitory mechanism of omega-3 fatty acids in pancreatic inflammation and apoptosis. *Ann N Y Acad Sci* **1171**, 421–427.

[46] Ebbesson SO, Risica PM, Ebbesson LO, Kennish JM, and Tejero ME (2005). Omega-3 fatty acids improve glucose tolerance and components of the metabolic syndrome in Alaskan Eskimos: the Alaska Siberia project. *Int J Circumpolar Health* **64**, 396–408.

[47] Browning LM, Krebs JD, Moore CS, Mishra GD, O'Connell MA, and Jebb SA (2007). The impact of long chain n-3 polyunsaturated fatty acid supplementation on inflammation, insulin sensitivity and CVD risk in a group of overweight women with an inflammatory phenotype. *Diabetes Obes Metab* **9**, 70–80.

[48] Barber M and Fearon K (2001). Tolerance and incorporation of a high-dose eicosapentaenoic acid diester emulsion by patients with pancreatic cancer cachexia. *Lipids* **36**, 347–351.

[49] Janakiram NB and Rao CV (2009). Role of lipoxins and resolvins as anti-inflammatory and proresolving mediators in colon cancer. *Cur Mol Med* **9**(5), 565–579.

[50] Lim K, Han C, Dai Y, Shen M, and Wu T (2009). Omega-3 polyunsaturated fatty acids inhibit hepatocellular carcinoma cell growth through blocking β-catenin and COX-2. *Mol Cancer Ther* **8**(11), 3046–3055.

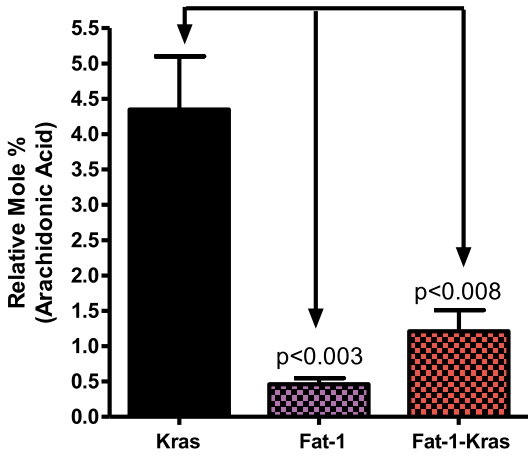

Figure W1. Effect of Fat-1 expression on AA. A significant decrease in the AA synthesis was observed in the pancreatic tissues of Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice.

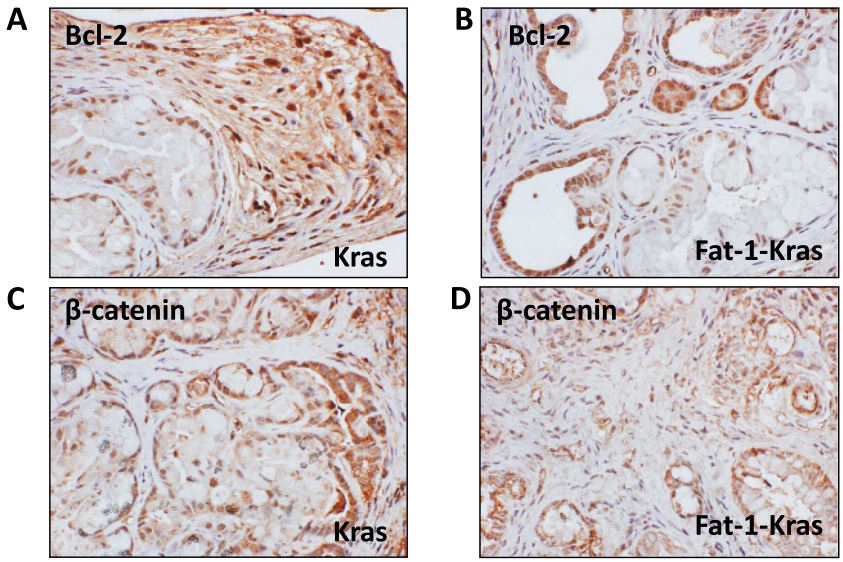

Figure W2. Immunohistochemical analysis of the effect of Fat-1 expression on expression of Bcl-2 and β-catenin in pancreatic tumors. Immunohistochemical analysis was performed with paraffin-embedded and microsectioned pancreatic tissues as described in Materials and Methods section. Immunohistochemical analysis of Bcl-2 (A and B) and β-catenin (C and D) expressions in PanIN lesions/PDAC. A and C represent pancreas from p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice, and B, D, and F represent pancreas from Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice. A significant decrease in the expression of Bcl-2 and β-catenin was seen in the pancreatic tissues of Fat-1–p48$^{Cre/+}$–LSL-Kras$^{G12D/+}$ mice.