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Consumption of High ω-3 fatty Acid Diet Suppressed Prostate Tumorigenesis in C3(1) Tag Mice

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Consumption of high ω-3 fatty acid diet suppressed prostate tumorigenesis in C3(1) Tag mice

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Prostate cancer incidence and mortality are high in the Western world and high ω-6/ω-3 PUFA in the Western diet may be a contributing factor. We investigated whether changing from a diet that approximates ω-6 fat content of the Western diet to a high ω-3 fat diet at adulthood might reduce prostate cancer risk. Female SV 129 mice that had consumed a high ω-6 diet containing corn oil for 2 weeks were bred with homozygous C3(1)Tag transgenic male mice. All male offspring were weaned to the corn oil diet (CO) until postpuberty when half of the male offspring were transferred to a high ω-3 diet containing canola oil and fish oil concentrate (FS). High ω-3 diet increased ω-3 and decreased ω-6 fat content of mice tissues. Average weights of prostate and genitourinary bloc were significantly lower in mice consuming high ω-3 diet at adulthood (CO-FS) than mice fed a lifetime high ω-6 diet (CO–CO). There was slower progression of tumorigenesis in dorsalateral prostate of CO-FS than in CO–CO mice. CO-FS mice had slightly lower plasma testosterone level at 24 and 40 weeks, significantly lower estradiol level at 40 weeks and significantly less expressed androgen receptor (AR) in the dorsalateral prostate at 40 weeks than CO–CO mice. Consumption of high ω-3 diet lowered the expression of genes expected to increase proliferation and decrease apoptosis in dorsalateral prostate. Our results suggest that consumption of high ω-3 diet slows down prostate tumorigenesis by lowering estradiol, testosterone and AR levels, promoting apoptosis and suppressing cell proliferation in C3(1)Tag mice.

Introduction

Prostate cancer has remained the most frequently diagnosed cancer and the second leading cause of cancer related death among men in the USA, accounting for 28% of total expected cancer incidence in men of USA in 2010 (1). In general, the incidence and mortality of prostate cancer are high in North America and Northern Europe but much lower in Japan and other Asian countries (2). Migration studies show that Asian men living in the USA have a lower risk of prostate cancer than the Caucasians but have a higher risk than their counterparts living in Asia (3). Japanese men that immigrate to the USA die of prostate cancer with increasing frequency as a function of the number of years of their residency (4). The major factor for this increased frequency in prostate cancer death is thought to be the Western diet.

Polyunsaturated fatty acids (PUFA) are a component of dietary fat reported from several investigations to influence the development of prostate cancer (5). In the past 100 years, the fatty acid composition of Western diets has witnessed a dramatic change, largely due to a high increase in the consumption of omega-6 (ω-6) PUFA from vegetable oils and red meat and less consumption of omega-3 (ω-3) PUFA (6). This has resulted in an ω-6/ω-3 ratio of 25:1 to 40:1 rather than near the ideal 1:1 in the US diets. Asian diets are lower in ω-6 and higher in ω-3 than the Western diet due to more consumption of fish and other sea products and low consumption of plant oils and red meat.

Several studies have investigated the effects of ω-3 and ω-6 fat on prostate tumor cells. Omega-3 PUFA [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] inhibit tumor cell growth in animal models and human prostate cell lines (7,8), whereas ω-6 PUFA [linoleic acid (LA) and arachidonic acid (AA)] increase growth of human prostate tumor cell lines (7,9). Epidemiologic studies also reported a decrease of metastatic prostate cancer risk and prostate cancer death in men who consume the most fish (10–13), a good source of EPA and DHA. LA is the most abundant ω-6 PUFA in the human diet. It is abundant in many plant oils such as corn oil, safflower oil and sunflower oil. LA is the precursor for the synthesis of AA, which is abundant in red meat and meat fat.

Prostate cancer burden continues to increase because of the aging and growing population as well as nutritional patterns that tend to increase the risk for the disease. There is the need to identify and establish factors that might prevent or slow the progression of prostate cancer. If beneficial, a dietary change that includes the reduction in the intake of ω-6 PUFA and increase the proportion of dietary ω-3 PUFA may therefore be a powerful tool for prevention of mortality from prostate cancer.

In this study, we tested the hypothesis that compared with a diet that approximates the ω-6 fatty acid content of the Western diet exposure to a diet with more ω-3 fatty acids during adulthood will slow the progression of prostate cancer and to identify the underlying molecular factors. The C3(1)Tag mouse was used for the study because it develops prostate cancer slowly and has a well-characterized disease progression making it suitable for prevention studies (14). We show that compared with a diet that is high in ω-6/ω-3 fat, consumption of low ω-6/ω-3 diet slows prostate carcinogenesis.

Materials and methods

Mice

Twenty female SV 129 mice, at 6 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA), quarantined for 2 weeks and transferred to a study room. Mice that bear a transgene for the SV40 large T antigen with a C3(1) rat prostatic steroid-binding protein promoter were obtained from Dr Jeffrey Green for breeding. The C3(1)Tag mouse model was developed in the laboratory of Dr Jeffrey E.Green of the National Cancer Institute. The male transgenic mice develop prostatic intraepithelial neoplasia (PIN) that progresses to prostate carcinoma due to expression of the large T antigen in the prostate (14). All mice were genotyped to confirm the presence of the transgene.

Abbreviations: AA, arachidonic acid; ADC, adenocarcinoma; AR, androgen receptor; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DL, dorsolateral prostate; HGPIN, high-grade prostatic intraepithelial lesion; GU, genitourinary; LA, linoleic acid; LGPIN, low-grade prostatic intraepithelial neoplasia; NOS, nitric oxide synthase; PCR, polymerase chain reaction; PIN, prostatic intraepithelial neoplasia; PCNA, proliferating cell nuclear antigen; PUFA, polyunsaturated fatty acid.

Study design and feeding

All female SV 129 mice were placed on a diet containing 10% wt/wt corn oil (high ω-6, control diet, ratio of ω-6/ω-3 was 50). After 2 weeks, they were bred with homozygous C3(1) Tag transgenic male mice. All male hemizygous offspring were weaned to the corn oil diet (CO) and consumed this diet until the postpuberty age of 7 weeks. Half of the offspring were retained on the corn oil diet to generate the high ω-6 CO–CO (mother—pup diet) group, whereas the remaining half were transferred to a higher ω-3 diet [(FO), test diet] to generate the CO-FS (mother—pup diet) group. The high ω-3 diet contained 5% canola oil (to provide adequate essential ω-6 fatty acid) and 5% fish oil concentrate (ratio of ω-6/ω-3 in the diet was 0.3). The offspring were housed not more than four in a cage, numbered for individual identification and weighed weekly. All animals were maintained in an isolated environment in barrier cages and fed the specified diet. Mice were fed *ad libitum* with fresh food 6 days per week and any leftover food in the cages was discarded.

The animal protocol was approved by the Marshall University school of Medicine Institutional Animal Care and Use Committee.

Diet

Diets were prepared in the animal diet prep room of Marshall University School of Medicine, using an AIN-76-A rodent diet modified to contain 10% total fat (Supplementary Table I is available at *Carcinogenesis* Online). The AIN-76-A diet is adequate for the supply of nutrition and growth of the mice (15). The control and test diets were isocaloric and isonutrient and are relevant to human consumption. Casein, corn starch and other dry ingredients for the diets (except sugar) were obtained from MP Biomedicals (Solon, Ohio), fish oil concentrate (containing 60% EPA + DHA) (Supplementary Table II is available at *Carcinogenesis* Online) were obtained from Zone Labs (Danvers, MA), whereas sugar and oil (100% canola oil or 100% corn oil) were obtained locally. Diets were prepared in batches as needed and stored in sealed containers at −20°C to prevent bacterial growth and fat oxidation in the food.

