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## **$\omega$ -3 polyunsaturated fatty acids and their cytochrome P450-derived metabolites suppress colorectal tumor development in mice**

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### **Abstract**

Many studies have shown that dietary intake of  $\omega$ -3 polyunsaturated fatty acids (PUFAs) reduces the risks of colorectal cancer, however the underlying mechanisms are not well understood. Here we used a LC-MS/MS-based lipidomics to explore the roles of eicosanoid signaling in the anti-colorectal cancer effects of  $\omega$ -3 PUFAs. Our results showed that dietary feeding of  $\omega$ -3 PUFAs-rich diets suppressed growth of MC38 colorectal tumor, and modulated profiles of fatty acids and eicosanoid metabolites in C57BL/6 mice. Notably, we found that dietary feeding of  $\omega$ -3 PUFAs significantly increased levels of epoxydocosapentaenoic acids (EDPs, metabolites of  $\omega$ -3 PUFA produced by cytochrome P450 enzymes) in plasma and tumor tissue of the treated mice. We further showed that systematic treatment with EDPs (dose = 0.5 mg/kg/day) suppressed MC38 tumor growth in mice, with reduced expressions of pro-oncogenic genes such as *c-myc*, *Axin2*, and *C-jun* in tumor tissues. Together, these results support that formation of EDPs might contribute to the anti-colorectal cancer effects of  $\omega$ -3 PUFAs.

### **Keywords**

$\omega$ -3 polyunsaturated fatty acids (PUFAs); docosahexaenoic acid (DHA); colorectal cancer; lipidomics; epoxydocosapentaenoic acids (EDPs)

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## Introduction

Every year, there are ~134,490 new cases and ~49,190 deaths from colorectal cancer, making colorectal cancer the second cause of cancer-related death in the United States [1]. Epidemiological and pre-clinical data support that dietary intake of  $\omega$ -3 polyunsaturated fatty acids (PUFAs), which are abundant in fish and fish oil supplements, may reduce risks of colorectal cancer. In contrast,  $\omega$ -6 PUFAs, which are commonly found in vegetable oils, are suggested to promote colorectal cancer [2–7]. The  $\omega$ -3 PUFAs can regulate a wide array of cancer-related pathways in colorectal cancer, such as cell cycle, apoptosis, NF- $\kappa$ B, Wnt signaling, and angiogenesis [8]. This is important because a typical Western diet contains 30–50 times more  $\omega$ -6 than  $\omega$ -3 PUFAs, though a ratio of  $\omega$ -6-to- $\omega$ -3 PUFAs of 1:1 was recommended for human consumption by many nutritional panels [9]. Therefore, it is of critical importance to validate the effects and mechanisms of  $\omega$ -3 PUFAs on colorectal cancer, which might help to effectively implement  $\omega$ -3 PUFAs for colorectal cancer prevention.

A general mechanism to explain the health benefits of  $\omega$ -3 PUFAs is that they compete with arachidonic acid (ARA, 20:4 $\omega$ -6) for the enzymatic metabolism by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes, leading to reduced formation of  $\omega$ -6-series eicosanoids which are predominately pro-inflammatory and pro-tumorigenic, and increased formation of  $\omega$ -3-series eicosanoid metabolites which are less-detrimental or even beneficial [10]. Recent research showed that  $\omega$ -3 PUFAs, such as eicosapentaenoic acid (EPA, 20:5 $\omega$ -3) and docosahexaenoic acid (DHA, 22:6 $\omega$ -3), are highly efficient alternative substrates of CYP enzymes [11], while they are known as relatively poor substrates of COX and LOX enzymes [12]. Notably, emerging animal and human studies showed that the CYP pathway is the dominant pathway in metabolizing  $\omega$ -3 PUFAs *in vivo* [13, 14]. We showed that epoxydocosapentaenoic acids (EDPs), which are CYP metabolites of DHA, potentially suppressed breast tumor growth and lung cancer metastasis through inhibiting angiogenesis *in vivo* [15]. In agreement with our finding, a recent study by Yanai et al. showed that systematic treatment with EDPs inhibited pathological angiogenesis in a mouse model of macular degeneration [16]. Together, these studies suggest that the CYP metabolites of  $\omega$ -3 PUFAs, such as EDPs, might play an important role in mediating the anti-cancer and anti-angiogenic effects of  $\omega$ -3 PUFAs.

Regarding colorectal cancer, previous studies have shown that eicosanoid metabolome is deregulated in colorectal cancer, and some eicosanoid biogenesis enzymes and associated metabolites play central roles in regulating colorectal cancer [17, 18]. However, how  $\omega$ -3 PUFAs modulate eicosanoid signaling in colorectal cancer is poorly characterized, in addition, the actions of  $\omega$ -3 PUFAs-derived eicosanoid metabolites on colorectal cancer are largely unknown. Here we used a LC-MS/MS-based lipidomics to explore the roles of eicosanoid signaling in the anti-colorectal cancer effects of  $\omega$ -3 PUFAs (see complete list of the eicosanoid metabolites analyzed by our method in supplemental information Table S1), using a xenograft MC38 colorectal cancer model in C57BL/6 mice. Our results showed that dietary feeding of  $\omega$ -3 PUFA-rich diets suppressed growth of colorectal tumor, and increased levels of EDPs in plasma and tumor tissue of the treated mice. We further found that systematic treatment with EDPs inhibited growth of colorectal cancer in mice. Together,

these results support that formation of EDPs could might to the anti-colorectal cancer effects of  $\omega$ -3 PUFAs.

