Effect of dietary polyunsaturated fatty acids on castration-resistant Pten-null prostate cancer

Shihua Wang1, Jiansheng Wu1, Janel Suburu1, Zhenhuan Gu1, Jiaozhong Cai1, Linara S.Axanova1, Scott D.Cramer1, Michael J.Thomas2, Donna L.Perry3, Iris J.Edwards3, Lorelei A.Mucci4, Jennifer A.Sinnott5, Massimo F.Loda6, Guangchao Sui1, Isabelle M.Berquin1 and Yong Q.Chen1,∗

1Department of Cancer Biology, 2Department of Biochemistry and 3Department of Pathology, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157, USA, 4Department of Epidemiology, 5Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115, USA and 6Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

To whom correspondence should be addressed. Tel: +1 336 713 7655; Fax: +1 336 713 7660; Email: ycqchen@wakehealth.edu

A common treatment of advanced prostate cancer involves the deprivation of androgens. Despite the initial response to hormonal therapy, eventually all the patients relapse. In the present study, we sought to determine whether dietary polyunsaturated fatty acid (PUFA) affects the development of castration-resistant prostate cancer. Cell culture, patient tissue microarray, allograft, xenograft, prostate-specific Pten knockout and omega-3 desaturase transgenic mouse models in conjunction with dietary manipulation, gene knockdown and knockout approaches were used to determine the effect of dietary PUFA on castration-resistant Pten-null prostate cancer. We found that deletion of Pten increased androgen receptor (AR) expression and Pten-null prostate cells were castration resistant. Omega-3 PUFA slowed down the growth of castration-resistant tumors as compared with omega-6 PUFA. Omega-3 PUFA decreased AR protein to a similar extent in tumor cell cytosolic and nuclear fractions but had no effect on AR messenger RNA level. Omega-3 PUFA treatment appeared to accelerate AR protein degradation, which could be blocked by proteasome inhibitor MG132. Knockdown of AR significantly slowed down prostate cancer cell proliferation in the absence of androgens. Our data suggest that omega-3 PUFA inhibits castration-resistant prostate cancer in part by accelerating proteasome-dependent degradation of the AR protein. Dietary omega-3 PUFA supplementation in conjunction with androgen ablation may significantly delay the development of castration-resistant prostate cancer in patients compared with androgen ablation alone.

Materials and methods

Transgenic mice
Prostate-specific Pten knockout and fat-1 transgenic mice were generated as described previously (19). Histopathological evaluation of mouse prostate tissues was performed by a board certified veterinary pathologist.

All animals were maintained in an isolated environment in barrier cages and fed the specified diet. Animal care was conducted in compliance with the state and federal Animal Welfare Acts and the standards and policies of the Department of Health and Human Services. The protocol was approved by our Institutional Animal Care and Use Committee.

Cell isolation and determination of androgen dependency
Pten+/−, Pten−/− and Pten+/+ mouse prostate epithelial cells were isolated from anterior prostates of 8 to10-week-old mice (19) as described previously (20). Cells were clonally selected using a serial dilution method (21) and Pten status was confirmed by genotyping and western blotting. To test for androgen dependence, cells (1 × 10⁶) were incubated with regular complete medium for 24 h. Cells were rinsed with phosphate-buffered saline twice and then incubated with phenol red-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% charcoal stripped fetal bovine serum (FBS) plus various amounts of R1881 (Sigma, St.Louis, MO). Cells were counted daily for 6 days. Three independent experiments were performed.

Pten knockdown and knockout in vitro
Pten−/− mouse prostate epithelial cells were infected with lentivirus expressing Pten targeting short hairpin RNA (shRNA) (GAA CCT GAT CAT TAT AGA TAT T) or control shRNA (GGG CCA TGG CAC GTA CGG CAA G). Infected cells were clonally selected using a serial dilution method (21) and confirmed by enhanced green fluorescent protein tag and western blotting of Pten. Pten+/− cells were isolated from anterior prostates of 8-week-old Pten floxed mice. In vitro deletion of Pten was achieved by infecting Pten+/− cells

Abbreviations: AR, androgen receptor; AA, arachidonic acid; BSA, bovine serum albumin; CE, collision energy; DHEA, dehydroepiandrosterone; DHA, docosahexaenoic acid; DMEM, Dulbecco’s modified Eagle’s medium; EGF, enhanced green fluorescent protein; FBS, fetal bovine serum; pAktS473, phospho-AktS473; PUFA, polyunsaturated fatty acid; shRNA, short hairpin RNA.
with a self-deleting Cre recombinase lentivirus (22). Cells were clonally selected using a serial dilution method and Pten status was confirmed by genotyping and western blotting.