Assessment of transgene expression

Ear punches stored at −20°C were digested as described previously (16) and assessed for the presence of SV40 Tag transgene by real time polymerase chain reaction (PCR) method, using an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA).

Body weights

To assess the effect of diet on body weight, mice body weights were recorded weekly and terminally during dissection.

Dissection and tissue collection

Mice were euthanized at 24 and 40 weeks of age. A 24-week time point was chosen as adequate time for early-stage disease progression in our heterozygote mice since previous studies indicated that C3(1)Tag male mice develop high-grade prostatic intraepithelial lesion (HGPIN) at 20 weeks (14). PIN is the primary precursor of prostate cancer. The HGPIN in C3(1)Tag male mice progresses to prostate carcinoma by 28 weeks (14). The 40-week time point therefore ensures advanced disease progression and provides for adequate time for intervention in our prevention study. After macroscopic evaluation during necropsy, the genitourinary (GU) apparatus [consisting of the anterior prostate, dorsolateral prostate (DL), ventral prostate, seminal vesicle, bladder, proximal ductal deference and proximal urethra] was dissected and weighed to assess the effect of diet on GU weight. The anterior prostate, ventral prostate and DL prostate lobes were dissected from the GU apparatus with the aid of a dissecting microscope and weighed to assess the effect of diet on prostate weight. GU apparatus were fixed in phosphate-buffered 10% paraformaldehyde for 4 h and transferred to 70% ethanol until ready for histopathological analyses. Mouse inguinal fat, liver and prostate were collected and stored at −80°C for other analyses.

Blood collection

Blood (~600 µl) was collected from each animal into ethylenediaminetetra-acetic acid vials by cardiac puncture at necropsy to assess testosterone and estrogen levels. Plasma was separated from blood samples by centrifugation and stored at −80°C until assayed.

Plasma measures

Total testosterone and estrogen were measured from plasma obtained from individual mice from CO–CO and CO-FS groups at 24 and 40 weeks, using the Testosterone EIA Kit or Estradiol EIA Kit respectively (Cayman Chemical Company, Ann Arbor, MI). Measurements were done in triplicate following the protocol provided in the kit.

Histopathological assessment

To assess cancer progression due to diet, GU apparatus previously fixed in paraformaldehyde and thereafter in ethanol were sent to the Rodent Histopa-thology Core of Dana-Farber/Harvard Cancer Center for processing and pathology diagnosis. Briefly, the prostatic complex (consisting of all prostate lobes) separated from each GU apparatus was paraffin embedded, sectioned on a slide (six sections per slide) and stained with hematoxylin and eosin. The animal identities were coded to avoid observer bias in the pathological diagnosis. Two independent evaluators analyzed all specimens. Observations were done for the presence and degree of epithelial hyperplasia and the presence of nuclear atypia in the epithelial cells in dorsalateral lobes of the prostate. Specimens with nuclear atypia were classified as low-grade prostatic intraepithelial neoplasia (LGPIN) or HGPIN lesions following the guideline established by the Mouse Models of Human Cancer Consortium Prostate cancer Committee (17). Scores from two evaluators were averaged.

Fatty acid composition analysis

The fatty acid compositions of prostate, liver and fat at 24 weeks were analyzed by gas chromatography. Extraction of fatty acids was done as described previously (16). Fatty acids suspended in iso-octane were separated and identified using a PerkinElmer Clarus 500 Gas Chromatography (Shelton, CT) with a Elite MWAX 30 m × 0.53 mm × 1.0 µm cross-linked polyethylene glycol Capillary Column, in the following conditions: 220°C for 100 min, injector and flame ion detector at 240°C, helium carrier gas flow rate of 2.0 ml/min. Peak identification was done using a customized standard (GLC #704) (Nu-Chek-Prep, Elysian, MN), which contains 10 fatty acids as methyl esters of stearate, oleate, linoleate, alpha linolenate, gamma linolenate, homo-gamma linolenate, arachidonate, eicosapentaenoate, docosapentaenoate and docosahexaenoate. Fatty acid methyl esters were estimated as the percentage of the methylated fatty acids (area under the curve).

Gene expression RT^2 PCR assay

Gene expression analysis of DL tissues from 24 weeks old mice consuming either high ω-3 or high ω-6 diets (three tissues per group) was done, using the Mouse Signal Transduction Pathway Finder™ RT^2 *profiler*™ PCR Array (PAMM-014) (SuperArray Bioscience Corporation, Frederick, MD). This array was chosen to identify genes in signal transduction pathways that are influenced by high ω-3 fat diet and that may be applicable to cancer development. Briefly, DL prostate was dissected in RNA stabilization reagent (Omega Bio-tek, Norcross, GA) and frozen at −80°C until ready to use. Total RNA was isolated from frozen DL prostate tissue with RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA), according to the manufacturer protocol. RNA yield, purity and integrity were determined using an Agilent 2100 Bioanalyzer (Santa Clara, CA). Further quality control was performed on all RNA samples with the RT^2 RNA QC PCR Arrays (SuperArray Bioscience Corporation). Complementary DNA was prepared from RNA using RT^2 First Strand Kit and amplified by real time PCR using SuperArray RT^2 qPCR Master Mix on ABI Prism 700 (Applied Biosystems), following the protocol provided by the manufacturer. Differential gene expression and statistical analysis of data were done using the SuperArray software.

Immunoblot analysis

Frozen DL prostate tissues (three to four per group) were homogenized in Tri Reagent (Sigma–Aldrich, St Louis, MO), followed by protein extraction according to protocol from the manufacturer. Protein concentration was determined by BCA protein assay kit (EMD Biosciences. Darmstad, Germany). Fifteen micrograms of protein were loaded onto a 4–20% Tris–HCl polyacrylamide gradient gel (Bio-Rad Laboratories, Hercules, CA), separated by electrophoresis and transferred onto a nitrocellulose membrane. Blots were blocked using 5% bovine serum albumin in Tris-Buffered Saline Tween-20 for 1 h at room temperature and thereafter probed with primary antibodies in blocking buffer overnight at 4°C against: inhibitor of NF-kappa-B kinase beta (IKKβ), B-cell leukemia/lymphoma 2 (BCL2) (Cell Signaling Technology); Nitric oxide synthase (NOS2) Type II (BD Biosciences), nuclear factor-kappaB (NF-κB, p65) (Santa Cruz Biotechnology) or NF-κB inhibitor alpha (IKα) (Abcam) for 1 h at room temperature. Beta-actin (Santa Cruz Biotechnology) was used to normalize protein loading. Membrane was then incubated with either horse-radish peroxidase conjugated antimouse (Santa Cruz Biotechnology) or anti-rabbit (PIERCE) secondary antibody as appropriate. A Chemiluminescence ECL Kit (PIERCE) was used for band detection. Densitometric analyses were carried out using ChemDoc XRS system (Bio-Rad Laboratories) to acquire the image, followed by image analysis with 'Quantity One' software, V.4.5.2 (Bio-Rad Laboratories).