## Materials and Methods

### Animal experiment of $\omega$ -3 PUFA-rich diet on MC38 colorectal cancer growth

We used three completely defined isocaloric diets containing 10 wt/wt% total fat to study the effects of  $\omega$ -3 PUFAs on colorectal cancer: control diet (ratio of  $\omega$ -6-to- $\omega$ -3 PUFA is  $\approx$  69.3:1), DHA diet (ratio of  $\omega$ -6-to- $\omega$ -3 PUFA is  $\approx$  1.26:1), and DHA-high diet (ratio of  $\omega$ -6-to- $\omega$ -3 PUFA is  $\approx$  0.56:1). The fat content of the control, DHA, and DHA-high diets was obtained by the addition to the respective diets of corn oil (Mazola<sup>®</sup>, ACH Food company, Inc., Cordova, TN, containing 100 ppm tocopherols as antioxidants, with peroxide value  $<$  1 meq/kg), or a blend of 38% and 62%, and 20.7% and 79.3% of oil from corn and algae (DHASCO<sup>®</sup>, DSM Nutritional Products, Columbia, MD, containing a mixture of 250 ppm ascorbyl palmitate and 200 ppm tocopherols as antioxidants, with peroxide value  $<$  5 meq/kg). The aliquoted corn oil or algae oil were stored at  $-80^{\circ}\text{C}$  until diet preparation, and the prepared diets were stored at  $-20^{\circ}\text{C}$  and changed every other day during animal feeding. The diet composition is shown in Table 1.

All procedures of animal care were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Massachusetts. Male C57BL/6 mice (age = 6 weeks) were purchased from Charles River Laboratories (Wilmington, MA). The mice were pre-fed with the experimental diets for three weeks, then 400,000 MC38 colorectal cancer cells (a gift from Prof. Ajit Varki at UCSD) in 100  $\mu\text{L}$  PBS were subcutaneously injected into each mouse to initiate primary tumor growth. Tumor sizes were measured using a caliper; after 15 days of cancer cell injection, the mice were sacrificed and the tumor tissues were dissected, weighted and subjected to biochemical analysis.

### LC-MS/MS-based Lipidomics analysis

The LC-MS/MS profiling of eicosanoid metabolites was performed as described in our previous report [19]. After animal sacrifice, the MC38 tumor tissues, colon tissues and plasma were harvested and subjected to LC-MS/MS-based lipidomics analysis. For plasma lipid metabolite extraction, about 250  $\mu\text{L}$  plasma were mixed with deuterated internal standards, then loaded onto pre-washed Waters<sup>®</sup> Oasis solid phase extraction (SPE) cartridges, washed with 95:5 water/methanol with 0.1% acetic acid, the analytes were eluted with methanol and ethyl acetate, dried using a centrifugal vacuum evaporator, then reconstituted in methanol for LC-MS/MS analysis. For extraction of eicosanoid metabolites from tissues, about 100 mg tissues mixed with an antioxidant solution (0.2 mg/mL butylated hydroxytoluene and 0.2 mg/mL triphenylphosphine in methanol), the deuterated internal standards, and 400  $\mu\text{L}$  extract solution (0.1% acetic acid with 0.2 mg/mL butylated hydroxytoluene in a methanol solution), were homogenized; the resulting homogenates were kept in  $-80^{\circ}\text{C}$  overnight. After centrifugation of the homogenates, the pellets were washed with methanol (containing 0.1% butylated hydroxytoluene and 0.1% acetic acid) and then combined with the supernatant. The eicosanoid metabolites in the combined solutions were

extracted using SPE columns, similar to the description above for the plasma lipid metabolite extraction. The LC-MS/MS analysis was carried out on an Agilent 1200SL HPLC system (Agilent, Santa Clara, CA) coupled to a 4000 QTRAP MS/MS (AB Sciex, Foster City, CA) as described in our previous report [19]. The peaks were identified according to the retention time and specific multiple reaction monitoring (MRM) transitions of the eicosanoid metabolite standards. The concentrations of the eicosanoid metabolites are calculated against the calibration curve with standards.

### GC-MS analysis of fatty acid composition

Total lipids from MC38 tumor tissues or diets were extracted as previously described [20], then treated with 3 N methanolic HCl at 55°C for 40 min to prepare the fatty acid methyl esters [21], which were dissolved in hexane and subjected to GC-MS analysis, using Shimadzu GC-MS-QP2010 SE (Tokyo, Japan). Oven conditions: initial temperature 50°C, temperature increase: 20°C/min to 200 °C, then increase 2°C/min to 220°C and held for 142.5 minutes. Other conditions: injector temperature 250 °C, detector temperature 250 °C, carrier gas helium, split ratio: 10:1. Column: Supelcowax 10 (fused silica), 100 m × 0.25 mm × 0.25 µm. The fatty acid methyl esters were identified by comparing with standards (Sigma-Aldrich, St. Louis, MO, or Nu-Chek Prep, Elysian, MN) or by their mass spectra, which were further compared to the NIST Mass Spectral library.

### Flow cytometry analysis

After animal sacrifice, the dissected tumor tissues were digested using enzymatic degradation solution (500 µg/ml collagenase, 500 µg/ml DNase, 100 µg/ml Hyaluronidase in HBSS) for 2 hours at room temperature, filtered through 70 µm cell sorters (BD Biosciences) to obtain single cell suspension, which were stained with FITC-conjugated anti-mouse CD45 antibody, APC-conjugated anti-mouse CD31 antibody, APC/Cy7 anti-mouse Ly-6G/Ly-6C (Gr-1) antibody, PerCP/Cy5.5-conjugated anti-mouse F4/80 antibody or isotype control antibody (R&D Systems). The stained cells were analyzed using BD LSRFortessa™ cell analyzer (BD Biosciences) and data were processed using FlowJo software. Endothelial cells were identified as CD45-, CD31+ cells, neutrophils were identified as CD45+, Gr1+ cells, and macrophages were identified as CD45+, F4/80+ cells.

### Animal experiment of EDPs on MC38 colorectal cancer growth

To test the effect of EDPs on colorectal cancer growth, C57BL/6 male mice were subcutaneously implanted with Osmotic mini-pumps (Durect, Cupertino, CA, catalog number 1004), which contained vehicle (a 1:1 vol/vol mixed solution of DMSO and PEG 400) or EDPs (dose of EDPs = 0.5 mg/kg body weight/day). After one week of mini-pump implantation, 400,000 MC38 colorectal cancer cells in 100 µL PBS were subcutaneously injected into each mouse to initiate primary tumor growth. Tumor sizing was measured using caliper, at the end of the experiment, the tumors were dissected, weighted and subjected to biochemical analysis. The EDPs are a synthetic mixture containing naturally occurring EDP regioisomers (7,8-, 10,11-, 13,14-, 16,17-, and 19,20-EDP), as we described [22].