**Allografts**

For the initial tumorigenesis assessment, 6 to 8-week-old male nude mice (NU/NU, strain code 088) were purchased from Charles River Laboratories (Wilmington, MA). Mice were castrated or sham operated. One week after castration, 2 × 10^7 mouse Pten<sup>−/−</sup> or Pten<sup>+/+</sup> cells mixed with an equal volume of Matrigel (BD Biosciences, San Jose, CA) were inoculated subcutaneously in pairs into each flank of castrated and non-castrated mice. Mice were terminated 3.5 weeks after tumor cell inoculation. For dietary PUFAs, experiments, 6 to 8-week-old male nude mice were randomly assigned to either omega-6 or omega-3 PUFAs diet group and castrated a week later. One week after castration, 5 × 10^7 mouse Pten<sup>−/−</sup> cells were randomly assigned to either omega-6 or omega-3 Matrigel were inoculated subcutaneously into each flank of mice. Tumor volume was measured twice a week and calculated with the formula: volume = 0.5236 × length × width^2 (13). Mice were euthanized 6.5 weeks after tumor cell inoculation. Tumors were harvested and weighed.

**Transgenic mouse models**

Two feeding schemes were used in our experiments. Prostate-specific Pten knockout mice were on chow until 2 months of age, castrated and then switched to omega-6 or omega-3 PUFAs diet. Alternatively, mice were fed omega-6 or omega-3 PUFAs diets after weaning, castrated at 2 months of age and continued on their respective diet after castration. Blood and prostate tissues were harvested when mice were euthanized at the age of 6 months.

**Diet**

Diet were prepared by the custom animal diet laboratory of the Animal Research Sources Program at Wake Forest University. The diets used were an omega-6 diet, based on a typical American diet consisting of an omega-6 to omega-3 ratio of 40:1, 397 kcal/100 g with 30% of energy from fat, 50% from carbohydrates and 20% from proteins, and an isocaloric omega-3 diet, which has a ratio of 1:1 (19). Because essential fatty acids cannot be completely removed from diet, we consider the omega-6 diet as a control for the omega-3 diet.

**Fatty acid and phospholipid analysis**

Fatty acid and glycerophospholipid analyses were performed as described previously (19).

**Androgen measurement**

Hundred microliter of plasma was added to 900 µl of water followed by 116 pg of D3-testosterone in 2 ml of ethyl acetate. Samples were mixed by vortexing and then rotated for 1 h at 4°C. The organic layer was removed and then dried under argon. After suspending the residue in 200 µl of methanol, the sample was transferred to a conical autosampler vial. Methanol was removed in a stream of argon and the residue resuspended in 50 µl of the liquid chromatograph injection solvent: H<sub>2</sub>O:MeOH 90:10, 0.1% formic acid. Samples were processed on a Symbiosis Pharma<sup>®</sup> System from Spark Holland and then analyzed on a Waters Quattro II triple quadrupole mass spectrometer. Sample vials were kept at 15°C before injection. Twenty-five microfilter sample aliquots were processed through Hypersil C18 (EC) cartridges. The eluted samples were separated at 40°C on a 2.1 × 50 mm Phenomenex Kinetex C18 column packed with 2.6 micron diameter particles having 100 Å pores. Running solvents for gradient liquid chromatograph separation were solvent A was 0.1% formic acid in water and solvent B was pure methanol. Separation was conducted at 100 µl/min with the following gradient: 100% A to 90% B at 10 min, hold at 90% B for 5 min and then back to 100% A at 16 min followed by a 14 min equilibration at 100% A. Column effluent was introduced into the Z-spray electrospray source of the mass spectrometer operated in the positive ion mode with a cone voltage of 35 V using selective reaction mode to monitor the following ion pairs: D3-testosterone as the internal standard. Results are shown in ng/ml of plasma.

**Immunohistochemistry**

Immunohistochemistry (IHC) staining was performed with a rabbit anti-Ki67 antibody (cat# ab15580, Abcam, Cambridge, MA), a rat anti-CD45R antibody (ab64100, Abcam) and a rabbit anti-Factor VIII antibody (cat# 18-0018, Invitrogen, Carlsbad, CA). All primary antibodies were incubated at 4°C overnight, followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody (cat# K4010, Dako, Carpinteria, CA); however, rat anti-CD45R antibody was followed first by a rabbit anti-IgG (Cat# 312005003, Jackson ImmunoResearch Laboratories, West Grove, PA) then by the horseradish peroxidase secondary antibody. All sections were visualized with DAB substrate (Dako), Total number and positively stained epithelial cells were enumerated using 20-fold magnified photographs from four areas of each section with the Image-Pro Plus 4.5 software (Image Processing Solutions, North Reading, MA). The Ki67 and CD45R were expressed as percentage of positive cells per hundred epithelial cells. The number of blood vessels stained by Factor VIII was manually counted by two individuals.