Immunohistochemistry

Expressions of androgen receptor (AR) and proliferating cell nuclear antigen (PCNA) were assessed in mouse DL prostate by immunohistochemistry. Paraffin sections of DL prostate (six sections per slide) were deparaffinized in xylene, hydrated in a series of graded ethanol and rinsed in distilled water. Antigen retrieval was done in a microwave oven using Vector Antigen

141

Unmasking solution (Vector Laboratories, Burlingame, CA). This was followed by blocking endogenous peroxidase activity using 3% hydrogen peroxide and Avidin/Biotin activity using Vector Avidin/Biotin blocking kit (Vector Laboratories) following the manufacturers protocols. After blocking, sections were treated with rabbit anti-AR (PG21; (Millipore, Temecula, CA) or mouse anti-PCNA (Biogenex, San Ramon, CA) and thereafter with secondary antibodies and vectastain Elite ABC kit (Vector Laboratories) for rabbit antibody or Vector M.O.M kit (Vector Laboratories) for mouse primary antibodies. Sections were incubated with 3, 3′-diaminobenzidine (Vector Laboratories) and counterstained using hematoxylin. The levels of proliferation or AR expression were compared between the two diet groups by counting the number of cells with PCNA or AR positively stained nucleus, respectively, per 1000 cells within viable areas of lesions. Apoptosis was assayed by morphological identification (nuclear and cellular condensation, fragmentation of nuclei and/or membrane blebbing and fragmentation of the cell into apoptotic bodies) of apoptotic cells (18). The numbers of cells undergoing apoptosis based on morphological indicators in 1000 epithelial cells of viable areas of lesions were counted to estimate an apoptotic index.

Statistics

Western blot protein expression levels, apoptosis, AR and PCNA levels, fatty acid composition, mean body weight gain, prostate and GU weight between diet groups and across time, were statistically analyzed by *t*-test. Testosterone and estradiol levels between diet groups and across time were analyzed by Mann–Whitney test. The differences in prostate lesion incidence between diet groups were assessed by Fisher's exact test. All statistical tests were performed using Prism software.

Results

Effect of diets on tissue lipid composition

The lipid composition of the prostate, liver and fat at 24 weeks of age were analyzed. Mice at this age consumed either high ω-3 diet or high ω-6 diet. As expected, the lipid composition of tissues reflected the lipid content of the diet (Supplementary Figure 1 is available at *Carcinogenesis* Online). The prostate, liver and fat of mice that consumed high ω-6 diet contained significantly more (by *t*-test at $P < 0.05$) LA than the prostate, liver and fat of mice that consumed high ω-3 diet. AA was significantly more (by *t*-test at $P < 0.05$) in the liver and prostate but not in the fat of mice that consumed the high ω-6 diet than mice that consumed high ω-3 diet. The liver, prostate and fat of mice that consumed the high ω-3 diet contained significantly more (by *t*-test at $P < 0.05$) ALA, EPA and DHA than the liver, fat and prostate of mice that consumed high ω-6 diet.

Effect of diets on body weight gain

To assess the effect of diet on body weight of mice, we weighed the mice weekly from the time of weaning to time of killing (40 weeks).
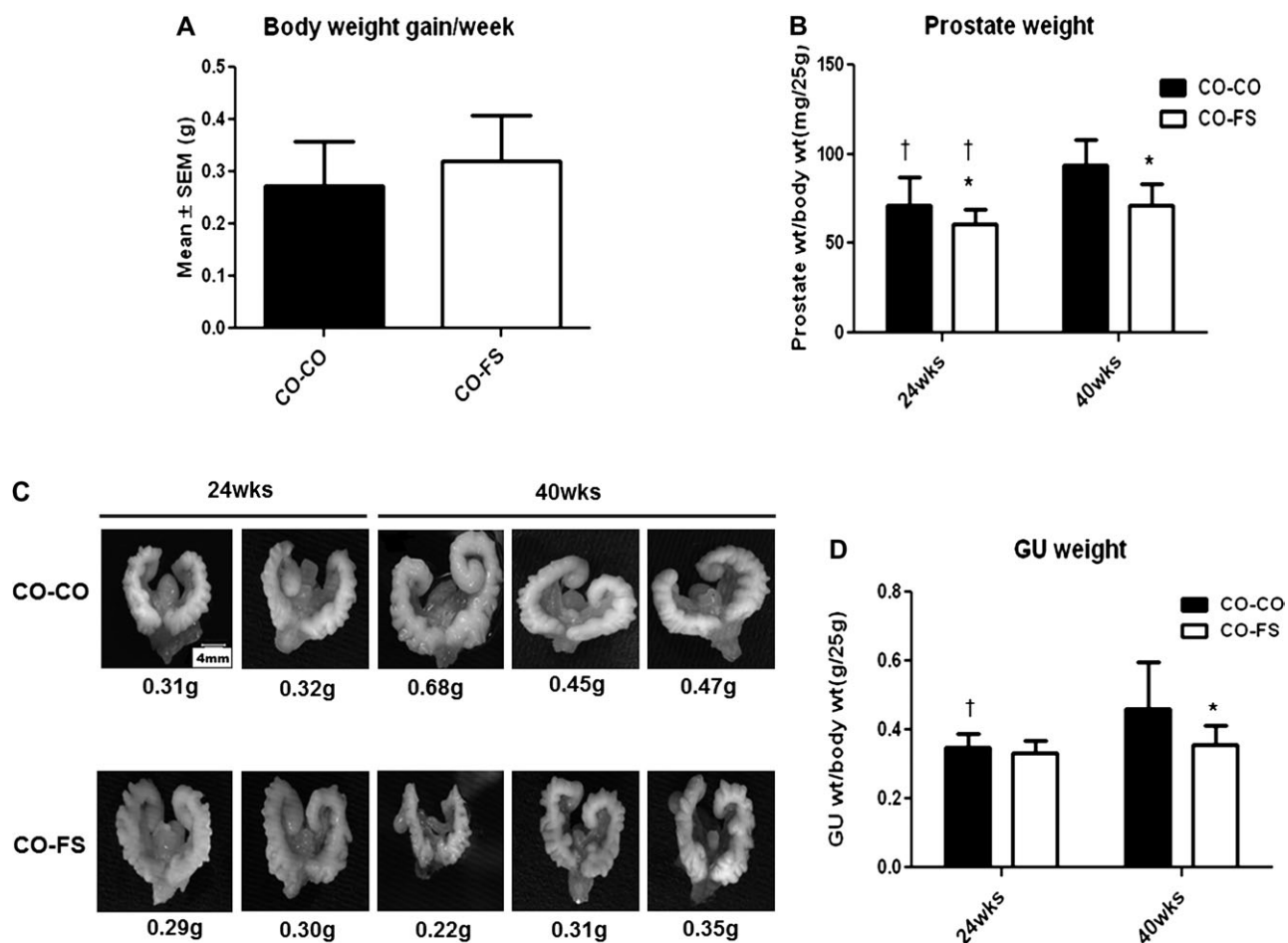

Fig. 1. Effect of diets on average body weight gain, prostate weight and GU weight between CO–CO and CO-FS mice. (**A**) Average body weight gain. Data are from measurements of body weight gain for individual mouse from the time of weaning to 40 weeks of age. Analysis by *t*-test showed no significant difference in body weight gain between the two groups at $P < 0.05$. $n = 9$/group. Bar is mean of body weight gain per week. (**B**) Prostate weight at 24 and 40 weeks of age. Mouse DL, anterior prostate and ventral prostate lobes were dissected and weighed, and the sums were expressed as milligrams per 25 grams body weight. The average prostate weight was significantly different between the two groups at 24 weeks ($n = 15$–19/group) and 40 weeks ($n = 12$/group) by *t*-test and at $P < 0.05$. Average prostate weight significantly increased in both groups with time by *t*-test at $P < 0.05$. **C** and **D**) GU weight at 24 and 40 weeks of age. Mouse GU were dissected at euthanasia and weighed. The weights were expressed as milligrams per 25 grams body weight. The average GU weight was not significantly different between the two groups at 24 weeks but significantly lower at 40 weeks in the CO-FS group than in the CO–CO group by *t*-test and at $P < 0.05$ ($n = 19$/group). Average GU weight increased significantly in CO–CO group with time. *Significantly different between groups, †significantly different across time at $P < 0.05$. Arrow bars represent SEM.