## Real-time PCR (RT-PCR) analysis

Total RNA was isolated from MC38 tumor tissues using TRIzol Reagent (Life technologies, Carlsbad, CA) according to manufacturer's instruction. Conversion of up to 2 µg of total RNA to single stranded cDNA was preformed using High-Capacity cDNA Reverse Transcription Kit (Life technologies, Carlsbad, CA) according to manufacturer's instruction. Quantitative RT-PCR was conducted using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Agawam, MA) on a DNA Engine Opticon® 2 System (Bio-Rad Laboratories, Hercules, CA) with specific mouse primers. The primers used in this research were: *C-myc* (sense) 5'-ATGCCCCCTCAACGTGAAGTTC-3' and (antisense) 5'-GTCGCAGATGAAATAGGGCTG-3',

*Axin2* (sense) 5'-TGACTCTCCTTCCAGATCCCA-3' and (antisense) 5'-TGCCCACACTAGGCTGACA-3', *C-jun* (sense) 5'-CCTTCTACGACGATGCCCTC-3' and (antisense) 5'-GGTTCAAGGTCATGCTCTGTTT-3', The results of target genes were normalized to *Gapdh* gene and expressed to the control group mice using the  $2^{-Ct}$  method. The primer to analyze *Gapdh* is (sense) 5'-AGGTCGGTGTGAACGGATTTG-3' and (antisense) 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

## Data Analysis

All data are expressed as the mean  $\pm$  standard error of the mean (SEM). Differences among Control, DHA and DHA-high diet feeding group were analyzed by one-way ANOVA and pairwise multiple comparisons among groups were performed by using Dunn's test. For the comparison between the control group and EDP group, Shapiro-Wilk test was used to verify the normality of data. When data were normally distributed, statistical significance was determined using two-side t-test; otherwise, significance was determined by Mann-Whitney U test. *P* values less than 0.05 are reported as statistically significant. All of these data analysis was performed by using SigmaPlot software (San Jose, CA).

## Results

### $\omega$ -3 PUFAs-rich diets inhibited growth of MC38 colorectal tumor in mice

We prepared three completely defined isocaloric diets containing 10 wt/wt% total fat (see diet composition in Table 1). For the control diet, corn oil was the only source of dietary fat, with a ratio of  $\omega$ -6-to- $\omega$ -3 PUFAs in the diet = 69.3:1 (the profile of fatty acids in diet was analyzed by GC-MS, see Table 1), to mimic a typical Western diet. For the DHA diet, the fat component was a mixture of 38% corn oil and 62% DHA-rich DHASCO® Algae oil, with a ratio of  $\omega$ -6-to- $\omega$ -3 PUFAs = 1.26:1. For the DHA-high diet, the fat component was a mixture of 20.7% corn oil and 79.3% DHASCO® oil, with a ratio of  $\omega$ -6-to- $\omega$ -3 PUFAs = 0.56:1 (see Table 1). We have designed these two  $\omega$ -3 PUFAs-rich diets, since a ratio of  $\omega$ -6-to- $\omega$ -3 PUFAs  $\approx$  1:1 was recommended for human consumption by nutritionists [23].

To study the effect of  $\omega$ -3 PUFAs on MC38 colorectal tumor growth, we pre-fed 6-week-old C57BL/6 mice with these three experimental diets for three weeks, then subcutaneously injected MC38 colorectal cancer cells into mice to initiate growth of colorectal tumor (see animal experimental scheme in Fig. 1A). Dietary feeding of DHA or DHA-high diets had

little effect on mouse body weight (Fig. S1A). Compared with control diet, both DHA and DHA-high diets suppressed growth of MC38 colorectal tumor in mice with similar inhibitory effects: at the end of the experiment, these two  $\omega$ -3 PUFAs-rich diets caused a ~50% reduction of tumor weight ( $P < 0.05$ , Fig. 1B–1D). ELISA analysis showed there was a decreased concentration of IL-6 in the plasma from the mice treated with DHA and DHA-high diets (Fig. S1B), which is consistent with previous studies for the anti-inflammatory effects of  $\omega$ -3 PUFAs [24]. Flow cytometry analysis of the single cell suspension from the dissected MC38 tumors showed that these two  $\omega$ -3 PUFAs-rich diets reduced the presence of endothelial cells (CD31+, CD45-) in tumor tissues ( $P < 0.05$ , Fig. 1E). This result is in agreement with previous studies which showed that  $\omega$ -3 PUFAs suppressed tumor angiogenesis, with reduced expression of vascular endothelial growth factor in colon cancer cells and decreased microvessel density in tumors [25].

### **$\omega$ -3 PUFAs-rich diets modulated fatty acid profiles in MC38 colorectal tumors**

We used GC-MS to analyze the profiles of fatty acids in the dissected MC38 colorectal tumors (Table 2). Dietary feeding of the DHA diet (ratio of  $\omega$ -6-to- $\omega$ -3 PUFAs = 1.26:1) significantly reduced levels of  $\omega$ -6 PUFAs such as ARA, and increased levels of  $\omega$ -3 PUFAs such as EPA and DHA in MC38 tumor tissues. Notably, the level of ARA in MC38 colorectal tumor tissues was reduced from  $9.96 \pm 1.71\%$  (mean  $\pm$  SEM, from mice fed on control diet, the result was expressed as % of ARA to the total fatty acids in MC38 tumor tissues) to  $2.89 \pm 0.38\%$  (from mice fed on DHA diet,  $P < 0.01$ ), representing a ~70% reduction of ARA in tumor tissues. The level of EPA was increased from  $0 \pm 0\%$  to  $1.27 \pm 0.19\%$  ( $P < 0.001$ ), and the level of DHA was increased from  $0.91 \pm 0.16\%$  to  $8.33 \pm 1\%$  ( $P < 0.001$ , Table 2). In contrast, the levels of other PUFAs, such as linoleic acid (LA, 18:2 $\omega$ -6) and  $\alpha$ -linoleic acid (ALA, 18:3 $\omega$ -3), were not significantly changed in the tumor tissues (see Table 2). Together, these results showed that dietary feeding of  $\omega$ -3 PUFAs-rich diet modulated profiles of PUFAs in MC38 colorectal tumor tissues, with reduced ARA and increased EPA and DHA.