**Phospho-Akt<sup>817</sup>/androgen receptor analysis in patient samples**

A large cohort of men with prostate cancer who are participants in the Physicians’ Health Study and Health Professionals Follow-up Study was assessed for the association between tumor expression of phospho-Akt<sup>817</sup> (pAkt<sup>817</sup>) and androgen receptor (AR) using immunohistochemical methods. The cases were included on ten tissue microarrays, aimed to include at least three 0.6 mm cores of tumor per case. Quantitative assessment of the markers was performed on the Ariol Image analysis system (Genetix Corp., San Jose, CA). pAkt<sup>817</sup> staining was divided into quartiles based on the distribution of percent of positive staining cells in the whole cohort. Generalized linear models were used to estimate the mean and standard error of the mean (SEM) of AR in tumors across categories of pAkt<sup>817</sup>. The ordinal variable of pAkt<sup>817</sup> quartiles was modeled to estimate a P value for trend.

**Western blotting**

Prostate tissues were homogenized or cells were lysed in 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride with 1X protease inhibitors and phosphatase inhibitors (Cat# 7878 and 04904901, Roche Applied Science, Indianapolis, IN). Western blotting was performed as described previously (19) with anti-Pten (cat# 9595), anti-pAkt<sup>817</sup> (cat# 20965), anti- Akt<sup>817</sup> (cat# 3787), anti-total Akt (cat# 9272) (Cell Signaling Technology, Danvers, MA), anti-androgen receptor (cat# sc-816), anti-Lamin B1 (cat# sc-20682), anti-GAPDH (cat# 84334) (Santa Cruz Biotechnology, Santa Cruz, CA) as well as anti-β-actin (cat# A5414, Sigma), anti-n-tubulin (cat# MCA77G) (ABD Serotec, Raleigh, NC). The density of bands from western blots was quantified using AlphaView software (ProteinSimple, Santa Clara, CA).

**PUFA treatment**

Pen-null human prostate cancer cells LNCaP and C4-2, a castration-resistant clone of LNCaP, as well as mouse prostate epithelial cells were maintained in advanced DMEM (Invitrogen) supplemented with 1% FBS. Cells were rinsed with phosphate-buffered saline twice and then incubated with phenol red-free DMEM supplemented with 1% charcoal-stripped FBS plus bovine serum albumin (BSA), 60 µM of BSA-conjugated arachidonic acid (AA) or docosahexaenoic acid (DHA) for 24–72 h.

**Cytosolic and nuclear fractionation**

Cells were first lysed in a lysis buffer [10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid pH 7.9, 150 mM NaCl, 0.5% CA630, 1X protease and phosphatase inhibitors (Roche), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>2</sub>VO<sub>4</sub>]. Nuclei were pelleted at 1000g for 10 min at 4°C and the supernatant was retained as the cytosolic fraction. After rinsing pellets with the lysis buffer twice, nuclear protein was extracted in a nuclear buffer (20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid pH 7.9, 420 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol bis(aminoethyl ether)-tetraacetic acid, 20% glycerol, 1X protease and phosphatase inhibitor, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>2</sub>VO<sub>4</sub>) with 3 × 10 s sonication at setting 10 in a Misonix XL-2000 sonicator (Qsonica, LLC, Newtown, CT).

**Real-time polymerase chain reaction**

Total RNA was extracted using TRIZOL (Invitrogen) and reverse transcribed with Superscript III Plus RNase H-Reverse Transcriptase (Invitrogen). Real-time polymerase chain reaction was performed with Platinum SYBR Green qPCR Supermix UDG (Invitrogen) on Bio-Rad iCycler (Hercules, CA). The primers were used to forward primer TTTGTTGAAGTGGCTGGTGG and reverse primer GCGTATTTGCCTGGAG and forward primer AGTATTGGTCCCTGAGG and reverse primer GCCATCATCAGTGGTTCTC and reverse primer CTGCCAATGGTTT for AR.