Control and test diets were designed to be nutritionally balanced and with the same percentage of energy from fat. There was no significant difference in body weight gain between mice consuming high ω-6 control diet (CO–CO group) and mice consuming high ω-3 test diet (CO-FS group) (Figure 1A).

Effect of diets on prostate weight and GU weight

We assessed the effect of diet on prostate and GU weights as an indicator of progression of prostate tumorigenesis. Two time points, 24 and 40 weeks, were considered for assessment of disease progression. The prostate of mice consuming the high ω-3 diet weighed significantly less than prostate of mice consuming the high ω-6 diet at 24 and 40 weeks (by t-test at $P < 0.05$) (Figure 1B). Mice that consumed high ω-3 diet had an increase of 10.21 mg in prostate weight between 24 and 40 weeks, whereas mice that consumed high ω-6 diet had an increase of 22.49 mg. Diet did not affect the GU weight significantly at 24 weeks (Figure 1C and D). The gross appearance of the GU apparatus (Figure 1C) was similar between the two diet groups at 24 weeks. In contrast, the gross appearance of the GU apparatus of mice that consumed high ω-3 diet appeared smaller and the average weight was significantly lower than GU apparatus of mice consuming high ω-6 diet at 40 weeks (Figure 1D).

Effect of diets on prostate histopathology

The histology of the prostate in C3(1) Tag mice of various ages has previously been documented, and it is known that on a usual diet, mice develop HGPIN in the prostate beginning ~20 weeks of age, which subsequently progresses to adenocarcinoma (ADC) with advancing age (14). DLs of C3(1) Tag mice were evaluated in CO–CO and CO-FS groups at 24 and 40 weeks of age. The degree and progression of epithelial hyperplasia and atypia were compared between the two diet groups (Figure 2). There was a slower progression of disease in mice consuming the high ω-3 diet than in mice consuming the high ω-6 diet at each time point (Figure 2). At 24 weeks, there was a significantly low proportion of LGPIN incidence (45% in CO–CO versus 64% in CO-FS) and significantly high proportion of HGPIN incidence (55% in CO–CO versus 36% in CO-FS) in CO–CO group compared with CO-FS group (by Fischer's exact test at $P < 0.05$). No ADCs were observed in mice at 24 weeks. There was a significantly lower proportion of LGPIN incidence and higher proportion of ADC incidence in CO–CO group compared with CO-FS group at 40 weeks (by Fischer's exact test at $P < 0.05$). At 40 weeks, the CO–CO group had 18% LGPIN, 46% HGPIN and 36% ADC, whereas the CO-FS group had 36% LGPIN, 46% HGPIN and 18% ADC.
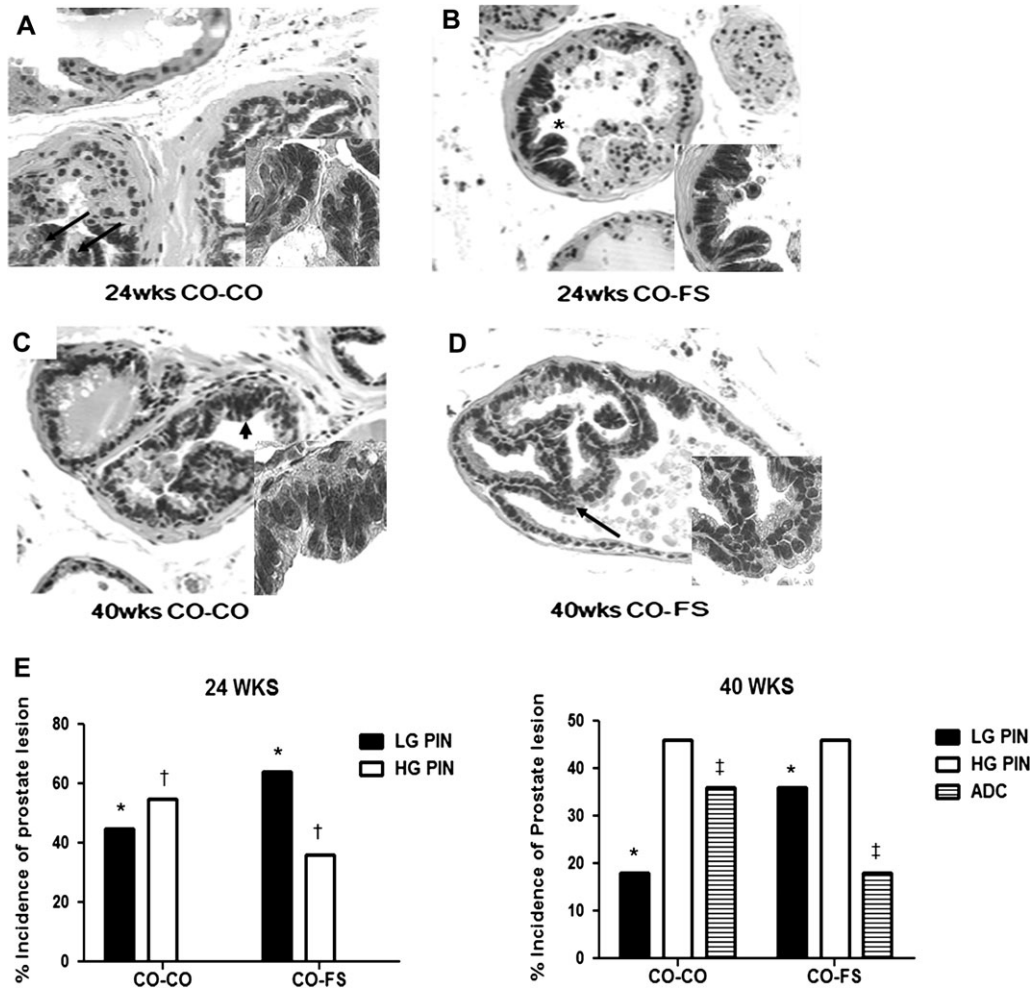

Fig. 2. Effect of diets on the incidence of PIN and ADC lesions in the DL prostate. (**A–D**) Representative hematoxylin- and eosin-stained sections from 24 to 40 weeks old mice. **A** and **C** illustrate the presence of LGPIN and HGPIN and ADC lesions as demonstrated by epithelial cells proliferation, hyperchromasia, nuclear enlargement and membrane irregularity. (**B** and **D**) Consumption of high ω-3 diet resulted in marked reduction of epithelial cells proliferation, hyperchromasia, nuclear enlargement and membrane irregularity. (**E**) Incidence of PIN lesions and ADC in CO–CO and CO-FS groups at 24 and 40 weeks of age. Prostate specimens were histologically examined for lesions by two independent evaluators and scores were averaged (n = 11–12/group). Pictures were taken at ×40 magnification (Star indicates LGPIN; arrow indicates HGPIN; arrow head indicates ADC. *, †, ‡ represent significant difference in LGPIN, HGPIN and ADC, respectively, between CO–CO and CO-FS group at $P < 0.05$).