### **$\omega$ -3 PUFAs-rich diets modulated profiles of eicosanoid metabolites in plasma, MC38 tumor and colon tissues**

After demonstrating that dietary feeding of  $\omega$ -3 PUFAs-rich diets modulated tissue profiles of ARA, EPA, and DHA, which are important precursors to generate eicosanoid metabolites [10], we studied the impacts of  $\omega$ -3 PUFAs-rich diets on profiles of eicosanoid metabolites in the treated mice. To this end, we used a LC-MS/MS-based lipidomics approach to analyze the profiles of eicosanoid metabolites extracted from plasma, MC38 tumor, and colon tissues of the treated mice. The MC38 colorectal tumor is a xenograft model and is not grown in colon tissues, we analyzed the profiles of eicosanoid metabolites in colon tissues to understand how  $\omega$ -3 PUFAs modulate colonic environment. A general trend was that dietary feeding of  $\omega$ -3 PUFAs-rich diets reduced levels of  $\omega$ -6-series metabolites, and increased levels of  $\omega$ -3-series metabolites in plasma and tissues (see complete results of LC-MS/MS analysis in supplemental information Table S2–4), which is in agreement with previous studies [10]. The details of the LC-MS/MS profiling are described below.



The CYP epoxygenases (mainly CYP2C and CYP2J isoforms) convert ARA, EPA, and DHA to fatty acid epoxides termed epoxyeicosatrienoic acids (EETs), epoxyeicosatetraenoic acids (EEQs), and EDPs respectively, which are further metabolized by soluble epoxide hydrolase (sEH) to generate the corresponding fatty acid diols (see scheme of CYP/sEH pathway in Fig. 2A) [26]. In the mice fed with control diet ( $\omega$ -6 PUFAs-rich), ARA-derived EETs are among the most abundant fatty acid epoxides in plasma (Fig. 2B). In contrast, in the plasma of mice fed with DHA and DHA-high diets, there was a dramatic reduction of EETs and an increase of EPA-derived EEQs and DHA-derived EDPs; notably, DHA-derived EDPs were among the most abundant fatty acid epoxides in plasma (Fig. 2B). We further analyzed whether there is a correlation of MC38 tumor volume from above diet experiment (see Fig. 1) with tumor or plasma concentrations of EDPs. The results showed that the tumor volume was inversely correlated with the tumor or plasma concentrations of EDPs (Fig. S2–3), supporting a critical role of EDPs in MC38 tumor growth. Consistent with the trend of fatty acid epoxides, dietary feeding of  $\omega$ -3 PUFAs-rich diets also reduced ARA-derived fatty acid diols termed dihydroxyeicosatrienoic acids (DHETs), and enhanced EPA-derived dihydroxyeicosatetraenoic acids (DiHETEs) and DHA-derived dihydroxydocosapentaenoic acids (DiHDPEs) (see Fig. 2B). Similar to the profiles of eicosanoid metabolites in plasma, we also found that dietary feeding of  $\omega$ -3 PUFAs-rich diets reduced levels of ARA-derived fatty acid epoxides and diols, and increased levels of EPA- and DHA-derived epoxides and diols in MC38 tumor and colon tissues (Fig. 2C–D).

Besides the CYP/sEH pathway, we also analyzed eicosanoid metabolites produced by COX and LOX enzymes, many of these metabolites play central roles in regulating inflammation and tumorigenesis [27, 28]. For COX pathway, dietary feeding of  $\omega$ -3 PUFAs-rich diets reduced levels of ARA-derived prostaglandins, notably,  $\omega$ -3 PUFAs caused ~80% reduction of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which has potent pro-inflammatory and pro-tumorigenic actions, in both MC38 tumor and colon tissues (Fig. 3A–C). For LOX pathway, dietary feeding of  $\omega$ -3 PUFAs-rich diets reduced ARA-derived hydroxyl fatty acids such as 11- and 15-hydroxyeicosatetraenoic acid (HETE), and increased EPA-derived 5- and 15-hydroxyeicosapentaenoic acid (HEPE) in plasma, MC38 tumor and colon tissues (see Fig. 3D–F). Together, these results showed that dietary feeding of  $\omega$ -3 PUFAs-rich diets caused a profound change of the eicosanoid metabolome, with reduced levels of ARA-derived metabolites, and increased levels of EPA- and DHA-derived metabolites.

### EDPs suppressed growth of MC38 tumor growth *in vivo*

Given that dietary feeding of  $\omega$ -3 PUFAs-rich diets dramatically increased concentrations of EDPs in plasma and tissues, we tested the effect of EDPs on MC38 tumor growth in C57BL/6 mice. Continuous infusion of synthetic EDPs (dose = 0.5 mg/kg/day) inhibited MC38 tumor growth in C57BL/6 mice, with ~50% reduction of tumor weight (Fig. 4A–B). Flow cytometry of the single cell suspension from the dissected MC38 tumors showed that EDPs slightly reduced infiltration of neutrophils and macrophages into tumor tissues ( $P < 0.05$ , Fig. 4C). RT-PCR analysis showed that EDPs reduced expressions of several genes related to cellular kinetics and tumorigenic Wnt pathway, such as *c-myc*, *Axin2*, and *C-jun* ( $P < 0.05$ , Fig. 4D), which suggests anti-tumor effect of EDPs and is consistent with

previous studies which showed that  $\omega$ -3 PUFAs have anti-cancer effect with inhibition of molecular pathways driven by  $\beta$ -catenin and c-Myc [8].