**AR protein stability assay**

C4-2 cells were incubated in phenol red-free DMEM supplemented with 1% charcoal-stripped FBS plus BSA, 60 µM AA or 60 µM DHA for 72 h and then cycloheximide (Sigma) or MG132 (Sigma) was added at the final concentration of 20 µg/ml or 10 µM. Cells were harvested after 1 or 2 h incubation and used for western blot analysis.
**AR knockdown and proliferation assays**

C4-2 human prostate cancer cells were infected with lentivirus expressing control shRNA or the AR targeting shRNA (GAG GCA CCT CTC TCA AGA GTT) (23). Infected cells were confirmed by enhanced green fluorescent protein tag and western blotting of AR.

For proliferation in culture, cells (6 × 10^3) were seeded onto 6 well plates in phenol red-free DMEM with 1% charcoal stripped FBS plus BSA or 60 μM DHA. Cells were counted after 24 h incubation and then counted every other day for 7 days. Two independent experiments were performed. For proliferation *in vivo*, 10 nude mice were fed with the omega-3 PUFA diet. All mice were castrated after 1 week on the diet. 5 × 10^3 AR shRNA or scrambled shRNA-infected C4-2 cells were mixed with an equal volume of Matrigel and inoculated subcutaneously into each flank of mice 2 weeks after castration. Tumor volume was measured once a week. Mice were euthanized 6 weeks after cancer cell inoculation.

**Statistics**

Quantitative data with two groups were tested by unpaired Student’s t-test using Excel software (Microsoft, Seattle, WA). Quantitative data of over two groups were initially evaluated by analysis of variance followed by Tukey test to evaluate pairwise comparisons using GraphPad Software (Abacus Concepts, Berkeley, CA). P < 0.05 was considered as significant.

**Results**

*The Pten/Akt pathway affects AR levels in prostate tumor cells*

An association between the loss of PTEN and emergence of castration-resistant tumors was observed in patients (4) and in *Pten* knock-out mouse models (12,13). Since the AR signaling pathway may play a role in castration resistance, AR expression was compared in *Pten* wild-type and *Pten*-null prostate cells. Prostate epithelial cells were isolated from mice with the following genotypes: *Pten<sup>L</sup>, Cre<sup>T</sup>* (equivalent to prostate-specific *Pten<sup>+/−</sup>*); *Pten<sup>+/−</sup>* and *Cre<sup>T</sup>*, *Pten<sup>+/−</sup>* and *Cre<sup>T</sup>* (**Figure 1A**). Interestingly, the level of AR protein was increased in *Pten<sup>−/−</sup>* and, to lesser extent, *Pten<sup>−/−</sup>* compared with *Pten<sup>+/+</sup>* cells (**Figure 1A**). To confirm this inverse correlation between Pten and AR, Pten expression was knocked down in the *Pten<sup>+/+</sup>* cells using Pten-specific shRNA or the *Pten* gene was deleted *in vitro* by Cre expression in *Pten<sup>L/+</sup>* cells. In either case, loss of Pten increased AR expression (**Figure 1B**). It is well known that deletion of *PTEN* activates Akt. Active Akt (pAkt<sup>S473</sup>) and AR proteins were assessed by IHC in a large cohort of prostate cancer patient samples from the Physicians’ Health Study and Health Professionals Follow-up Study. A significant positive association was found between prostate tumor expression of pAkt<sup>S473</sup> and expression of AR (P for trend < 0.001) (**Figure 1C**). Men in the top quartile of pAkt<sup>S473</sup> in their tumors had a 20% increase in expression of AR compared with those in the bottom quartile of pAkt<sup>S473</sup> staining. Therefore, *in vitro*, animal and patient data collectively indicate that the Pten/Akt pathway affects AR levels in prostate tumor cells.

*Pten-null prostate tumor cells are castration resistant*

To assess their androgen responsiveness, *Pten<sup>+/+</sup>* and *Pten<sup>−/−</sup>* prostate cells were grown in culture or as allografts in the presence or absence of androgens. *Pten<sup>−/−</sup>* cells did not proliferate in charcoal stripped medium nor did they respond to R1881, a synthetic androgen analog. However, *Pten<sup>−/−</sup>* cells could proliferate slowly in charcoal stripped medium and more rapidly in response to R1881 (Supplementary Figure 1A is available at Carcinogenesis Online). *Pten<sup>+/+</sup>* cells also did not grow in castrated or intact male nude mice as allografts. In contrast, *Pten<sup>−/−</sup>* cells formed tumors in both castrated and intact male nude mice (Supplementary Figure 1B is available at Carcinogenesis Online). Thus, *Pten*-null prostate tumor cells are androgen responsive and castration resistant.