Effect of diets on plasma estrogen and testosterone levels and expression of AR

Plasma testosterone and estradiol levels were measured by enzyme immunoassays. Testosterone levels were not significantly different
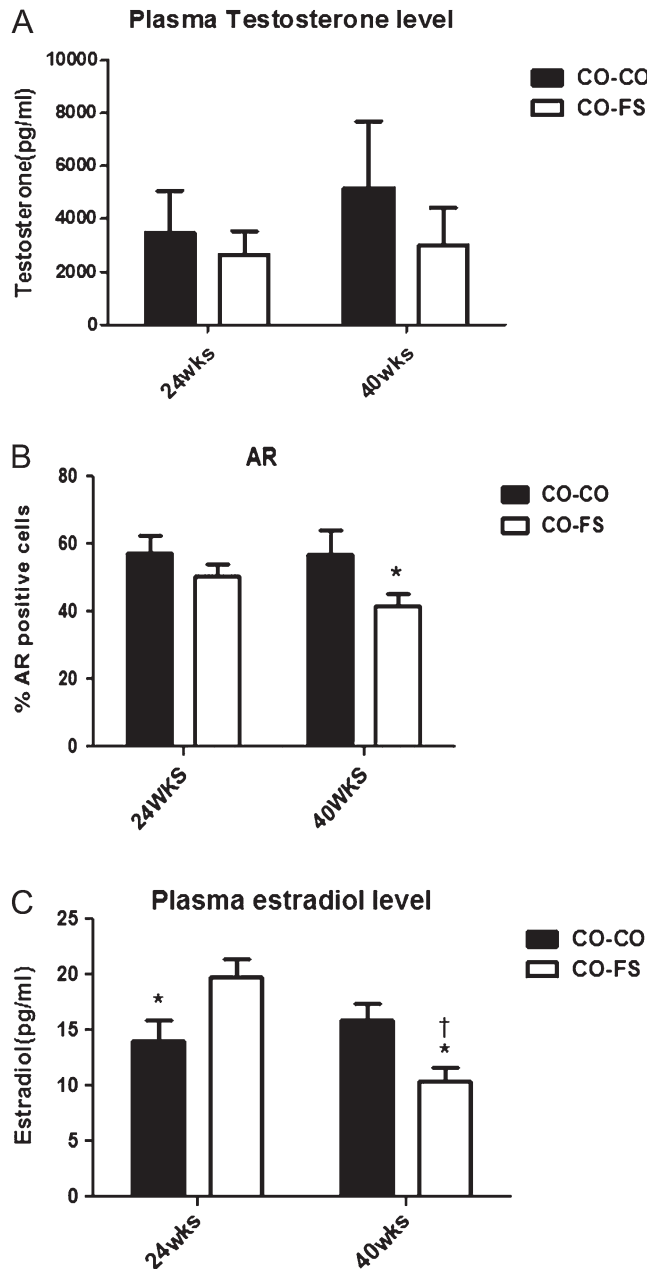
Fig. 3. Effect of diets on plasma testosterone and estradiol levels. Mice from both diet groups were killed at 24 and 40 weeks of age. Plasma was obtained for individual assay from blood collected from mice. (**A**) The level of testosterone was slightly less in mice from CO-FS group than mice from CO–CO group at 24 and 40 weeks ($n = 12$–19/group) and increased slightly as mice progressed in age in the CO–CO group (Mann–Whitney test at $P < 0.05$). (**B**) Expression of AR was lower significantly in the CO-FS group than the CO–CO group at 40 weeks by *t*-test, $n = 5$/group. A total of 1000 epithelial cells within viable areas of lesions per slide were counted for AR expression assay. (**C**) Estradiol levels were significantly different between the two groups at 24 and 40 weeks by Mann–Whitney test at $P < 0.05$ ($n = 15$–20/group). Estradiol level increased slightly as mice progressed in age in CO–CO group. Estradiol level was significantly less in CO-FS group than CO–CO group at 40 weeks and decreased significantly as mice progressed in age in CO-FS group. *Significantly different at $P < 0.05$, †significantly different across time at $P < 0.05$. Arrow bars represent SEM.

due to age or diet (Figure 3A). To further test the effect of diet on testosterone action, AR expression in the DL was analyzed by immunohistochemistry. The expression level of AR in DL was significantly higher in mice consuming the high ω-6 diet than in mice consuming the high ω-3 diet at 40 weeks but not at 24 weeks (Figure 3B). There were significant differences in the levels of estradiol in mice consuming high ω-6 diet compared with mice consuming high ω-3 diet at 24 and 40 weeks of age (Figure 3C). Estradiol level was significantly increased in mice that consumed high ω-3 diet compared with mice that consumed high ω-6 diet at 24 weeks. However, at 40 weeks, estradiol level was significantly less in mice that consumed high ω-3 diet (10.45 pg/ml) compared with mice that consumed high ω-6 diet (15.89 pg/ml). Consumption of high ω-3 diet resulted in a significant decrease in estradiol level as mice progressed in age, whereas consumption of high ω-6 diet resulted in slight increase in estradiol level as mice progressed in age.

Effect of diets on gene and protein expression in the DL prostate at 24 weeks of age

To investigate the molecular mechanism by which high ω-3 diet may be slowing down the progression of prostate tumorigenesis in the DL prostate, we analyzed the expression of several genes involved in signal transduction pathways. We chose the 24 weeks time point because mice are in the early stages of prostate carcinogenesis, therefore gene expression difference due to the diet and not to the tumor

Table I. Gene expression in DL at 24 weeks of age

Gene name	Symbol	Fold difference CO-FS versus CO–CO
Activating transcription factor 2	Atf2	4.91
Ornithine decarboxylase, structural 1	Odc1	2.19
Etoposide induced 2.4 mRNA	Ei24	3.91
Cyclin-dependent kinase 2	Cdk2	2.37
Glycogen synthase 1, muscle	Gys1	2.11
Hexokinase 2	Hk2	2.25
Jun oncogene	Jun	2.54
B-cell leukemia/lymphoma 2	Bcl2	−9.94
Bone morphogenetic protein 2	Bmp2	−2.81
Chemokine (C-C motif) ligand 2	Ccl2	−3.56
Chemokine (C-X-C motif) ligand 9	Cxcl9	−3.64
Cytochrome P450, family 19, subfamily a, polypeptide 1	Cyp19a1	−2.26
Early growth response 1	Egr1	−2.45
Engrailed 1	En1	−3.18
Fas ligand (TNF superfamily, member 6)	Fasl	−2.98
Fatty acid synthase	Fasn	−2.15
Fibroblast growth factor 4	Fgf4	−4.44
Forkhead box A2	Foxa2	−3.87
Homeo box A1	Hoxa1	−3.31
Insulin-like growth factor binding protein 3	Igfbp3	−2.15
Interleukin 1 alpha	Il1a	−21.61
Lymphoid enhancer binding factor 1	Lef1	−3.1
Matrix metallopeptidase 10	Mmp10	−2.5
Peroxisome proliferator activated receptor gamma	Pparg	−7.6
Retinol binding protein 1, cellular	Rbp1	−2.08
Transcription factor 7, T-cell specific	Tcf7	−2.51
Telomerase reverse transcriptase	Tert	−3.35
Prostate transmembrane protein, androgen induced 1	Pmepa1	−3.41
Vascular cell adhesion molecule 1	Vcam1	−2.35
WNT1 inducible signaling pathway protein 1	Wisp1	−9.67
Wingless-related MMTV integration site 2	Wnt2	−2.66