## Discussion

To date, there is a fair amount of studies to demonstrate the anti-colorectal cancer effects of  $\omega$ -3 PUFAs [2–7]; however, the underlying mechanisms remain largely unknown. A general theory to explain the health benefits of EPA and DHA is that they can efficiently compete with ARA at almost every step of eicosanoid biogenesis, leading to reduced formation of ARA-derived eicosanoids which are predominately pro-inflammatory and/or pro-tumorigenic, and increased formation of EPA- and DHA-derived metabolites which have less-detrimental or beneficial actions [10]. Here we used a LC-MS/MS-based lipidomics to explore the roles of eicosanoid signaling in the anti-colorectal cancer effects of  $\omega$ -3 PUFAs. The LC-MS/MS analysis showed that dietary feeding of  $\omega$ -3 PUFA-rich diets caused a dramatic modulation of the eicosanoid profiles in circulation and in colorectal tumor tissues, with reduced levels of  $\omega$ -6-series metabolites and increased levels of  $\omega$ -3-series metabolites. A central finding of our study is that dietary feeding of  $\omega$ -3 PUFAs-rich diets dramatically increased levels of EDPs in both plasma and MC38 colorectal tumor of the treated mice. In addition, systematic treatment with EDPs suppressed growth of MC38 colorectal tumor in mice. Together, these results support that formation of EDPs could contribute the anti-colorectal cancer effects of  $\omega$ -3 PUFAs, and EDPs might serve as a biomarker for the anti-cancer effects of  $\omega$ -3 PUFAs.

Regarding the biological effects of EDPs, our results further support the anti-cancer effects of EDPs. Our previous study showed that stabilized EDPs suppressed breast tumor growth and Lewis lung carcinoma metastasis in mice [15]. However, in our previous study, co-administration of a pharmacological inhibitor of soluble epoxide hydrolase (sEH, the major enzyme in degrading EDPs) was required to stabilize low-dose EDP (dose of EDP = 0.05 mg/kg/day in our previous study) in circulation, in order to demonstrate the anti-cancer and anti-metastatic effects of EDP *in vivo* [15]. Based on our previous study, here we hypothesize that administration of EDP alone, at a higher dose, inhibits tumor growth in mice. Indeed, our result showed that administration of EDPs alone, at a higher dose than that of our previous experiment (dose of EDPs in this study = 0.5 mg/kg/day), significantly inhibited MC38 colorectal tumor growth in mice, further validating the anti-cancer effects of EDPs *in vivo*. Several animal and human studies have shown high levels of EDPs in circulation upon dietary intake of  $\omega$ -3 PUFAs [13, 14], supporting that EDPs could serve as a reliable biomarker for exposure to  $\omega$ -3 PUFAs. In addition, in the diet experiment, we found that high concentration of EDPs in tumor or plasma were correlated with decreased tumor sizes (Fig. S2–3), further supporting a critical role of EDPs in the anti-cancer effects of  $\omega$ -3 PUFAs. A limitation of current study is that we only tested the effects of exogenously administered EDPs, the biological effects of endogenous EDPs remain largely unknown. Further studies are needed to better characterize the actions of EDPs, in order to facilitate the development of potential biomarkers of  $\omega$ -3 PUFAs.

Many studies support that  $\omega$ -3 PUFAs reduce the risks of colon inflammation [29–31] and colon cancer [2–7]. However, there are inconsistent results from animal and human studies,



which showed that  $\omega$ -3 PUFAs had no effect [32, 33] or detrimental effects [34, 35], making it difficult to implement  $\omega$ -3 PUFAs for disease prevention. The mixed results for the health-promoting effects of  $\omega$ -3 PUFAs is a major barrier to effectively implement  $\omega$ -3 PUFAs for disease prevention. Based on our studies, polymorphisms in the genes encoding enzymes in CYP pathway may affect the metabolism of  $\omega$ -3 PUFAs [36–42], impacting the generation of bioactive lipid metabolites, and thereby contributing to observed inter-individual variations to  $\omega$ -3 PUFA supplementation [43]. For example, based on our findings, people carrying Lys55Arg and Cys154Tyr mutations of soluble epoxide hydrolase (sEH, the major enzyme to degrade EDPs), which lead to higher sEH enzymatic activities [42], might have lower tissue levels of EDPs, and thus poorer anti-cancer responses upon dietary  $\omega$ -3 PUFA supplementation. Such mechanistic knowledge, together with utilization of nutrigenomic and metabolomic approaches, could lead to targeted human trials to better understand the metabolic individuality and nutrition effects of  $\omega$ -3 PUFAs [43, 44].

A limitation of our study is that we used a xenograft model of MC38 colorectal cancer, which has many limitations to mimic the complicated process of colorectal carcinogenesis. To partially address this issue, we also analyzed the effects of  $\omega$ -3 PUFAs-rich diets on colonic profiles of eicosanoid metabolites, and observed a similar pattern that  $\omega$ -3 PUFAs-rich diets reduced levels of  $\omega$ -6-series metabolites, and increased levels of  $\omega$ -3-series metabolites. Further studies using advanced colon cancer model are needed to better understand how  $\omega$ -3 PUFAs modulate colonic eicosanoid signaling to reduce risks of colon cancer.