*Omega-3 PUFA slows down the growth of castration-resistant tumors*

To determine the effects of diet on the growth of castration-resistant prostate cancer allografts, 8-week-old nude mice were placed on the
omega-3 or omega-6 diet 1 week before undergoing castration and then inoculated with $Pten^{-/-}$ cells 1 week after surgery. Tumors were measured for volume weekly and then dissected and weighed 6 weeks after cell inoculation. Allografts from mice on the omega-3 diet grew to less than half the average volume (Figure 2A) and weight (Figure 2B) of those on the omega-6 control diet.

Androgens, namely testosterone (T), dihydrotestosterone and DHEA were also measured in the plasma. Non-castrated male mice had variable levels of T, which is consistent with a previous report (24). As expected, castrated mice had no detectable levels of T (Figure 2A). In fact, no T, DHT or DHEA were detectable in castrated mice on either omega-3 or omega-6 diet.

Fig. 2. Differential effect of omega-3 and omega-6 PUFA on the development of castration-resistant prostate tumors. (A) Tumor volume in allograft model: $Pten^{-/-}$ cells were inoculated in castrated mice as described earlier. Plasma testosterone level was measured and confirmed the success of castration (N1–N3: non-castrated, C1–C3: castrated). Mice were fed omega-3 or omega-6 diet (6 mice and 12 allografts per group). Tumor volumes were monitored twice a week. Compared with omega-6 diet group, omega-3 diet slowed down the growth of castration-resistant tumors significantly (6-week data point, $P<0.014$, Student’s t-test). (B) Tumor weight in allograft model: at the end of 6 weeks, tumors were dissected and weighed. Compared with omega-6 diet group, omega-3 diet reduced the weight of castration-resistant tumors significantly ($P<0.015$, Student’s t-test). Pictures of representative tumor-bearing nude mice are shown. (C) Tumor weight in transgenic model: prostate-specific $Pten$ knockout mice were placed on omega-3 or omega-6 diet after weaning, castrated at the age of 2 months and terminated at the age of 6 months (left panel, five mice per group). Alternatively, prostate-specific $Pten$ knockout mice were placed on chow diet for 2 months to allow tumor formation and then castrated and fed omega-3 or omega-6 diet for 4 months (middle panel, nine mice per group). Compared with omega-6 diet group, omega-3 diet significantly delayed the development of castration-resistant tumors. Finally, fat-1 transgene was bred into the prostate-specific $Pten$ knockout genetic background. Mice with or without fat-1 were placed on chow diet for 2 months and then on omega-6 diet after castration (right panel, 6–12 mice per group). Significantly, smaller prostate tumors were seen in fat-1 transgenic mice compared with the littermate controls ($P<0.003$, Student’s t-test).
To more closely model the development of castration-resistant prostate cancer in patients, we used prostate-specific Pten knockout mice. In an initial experiment, Pten<sup>−/−</sup> mice were placed on omega-3 or omega-6 diet at weaning (3 weeks of age), castrated at the age of 2 months and terminated at the age of 6 months. Mouse prostates were dissected and weighed. Castration-resistant tumors developed in all mice, but were 30% smaller in mice on the omega-3 compared with the omega-6 control diet (Figure 2C, left panel). Since PUFA also affects primary prostate tumor growth (19), prostate-specific Pten knockout mice were fed chow diet prior to castration to allow tumor formation under the same condition for both groups, then castrated at 2 months of age and subsequently fed the omega-3 or omega-6 diet for 4 months. Mouse prostates were dissected and weighed at 6 months of age. A similar reduction in castration-resistant prostate tumor weights was observed in mice on the omega-3 compared with mice on the omega-6 control diet (Figure 2C, middle panel).

In addition, to confirm that omega-3 PUFA is responsible for the observed effect on prostate tumor growth, the fat-1 transgene (14), encoding an omega-3 desaturase from Caenorhabditis elegans that converts omega-6 into omega-3 fatty acids (19), was bred into the prostate-specific Pten knockout genetic background. Mice with or without the fat-1 transgene were both maintained on chow diet for 2 months and then switched to the omega-6 diet after castration. Significantly, smaller prostate tumors were observed in fat-1 transgenic mice compared with the littermate controls (Figure 2C, right panel). Data from all three models (allograft, Pten<sup>−/−</sup> and Pten<sup>−/−</sup>; fat<sup>1</sup>) indicate that omega-3 PUFA slows down growth of the castration-resistant prostate tumors.