Mice were placed on either corn oil diet or transferred to fish oil diet at 7 weeks of age. Gene expression in the DL prostate of mice at 24 weeks of age was performed by real time reverse transcriptase. Genes with >2-fold differences in mRNA expression are shown. Results are from three replicates for each group.
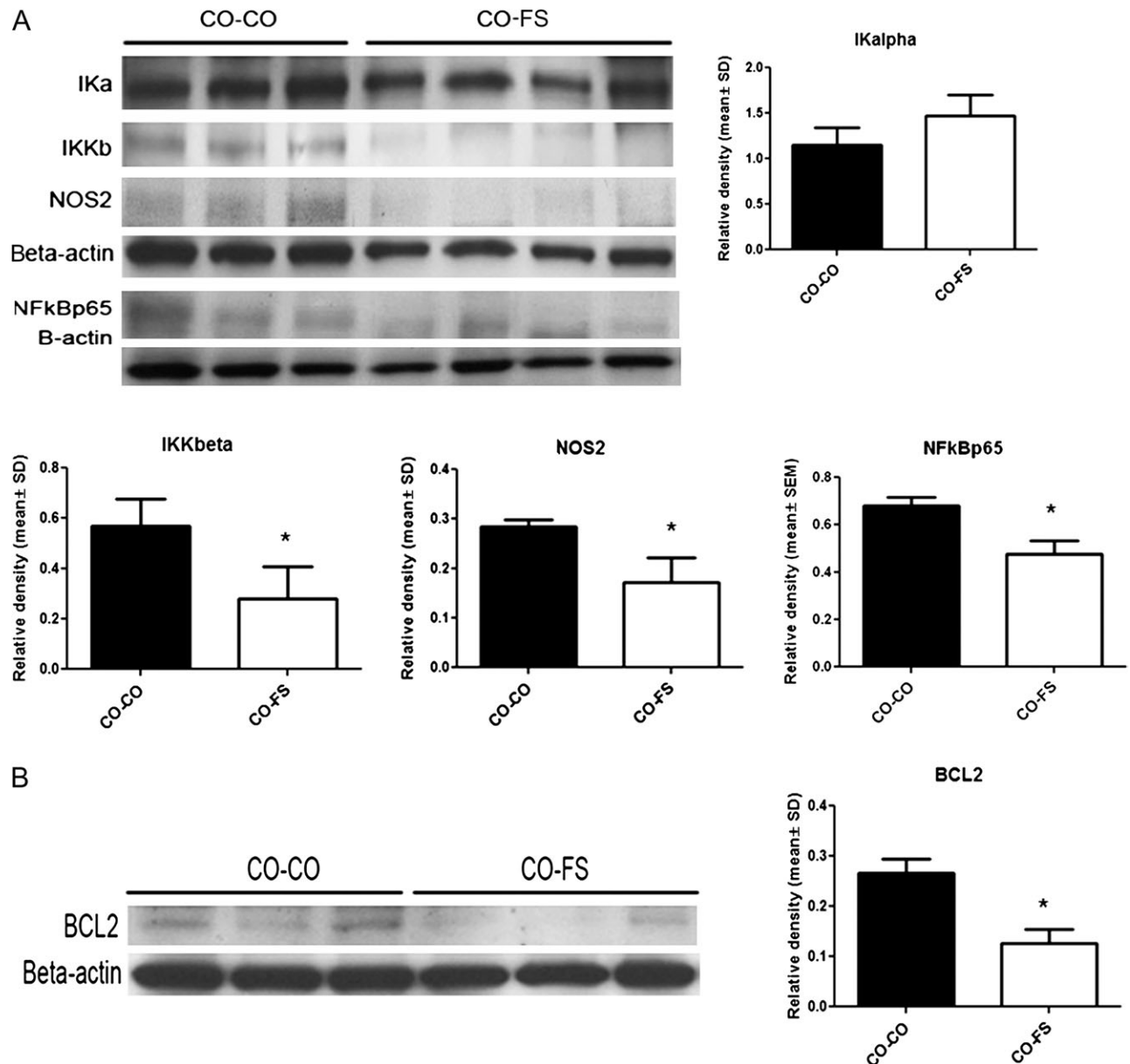
Fig. 4. Effect of diets on proteins in NF-κB and apoptosis pathways in DL prostate. (**A**) Protein expression of NF-κBp65, IKα, IKKβ and NOS2 in the DL prostate and their corresponding densitometric analyses in 24 weeks old mice. Expression of IKα protein was slightly higher, whereas the expressions of NF-κBp65, IKKβ and NOS2 proteins were significantly lower in DL prostate of mice that consumed high ω-3 fat diet than mice that consumed high ω-6 fat diet. (**B**) Protein expression of BCL2 in the DL prostate and the densitometric analysis in 24 weeks old mice. Expression of BCL2 protein was lower significantly in the DL prostate of mice that consumed high ω-3 fat diet compared with mice that consumed high ω-6 fat diet. Equal loading of protein in the lanes was confirmed by β-actin. Values are relative density corrected for β-actin. *Significantly different at $P < 0.05$, $n = 3–5$/group. Arrow bars represent SD or SEM where applicable.

can be assessed. A total of 84 genes was quantitatively assayed using the Mouse Signal Transduction Pathway Finder™ RT² *profiler*™ PCR Array. Table I presents 31 genes with >2-fold differences (using SuperArray statistical analyses software) between CO-FS and CO–CO group at 24 weeks of age. We further analyzed the genes using the Ingenuity Systems software (IPA 8.0), and this showed that a number of the altered genes are associated with NF-κB and apoptosis pathways (data not shown). We chose NF-κB inhibitor alpha (IKα), IkB kinase beta (IKKβ), NF-κBp65 and NOS2 from NF-κB pathway and B-cell leukemia/lymphoma 2 (BCL2) from apoptosis pathway for Western blot analysis. Western blot results showed the expressions of Ikα protein (Figure 4A) to be slightly higher and BCL2 protein (Figure 4B) to be significantly lower due to consumption of high ω-3 diet. Expressions of NF-κBp65, IKKβ and NOS2 proteins were significantly lower due to consumption of high ω-3 diet (Figure 4A).

Effect of diets on cell proliferation and apoptosis in the DLs

We further tested the molecular mechanism by which diet may be influencing the progression of prostate tumorigenesis by comparing the proliferation and apoptosis in the areas of lesions in DL prostate of mice from the two groups. Proliferation was significantly lower (Figure 5E) and apoptosis was significantly higher (Figure 5J) in prostate epithelial cells in mice that consumed high ω-3 diet compared with mice that consumed high ω-6 diet.