In conclusion, our studies showed that dietary feeding of  $\omega$ -3 PUFAs-rich diets dramatically modulated profiles of eicosanoid metabolites in a mouse model of colorectal cancer. Among the altered eicosanoid metabolites, DHA-derived EDPs could play critical roles in mediating the anti-colorectal cancer effects of  $\omega$ -3 PUFAs. Further studies are needed to better characterize the roles of EDPs in health benefits of  $\omega$ -3 PUFAs, in order to establish potential biomarkers for effective implementation of  $\omega$ -3 PUFAs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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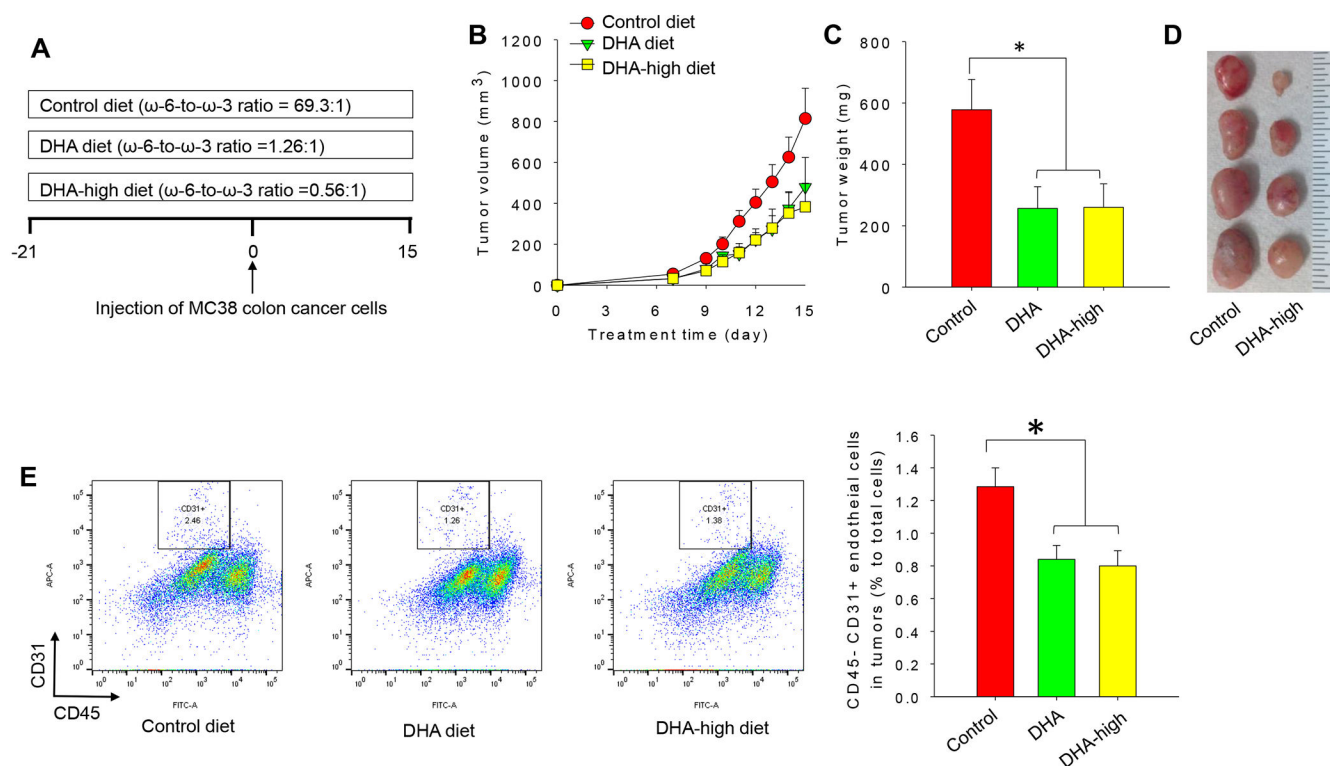
## References

- [1]. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin.* 2016;66:7–30. [PubMed: 26742998]
- [2]. Sasazuki S, Inoue M, Iwasaki M, Sawada N, Shimazu T, Yamaji T, et al. Intake of n-3 and n-6 polyunsaturated fatty acids and development of colorectal cancer by subsite: Japan Public Health Center-based prospective study. *Int J Cancer.* 2011;129:1718–29. [PubMed: 21120874]
- [3]. Murff HJ, Shrubsole MJ, Cai Q, Smalley WE, Dai Q, Milne GL, et al. Dietary intake of PUFAs and colorectal polyp risk. *Am J Clin Nutr.* 2012;95:703–12. [PubMed: 22277551]