**Dietary PUFA is efficiently incorporated into tumor tissues**

To assess the efficiency of dietary PUFA intake, fatty acid profiles were analyzed in allografts and prostate tissues from Pten<sup>−/−</sup> mice on omega-3 and omega-6 diets. Total fatty acid measurements indicate that tumors from mice on the omega-3 diet contained substantially higher levels of eicosapentaenoic acid (EPA 20:5n-3), docosapentaenoic acid (DPA 22:5n-3) and docosahexaenoic acid (DHA 22:6n-3), whereas higher levels of linoleic acid (LA 18:2n-6) and arachidonic acid (AA 20:4n-6) were found in tumors from mice on the omega-6 diet (Supplementary Figure 2A is available at Carcinogenesis Online).

Dietary PUFA was incorporated into glycerophospholipids. Tissues from mice on omega-3 diet had high amounts of eicosapentaenoic acid- and DHA-containing phosphatidylcholine (16:0, 20:5; 16:0, 22:6 and 18:0, 22:6), phosphatidylethanolamine (16:0, 20:5; 16:0, 22:6; 18:0, 22:6 and 18:1, 22:6), phosphatidylinositol (18:0, 20:5; 20:0, 20:5; 16:0, 22:6 and 18:0, 22:6) and phosphatidylserine (20:0, 20:5; 18:0, 22:6). In contrast, tissues from mice on omega-6 diet had high amounts of AA-containing phosphatidylcholine (16:0, 20:4; 18:0, 20:4 and 20:0, 20:4), phosphatidylethanolamine (16:0, 20:4; 18:0, 20:4 and 20:0, 20:4), phosphatidylinositol (16:0, 20:4; 18:0, 20:4 and 20:0, 20:4) and phosphatidylserine (18:0, 20:4; 20:0, 20:4) (Supplementary Figure 2B is available at Carcinogenesis Online).

**Omega-3 PUFA, compared with omega-6 PUFA, reduces tumor pathological progression, proliferation, angiogenesis and inflammation**

In allografts, all tumors from mice fed the omega-3 diet for 6 weeks had >10% of necrotic areas, whereas none of the tumors from mice fed the omega-6 diet had any necrosis at this time point (Figure 3A). In transgenic mice, no necrosis was detected in tumors from mice fed either the omega-3 or omega-6 diet for 6 months (Figure 3A). Allografts were well-circumscribed nodules composed of fusiform cells with no obvious invasion of the surrounding tissues. However, most of the castration-resistant tumors in transgenic mice invaded into the surrounding tissues at 6 months of age regardless of diet type (Figure 3A).
As mentioned above, the \textit{Pten}^{-/-} allografts had fusiform or mesenchymal morphology. \textit{Pten}^{-/-} epithelial cells, after propagation in culture, acquired changes resembling an epithelial–mesenchymal transition. Cells exhibited elongated morphology were negative for E-cadherin and pan-cytokeratin but expressed N-cadherin (Chen unpublished results). To further substantiate that these changes are associated with the loss of the \textit{Pten}, \textit{Pten}^{-/-} prostate epithelial cells were isolated, infected with Cre-expressing lentivirus in culture and \textit{Pten}^{-/-}/Cre were incubated with AA, DHA or BSA. AR protein expression was determined by western blotting. AR protein expression was determined by western blotting. AR messenger RNA was quantified by real-time reverse transcription–polymerase chain reaction. DHA treatment reduced the level of AR protein, but not messenger RNA levels. Bars are standard deviations. AR messenger RNA in C4-2 cells was quantified by real-time reverse transcription–polymerase chain reaction. DHA treatment reduced AR expression in transgenics (\(P = 0.02\), Student’s \(t\)-test). A similar trend was seen in allografts. Bars are standard deviations. AR messenger RNA was quantified by real-time reverse transcription–polymerase chain reaction. No statistically significant difference was seen between tumor samples from mice on omega-3 and omega-6 diet. (B) Effects on multiple cell lines: human PTEN-negative prostate tumor cells (C4-2 and LNCaP), mouse \textit{Pten}-null cells (\textit{Pten}^{-/-}) and \textit{in vitro} \textit{Pten}-deleted mouse prostate cells (\textit{Pten}^{-/-}/Cre) were incubated with AA, DHA or BSA. AR protein expression was determined by western blotting. AR messenger RNA in C4-2 cells was quantified by real-time reverse transcription–polymerase chain reaction. DHA treatment reduced AR protein, but not messenger RNA levels. Bars are standard deviations. (C) Kinetics: C4-2 cells were treated with AA, DHA or BSA for 24, 48 and 72 h. AR protein expression was determined by western blotting. DHA treatment reduced the level of AR protein. (D) Subcellular distribution of AR protein: C4-2 cells were treated with AA, DHA or BSA for 72 h. Cytosolic and nuclear fractions were prepared and AR protein expression was determined by western blotting. Lamin B1 and \(\alpha\)-tubulin were used as loading controls for nuclear and cytosolic fractions, respectively. DHA treatment reduced the level of AR protein to a similar extent in both fractions, suggesting that DHA had no effect on AR nuclear translocation. Analysis of variance was used to assess the significance of data. \(P < 0.05\) was considered as significant.