Discussion

One obvious characteristic of the Western diet is the high intake of high ω-6 fat, which has been associated with high prostate cancer risk in epidemiologic studies (19–21). These studies also suggest that
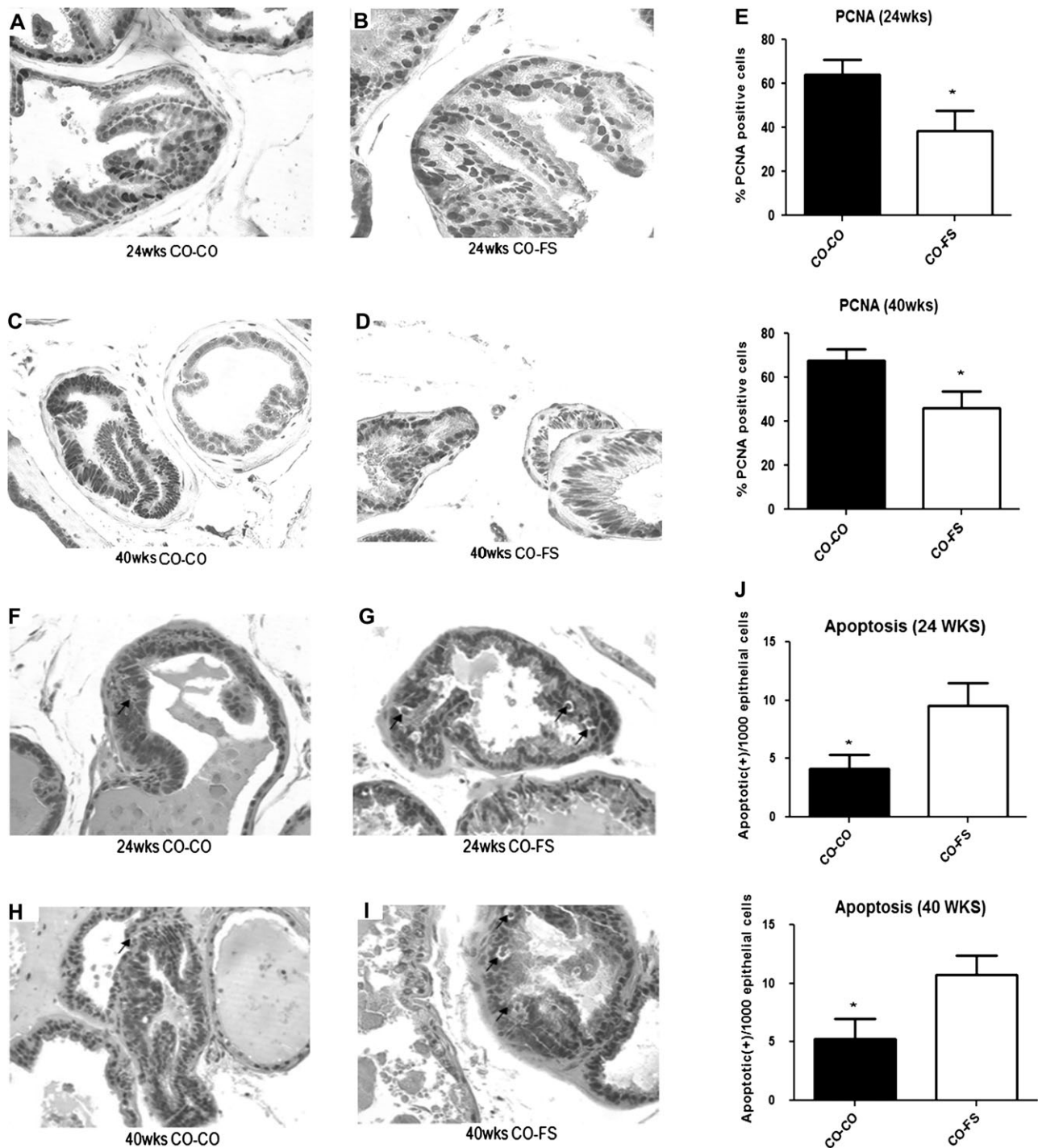
Fig. 5. Effect of diets on the extent of proliferation and apoptosis in DL prostate. DL prostate sections from the two groups were assessed for proliferation by immunohistochemistry analysis of PCNA at 24 and 40 weeks (**A–D**). Proliferation was significantly less in mice that consumed high ω-3 diet compared with mice that consumed high ω-6 diet by *t*-test at $P < 0.05$ (**E**). Apoptosis was assessed by morphological identification of apoptotic cells in hematoxylin- and eosin-stained sections (**F–I**) and was significantly greater in mice that consumed high ω-3 diet compared with mice that consumed high ω-6 diet by *t*-test at $P < 0.05$ (J). A total of 1000 epithelial cells within viable areas of lesions per slide were counted for proliferation and apoptosis assays. $n = 5$/group for proliferation assay, $n = 7$/group for apoptosis assay. Pictures were taken at ×40 magnification. Arrow indicates apoptotic cells. Arrow bars represent SEM. *Significantly different at $P < 0.05$.

consumption of fish or fish oil, which are very high in ω-3 fatty acids, may reduce prostate cancer risk (10,21). We investigated the role of high ω-3/ω-6 diet in the prevention of prostate cancer risk and the molecular mechanism, which might be involved. The 24 and 40 weeks intervention time points were chosen as adequate time for early-stage disease progression and advanced disease progression since studies indicate that C3(1)Tag male mice on a usual diet develop HGPIN

beginning ∼20 weeks and progresses to prostate carcinoma from 28 weeks (14,22). We chose the C3(1) Tag model for this study for its well characterized disease progression from normal to PIN to ADC (14).

A switch from high ω-6 diet to a high ω-3 diet at postpuberty was chosen for this study because adults are able to make diet choices. Postpuberty consumption of high ω-3 fat diet significantly slowed down the progression of prostate tumorigenesis in mice as observed

by the slowed development of high-grade prostate lesions in mice that consumed high ω-3 diet compared with mice that consumed high ω-6 diet. The ability of high ω-3 diet to slow promotion of tumorigenesis in our mouse model was also observed as less GU and prostate weights in mice that consumed high ω-3 diet. At the molecular level, mice that consumed high ω-3 diet showed greater apoptosis and less proliferation in epithelial cells of the prostate as confirmed by less expression of the antiapoptotic gene BCL2 at messenger RNA and protein levels and less staining for PCNA compared with mice that consumed high ω-6 diet. The EPA and DHA in the high ω-3 diet were accumulated in the prostate of mice that consumed high ω-3 diet, which likely induced suppression of disease progression. This is supported by Rose et al. (7), indicating that EPA and DHA suppress proliferation and promote apoptosis in prostate cancer cells.

To identify the underlying molecular factors by which high ω-3 diet may be slowing down the progression of prostate tumorigenesis, we analyzed for the expression of some genes involved in NF-κB pathway because further analysis of the gene array into pathway-focused array (IPA 8.0) showed the NF-κB pathway to be differentially activated. NF-κB is a key mediator of survival, and is upregulated during prostate cancer progression (23,24). NF-κB is complexed with the inhibitor of NF-κB (IK) in the cytosol. When activated, the inhibitor of NF-κB kinase (IKK) phosphorylates the IK protein, resulting in the dissociation of IK from NF-κB. The activated NF-κB is then translocated into the nucleus to regulate the expression of downstream genes. The expression of IK was slightly higher and the expression of IKK was significantly lower in mice that consumed high ω-3 fat diet as shown in the Western blot results (Figure 4A). The messenger RNA expression fold for IK and IKK was 1.2 and −1.97 (CO-FS versus CO–CO), respectively. The expressions of NF-κBp65 subunit and NOS2, a downstream gene in the NF-κB pathway, were significantly less in mice that consumed high ω-3 fat diet. NOS2 is highly expressed in prostate tumors (25).