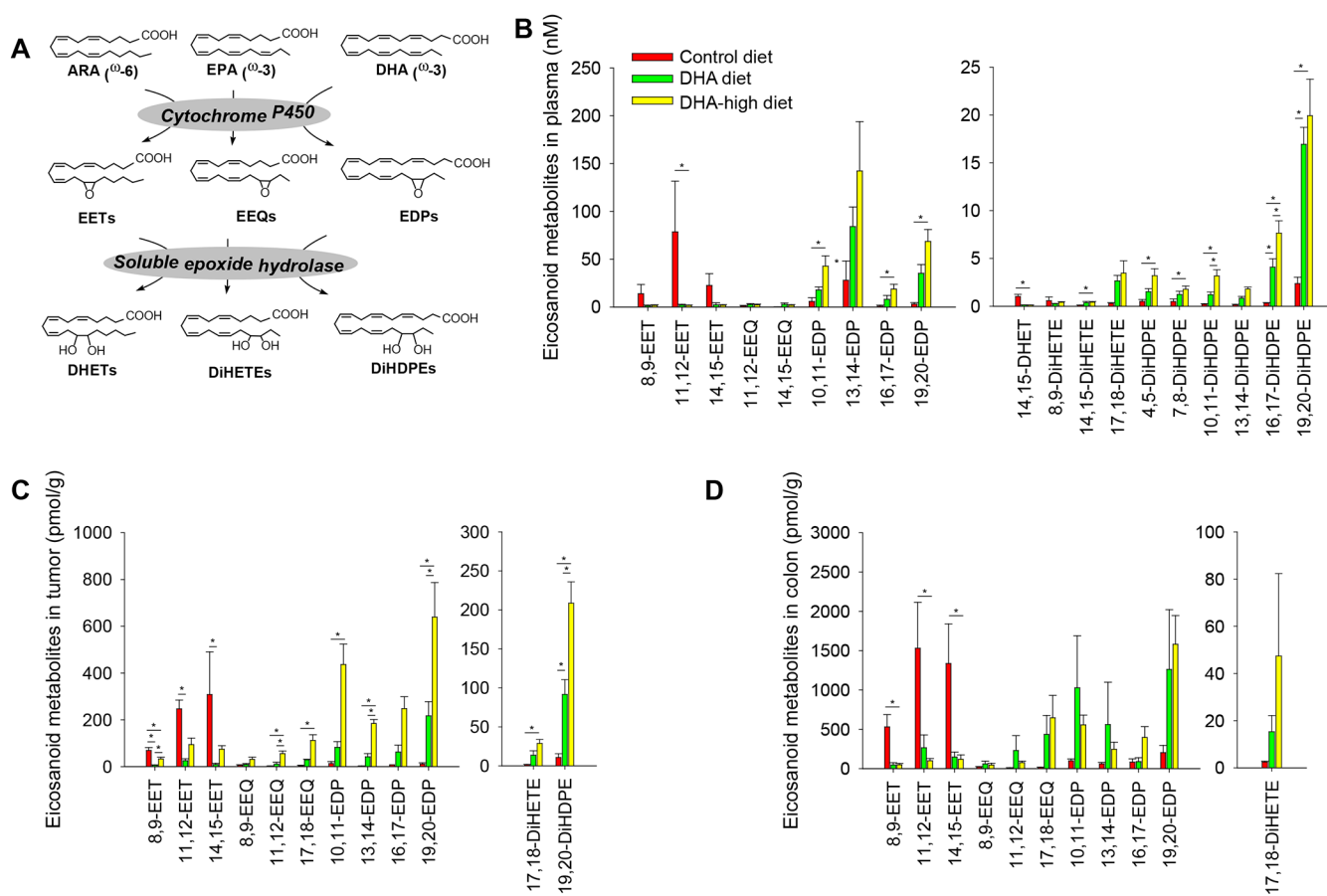
- [4]. Kim S, Sandler DP, Galanko J, Martin C, Sandler RS. Intake of polyunsaturated fatty acids and distal large bowel cancer risk in whites and African Americans. *Am J Epidemiol*. 2010;171:969–79. [PubMed: 20392864]
- [5]. Hall MN, Chavarro JE, Lee IM, Willett WC, Ma J. A 22-year prospective study of fish, n-3 fatty acid intake, and colorectal cancer risk in men. *Cancer Epidemiol Biomarkers Prev*. 2008;17:1136–43. [PubMed: 18483335]
- [6]. Schloss I, Kidd MS, Tichelaar HY, Young GO, O’Keefe SJ. Dietary factors associated with a low risk of colon cancer in coloured west coast fishermen. *S Afr Med J*. 1997;87:152–8. [PubMed: 9107220]
- [7]. Pot GK, Geelen A, van Heijningen EM, Siezen CL, van Kranen HJ, Kampman E. Opposing associations of serum n-3 and n-6 polyunsaturated fatty acids with colorectal adenoma risk: an endoscopy-based case-control study. *Int J Cancer*. 2008;123:1974–7. [PubMed: 18661525]
- [8]. Calviello G, Serini S, Piccioni E. n-3 Polyunsaturated Fatty Acids and the Prevention of Colorectal Cancer: Molecular Mechanisms Involved. *Current Medicinal Chemistry*. 2007;14:3059–69. [PubMed: 18220742]
- [9]. Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*. 2002;56:365–79. [PubMed: 12442909]
- [10]. Wang W, Zhu J, Lyu F, Panigrahy D, Ferrara KW, Hammock B, et al. omega-3 polyunsaturated fatty acids-derived lipid metabolites on angiogenesis, inflammation and cancer. *Prostaglandins Other Lipid Mediat*. 2014;113–115:13–20.
- [11]. Arnold C, Markovic M, Blossey K, Wallukat G, Fischer R, Dechend R, et al. Arachidonic acid-metabolizing cytochrome P450 enzymes are targets of omega-3 fatty acids. *J Biol Chem*. 2010;285:32720–33. [PubMed: 20732876]
- [12]. Jump DB. The biochemistry of n-3 polyunsaturated fatty acids. *J Biol Chem*. 2002;277:8755–8. [PubMed: 11748246]
- [13]. Fischer R, Konkel A, Mehling H, Blossey K, Gapelyuk A, Wessel N, et al. Dietary Omega-3 Fatty Acids Modulate the Eicosanoid Profile in Man Primarily via the CYP-epoxygenase Pathway. *J Lipid Res*. 2014.
- [14]. Zivkovic A, Yang J, Georgi K, Hegedus C, Nording M, O’Sullivan A, et al. Serum oxylipin profiles in IgA nephropathy patients reflect kidney functional alterations. *Metabolomics*. 2012;8:1102–13. [PubMed: 23833568]
- [15]. Zhang G, Panigrahy D, Mahakian LM, Yang J, Liu JY, Stephen Lee KS, et al. Epoxy metabolites of docosahexaenoic acid (DHA) inhibit angiogenesis, tumor growth, and metastasis. *Proc Natl Acad Sci U S A*. 2013;110:6530–5. [PubMed: 23553837]
- [16]. Yanai R, Mulki L, Hasegawa E, Takeuchi K, Sweigard H, Suzuki J, et al. Cytochrome P450-generated metabolites derived from omega-3 fatty acids attenuate neovascularization. *Proc Natl Acad Sci U S A*. 2014;111:9603–8. [PubMed: 24979774]
- [17]. Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer*. 2010;10:181–93. [PubMed: 20168319]
- [18]. Cathcart MC, Lysaght J, Pidgeon GP. Eicosanoid signalling pathways in the development and progression of colorectal cancer: novel approaches for prevention/intervention. *Cancer Metastasis Rev*. 2011;30:363–85. [PubMed: 22134655]
- [19]. Yang J, Schmelzer K, Georgi K, Hammock BD. Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem*. 2009;81:8085–93. [PubMed: 19715299]
- [20]. Folch J, Lees M, Sloane-Stanley G. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem*. 1957;226:497–509. [PubMed: 13428781]
- [21]. Park Y, Albright KJ, Cai ZY, Pariza MW. Comparison of methylation procedures for conjugated linoleic acid and artifact formation by commercial (trimethylsilyl) diazomethane. *Journal of Agricultural and Food Chemistry*. 2001;49:1158–64. [PubMed: 11312828]
- [22]. Wagner K, Lee KS, Yang J, Hammock BD. Epoxy fatty acids mediate analgesia in murine diabetic neuropathy. *Eur J Pain*. 2016.
- [23]. Simopoulos AP, Leaf A, Salem N., Jr. Essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. *Ann Nutr Metab*. 1999;43:127–30. [PubMed: 10436312]