\textbf{Omega-3 PUFA reduces AR protein level in castration-resistant tumor cells}

Deletion of \textit{Pten} increases AR expression, which may be partly responsible for the castration resistance of tumors. We asked whether omega-3 PUFA slows down the development of castration-resistant prostate tumor by affecting the AR signaling pathway. AR expression was examined in prostate tumors from mice on different diets. Tissues from transgenic mice on the omega-3 diet had significantly reduced levels of the AR protein compared with mice on the omega-6 diet (Figure 4A). A similar trend was observed in allograft tissues (Figure 4A) as well as \textit{Pten}-null cells treated with DHA (Figure 4B).

To understand better the mechanism of AR downregulation by omega-3 PUFA, AR messenger RNA was quantified by real-time reverse transcription–polymerase chain reaction. No significant difference in AR messenger RNA levels was seen in prostate tissues from transgenic mice on either the omega-3 or omega-6 diet (Figure 4A) nor in cells treated with AA and DHA (Figure 4B). Instead, omega-3 PUFA appears to downregulate AR protein rather than messenger RNA. By 48–72h, DHA treatment significantly reduced
the AR protein level compared with AA or BSA treatment (Figure 4C).
To determine if the effect of DHA might be mediated in part by a change in subcellular distribution of AR, we determined AR protein levels in cytosolic and nuclear fractions isolated from BSA-, AA- or DHA-treated C4-2 cells (Figure 4D). DHA treatment reduced the levels of AR protein to a similar extent in both fractions, suggesting that DHA had no significant effect on nuclear translocation of the protein.

Omega-3 PUFA increases proteasome-dependent degradation of AR protein
To assess the AR protein degradation rate, C4-2 cells were treated with BSA, AA or DHA and then the protein synthesis inhibitor cycloheximide. AR protein from BSA- and AA-treated cells had a similar degradation rate with slopes of −0.28 and −0.27, respectively. In DHA-treated cells, however, AR appeared to degrade faster with a slope of −0.35 (Figure 5A). AR protein degradation was proteasome dependent since incubation of BSA-, AA- or DHA-treated C4-2 cells (Figure 4D). DHA treatment reduced the levels of AR protein to a similar extent in both fractions, suggesting that DHA had no significant effect on nuclear translocation of the protein.

AR knockdown inhibits proliferation of castration-resistant tumor cells
To further demonstrate its role in castration resistance, AR was knocked down in C4-2 cells (Figure 6A) and cell proliferation was measured in the absence of androgens. AR knockdown significantly reduced cell proliferation in medium with charcoal stripped FBS. Cell proliferation was inhibited in medium with charcoal stripped FBS plus 60 μM DHA and AR knockdown further diminished cell proliferative capacity in culture (Figure 6A). AR knockdown also significantly reduced tumor growth in xenografts as measured by volume and weight (Figure 6B).

Discussion
Diet is a potential modulator of prostate cancer. Humans obtain omega-3 and omega-6 PUFAs entirely from diet or dietary supplements (25). Epidemiological studies suggest that consumption of fish or fish oil reduces prostate cancer incidence (15,16), as determined by one of the largest studies following 6272 men over 30 years (16). Additionally, patients with benign prostatic hyperplasia and prostate cancer were reported to have significantly decreased omega-3 and increased omega-6 PUFA levels in the serum compared with the control group (17). Therefore, omega-3 PUFA may be protective against prostate cancer development in patients.