We also investigated the effect that high ω-3 diet may have on testosterone and estradiol levels in circulation. Testosterone has been vigorously studied because of its role in promoting prostate cancer, but the role of estrogen in prostate disease process has received very little attention despite reports that estrogen may promote prostate tumorigenesis (26–29). Serum testosterone level declines in the aging male, whereas estrogen level increases and remains constant (30,31). However, rising estradiol levels may increase prostate sensitivity to testosterone via upregulation of AR (32,33). We showed that consumption of high ω-3 diet resulted in significantly lower estradiol level at 40 weeks and slightly lower testosterone level with a concomitant significant decrease in the expression level of AR at 40 weeks, suggesting a decrease in androgen stimulation due to consumption of high ω-3 diet.

We conclude that high ω-3 diet slowed prostate tumorigenesis in C3(1) Tag mouse model, and the mechanisms involved are due to lower levels of testosterone and estradiol resulting in lower expression of AR and less expressions of genes in the NF-κB and the antiapoptotic BCL2 pathways. These molecular changes suppressed proliferation and promote apoptosis in the epithelial cells in areas of lesions in mouse dorsalateral prostate. By extrapolation of our findings to humans, a dietary change by humans that involves consumption of more ω-3 fat such as obtained from fish and fish oil, instead of corn oil with little or no ω-3 fat, may be helpful in the suppression and prevention of prostate cancer risk, especially for those who are at the high risk of developing the disease.

Supplementary material

Supplementary Tables I and II and Figure 1 can be found at http://carcin.oxfordjournals.org.

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References

1. Jemal,A. *et al.* (2010) Cancer statistics. *CA Cancer J. Clin.*, **60**, 277–300.
2. Jemal,A. *et al.* (2011) Global cancer statistics. *CA Cancer J. Clin.*, **61**, 69–90.
3. Platz,E. *et al.* (2006) Prostate cancer. 3rd edn In Schottenfeld, D and Fraumeni, JF. Jr (eds) *Cancer Epidemiology and Prevention*. Oxford University Press, Oxford, UK, 1128–1150[59].
4. Astorg,P. (2004) Dietary N-6 and N-3 polyunsaturated fatty acids and prostate cancer risk: a review of epidemiological and experimental evidence. *Cancer Causes Control*, **15**, 367–386.
5. Pandalai,P.K. *et al.* (1996) The effects of omega-3 and omega-6 fatty acids on in vitro prostate cancer growth. *Anticancer Res.*, **16**, 815–820.
6. Weaver,K.L. *et al.* (2009) Effect of dietary fatty acids on inflammatory gene expression in healthy humans. *J. Biol. Chem.*, **284**, 15400–15407.
7. Rose,D.P. *et al.* (1991) Effects of fatty acids and eicosanoid synthesis inhibitors on the growth of two human prostate cancer cell lines. *Prostate*, **18**, 243–254.
8. Rose,D.P. *et al.* (1999) Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol. Ther.*, **83**, 217–244.
9. Connolly,J.M. *et al.* (1997) Effects of dietary fatty acids on DU145 human prostate cancer cell growth in athymic nude mice. *Nutr. Cancer*, **29**, 114–119.
10. Terry,P. *et al.* (2001) Fatty fish consumption and risk of prostate cancer. *Lancet*, **357**, 1764–1766.
11. Tzonou,A. *et al.* (1999) Diet and cancer of the prostate: a case-control study in Greece. *Int. J. Cancer*, **80**, 704–708.
12. Hedelin,M. *et al.* (2007) Association of frequent consumption of fatty fish with prostate cancer risk is +modified by COX-2 polymorphism. *Int. J. Cancer*, **120**, 398–405.
13. Chavarro,J.E. *et al.* (2008) A 22-y prospective study of fish intake in relation to prostate cancer incidence and mortality. *Am. J. Clin. Nutr.*, **88**, 1295–1303.
14. Shibata,M.A. *et al.* (1996) Progression of prostatic intraepithelial neoplasia to invasive carcinoma in C3(1)/SV40 large T antigen transgenic mice: histopathological and molecular biological alterations. *Cancer Res.*, **56**, 4894–4903.
15 American Institute of Nutrition. (1977) Report of the American Institute of Nutrition *ad hoc* committee on standards for nutritional studies. *J. Nutr.*, **107**, 1340–1348.
16. Ion,G. *et al.* (2010) Maternal consumption of canola oil suppressed mammary gland tumorigenesis in C3(1) TAg mice offspring. *BMC Cancer*, **10**, 81.
17. Shappell,S.B. *et al.* (2004) Prostate pathology of genetically engineered mice: definitions and classification. The consensus report from the bar Harbor meeting of the mouse models of human cancer Consortium prostate pathology committee. *Cancer Res.*, **64**, 2270–2305.
18. Narayanan,B.A. *et al.* (2004) Regression of mouse prostatic intraepithelial neoplasia by nonsteroidal anti-inflammatory drugs in the transgenic adenocarcinoma mouse prostate model. *Clin. Cancer Res.*, **10**, 7727–7737.
19. Lanier,A.P. *et al.* (1980) Cancer in Alaskan Indians, Eskimos, and Aleuts. *J. Natl Cancer Inst.*, **65**, 1157–1159.
20. Giovannucci,E. *et al.* (1993) A prospective study of dietary fat and risk of prostate cancer. *J. Natl Cancer Inst.*, **85**, 1571–1579.
21. Augustsson,K. *et al.* (2003) A prospective study of intake of fish and marine fatty acids and prostate cancer. *Cancer Epidemiol. Biomarkers Prev.*, **12**, 64–67.

22. Maroulakou,I.G. *et al*. (1994) Prostate and mammary adenocarcinoma in transgenic mice carrying a rat C3(1) simian virus 40 large tumor antigen fusion gene. *Proc. Natl Acad. Sci. USA*, **91**, 11236–11240.

23. Suh,J. *et al*. (2004) NF-kappaB activation in human prostate cancer: important mediator or epiphenomenon? *J. Cell. Biochem.*, **91**, 100–117.

24. Huang,S. *et al*. (2001) Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene*, **20**, 4188–4197.

25. Uotila,P. *et al*. (2001) Increased expression of cyclooxygenase-2 and nitric oxide synthase-2 in human prostate cancer. *Urol. Res.*, **29**, 23–28.

26. Henderson,B.E. *et al*. (1988) The early in utero oestrogen and testosterone environment of blacks and whites: potential effects on male offspring. *Br. J. Cancer*, **57**, 216–218.

27. Thomas,J.A. *et al*. (1994) Effects of estrogens on the prostate. *J. Androl.*, **15**, 97–99.

28. DeKlerk,D.P. *et al*. (1979) Comparison of spontaneous and experimentally induced canine prostatic hyperplasia. *J. Clin. Invest.*, **64**, 842–849.

29. Ho,S.M. (2004) Estrogens and anti-estrogens: key mediators of prostate carcinogenesis and new therapeutic candidates. *J. Cell. Biochem.*, **91**, 491–503.

30. Kaufman,J.M. *et al*. (2005) The decline of androgen levels in elderly men and its clinical and therapeutic implications. *Endocr. Rev.*, **26**, 833–876.

31. Vermeulen,A. *et al*. (2002) Estradiol in elderly men. *Aging Male*, **5**, 98–102.

32. Moore,R.J. *et al*. (1979) Regulation of cytoplasmic dihydrotestosterone binding in dog prostate by 17 beta-estradiol. *J. Clin. Invest.*, **63**, 351–357.

33. Trachtenberg,J. *et al*. (1980) Androgen- and estrogen-receptor content in spontaneous and experimentally induced canine prostatic hyperplasia. *J. Clin. Invest.*, **65**, 1051–1059.