Following our previous study on the influence of PUFA on prostate tumor development (19), we sought to determine whether dietary PUFA can also affect castration resistance of Pten-null prostate cancer. The data presented here indicate that the omega-3 diet slows down, compared with the omega-6 control diet, the growth of castration-resistant tumors. Consistent with our observation, a recent study showed that supplementation of the AIN-76A-based diet with a single omega-3 PUFA, eicosapentaenoic acid, significantly delays the androgen independent relapse of human prostate cancer xenografts (26). In addition to tumor weight, omega-3 and omega-6 PUFA also
differentially affected the histology, proliferation, necrosis, angiogenesis, inflammation and possibly invasion of castration-resistant tumors (Figure 3). Necrosis was seen in tumors from allograft mice fed omega-3 diet, but not in tumors from transgenic mice, due perhaps to the larger mass of tumors in allografts compared with orthotopic prostate tumors in the transgenic mouse model. In primary Pten-null prostate tumors, omega-3 PUFA induced apoptosis in a Bad-dependent manner (19). In castration-resistant tumors, however, we detected no significant differences in apoptosis based on cleaved caspase-3 IHC staining between tumors from mice fed omega-3 and omega-6 diets. Instead, tumors from mice fed the omega-3 diet had smaller proliferative fractions compared with mice fed the omega-6 diet. This discrepancy in proliferation and apoptosis may be due, in part, to an altered cellular response of castration-resistant tumor since these tumor cells have undergone massive apoptosis after androgen withdrawal. We noticed that the number of CD3+ T and CD45R+ B lymphocytes was significantly reduced in the primary prostate tumors from transgenic mice fed the omega-3 compared with omega-6 diet (Chen unpublished results). In the case of castration-resistant tumors, a difference in CD45R+ B lymphocytes was observed (Figure 3D). Taken together with the recent observation that B-cell-derived lymphotxin promotes castration-resistant prostate cancer (27), this suggests that omega-3 PUFA may act in part by reducing B lymphocytes in castration-resistant cancer.

Previously, it was believed that hormone-refractory prostate cancer was androgen independent and thus that the AR was dispensable. However, evidence suggests that AR is not only present in >90% of hormone-refractory prostate tumors (28,29) but also amplified or overexpressed in 50% of bone metastases (30) and in 20–30% of recurrent hormone-refractory prostate cancers (31–34). To more accurately describe this clinical condition, ‘castration resistant’ has recently replaced ‘hormone refractory’ or ‘androgen independent’. Interestingly, we observed an increase in the AR protein level in Pten-null prostate tumor cells (Figure 1A), which may explain the association between Pten loss and development of castration resistance. Indeed, AR knockdown significantly impaired the tumorigenesis of Pten-null prostate cells in female SCID mice (13). We demonstrated that omega-3 PUFA slowed down, compared with omega-6 PUFA, the growth of castration-resistant tumors, and this differential effect may be due, in part, to their effect on the level of AR proteins (Figure 4). A report suggests that omega-3 PUFA affects AR-dependent gene expression (35). We found that AR protein was similarly reduced in both the cytosolic and nuclear fractions. However, AR-dependent gene expression was not assessed. The reduction in AR proteins appears to be due to an increase in proteasome-dependent degradation.

A recent study suggests that castration-resistant growth is an intrinsic property of Pten-null prostate cancer cells. Loss of *PTEN* suppresses androgen-responsive gene expressions and deletion of *Ar* in mouse epithelium promotes the proliferation of Pten-null cancer cells (36). This seems to be difficult to reconcile with the fact that the vast majority of advanced prostate cancer cases are AR positive, a third of which have AR overexpression, and that up to 70% of advanced prostate cancer cells have deletion of *PTEN*. Our data show that knockdown or knockout of *Pten* increases AR protein expression in prostate epithelial cells (Figure 1) and *Pten*−/− cells are castration-resistant (Supplementary Figure 1 is available at Carcinogenesis Online). Knockdown of AR inhibits prostate Pten-null cell proliferation (Figure 6), suggesting a critical role of AR in castration resistance. It is possible that deletion of the Ar gene can select a population of castration-resistant tumor cells that is independent of AR.

Prostate cancer is usually diagnosed in aged men and tumor cell proliferation is typically slow. Preventing or slowing down the local regrowth of tumors and the development of castration-resistant lesions could significantly extend patient lives. Our data suggest that dietary omega-3 PUFA supplementation in conjunction with androgen ablation could significantly delay the development of castration-resistant Pten-null prostate cancer compared with androgen ablation alone. Furthermore, one prominent side effect of androgen ablation is osteoporosis, and higher omega-3 to omega-6 ratios have been linked to increased bone mineral density in older adults (37). Therefore, omega-3 PUFA may not only delay the development of castration-resistant prostate cancer but could also have a salutary effect on bone density in patients treated with hormonal ablation.

### Supplementary material

Supplementary Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/.

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References


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