- [24]. Wang W, Zhu J, Lyu F, Panigrahy D, Ferrara KW, Hammock B, et al. Omega-3 Polyunsaturated fatty acids-derived lipid metabolites on angiogenesis, inflammation and cancer. *Prostaglandins Other Lipid Mediat.* 2014;113–115C:13–20.
- [25]. Calviello G, Di Nicuolo F, Gragnoli S, Piccioni E, Serini S, Maggiano N, et al. n-3 PUFAs reduce VEGF expression in human colon cancer cells modulating the COX-2/PGE2 induced ERK-1 and -2 and HIF-1alpha induction pathway. *Carcinogenesis.* 2004;25:2303–10. [PubMed: 15358633]
- [26]. Zhang G, Kodani S, Hammock BD. Stabilized epoxygenated fatty acids regulate inflammation, pain, angiogenesis and cancer. *Prog Lipid Res.* 2014;53:108–23. [PubMed: 24345640]
- [27]. Dennis EA, Norris PC. Eicosanoid storm in infection and inflammation. *Nat Rev Immunol.* 2015;15:511–23. [PubMed: 26139350]
- [28]. Wang D, DuBois RN. The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene.* 2009;29:781–8. [PubMed: 19946329]
- [29]. Aslan A, Triadafilopoulos G. Fish oil fatty acid supplementation in active ulcerative colitis: a double-blind, placebo-controlled, crossover study. *Am J Gastroenterol.* 1992;87:432–7. [PubMed: 1553930]
- [30]. Salomon P, Kornbluth AA, Janowitz HD. Treatment of ulcerative colitis with fish oil n--3-omega-fatty acid: an open trial. *J Clin Gastroenterol.* 1990;12:157–61. [PubMed: 2109004]
- [31]. Belluzzi A, Boschi S, Brignola C, Munarini A, Cariani G, Miglio F. Polyunsaturated fatty acids and inflammatory bowel disease. *Am J Clin Nutr.* 2000;71:339S–42S. [PubMed: 10617993]
- [32]. Akedo I, Ishikawa H, Nakamura T, Kimura K, Takeyama I, Suzuki T, et al. Three cases with familial adenomatous polyposis diagnosed as having malignant lesions in the course of a long-term trial using docosahexanoic acid (DHA)-concentrated fish oil capsules. *Jpn J Clin Oncol.* 1998;28:762–5. [PubMed: 9879296]
- [33]. Kobayashi M, Tsubono Y, Otani T, Hanaoka T, Sobue T, Tsugane S. Fish, long-chain n-3 polyunsaturated fatty acids, and risk of colorectal cancer in middle-aged Japanese: the JPHC study. *Nutr Cancer.* 2004;49:32–40. [PubMed: 15456633]
- [34]. Stern MC, Butler LM, Corral R, Joshi AD, Yuan JM, Koh WP, et al. Polyunsaturated fatty acids, DNA repair single nucleotide polymorphisms and colorectal cancer in the Singapore Chinese Health Study. *J Nutrigenet Nutrigenomics.* 2009;2:273–9. [PubMed: 20559012]
- [35]. Woodworth HL, McCaskey SJ, Duriancik DM, Clinthorne JF, Langohr IM, Gardner EM, et al. Dietary fish oil alters T lymphocyte cell populations and exacerbates disease in a mouse model of inflammatory colitis. *Cancer Res.* 2010;70:7960–9. [PubMed: 20798218]
- [36]. Srivastava PK, Sharma VK, Kalonia DS, Grant DF. Polymorphisms in human soluble epoxide hydrolase: effects on enzyme activity, enzyme stability, and quaternary structure. *Arch Biochem Biophys.* 2004;427:164–9. [PubMed: 15196990]
- [37]. Dreisbach AW, Japa S, Sigel A, Parenti MB, Hess AE, Srinouanprachanh SL, et al. The Prevalence of CYP2C8, 2C9, 2J2, and soluble epoxide hydrolase polymorphisms in African Americans with hypertension. *Am J Hypertens.* 2005;18:1276–81. [PubMed: 16202848]
- [38]. Spiecker M, Darius H, Hankeln T, Soufi M, Sattler AM, Schaefer JR, et al. Risk of coronary artery disease associated with polymorphism of the cytochrome P450 epoxygenase CYP2J2. *Circulation.* 2004;110:2132–6. [PubMed: 15466638]
- [39]. Lee CR, North KE, Bray MS, Fornage M, Seubert JM, Newman JW, et al. Genetic variation in soluble epoxide hydrolase (EPHX2) and risk of coronary heart disease: The Atherosclerosis Risk in Communities (ARIC) study. *Hum Mol Genet.* 2006;15:1640–9. [PubMed: 16595607]
- [40]. Wei Q, Doris PA, Pollizotto MV, Boerwinkle E, Jacobs DR, Jr, Siscovick DS, et al. Sequence variation in the soluble epoxide hydrolase gene and subclinical coronary atherosclerosis: interaction with cigarette smoking. *Atherosclerosis.* 2007;190:26–34. [PubMed: 16545818]
- [41]. Fornage M, Boerwinkle E, Doris PA, Jacobs D, Liu K, Wong ND. Polymorphism of the soluble epoxide hydrolase is associated with coronary artery calcification in African-American subjects: The Coronary Artery Risk Development in Young Adults (CARDIA) study. *Circulation.* 2004;109:335–9. [PubMed: 14732757]
- [42]. Przybyla-Zawislak BD, Srivastava PK, Vazquez-Matias J, Mohrenweiser HW, Maxwell JE, Hammock BD, et al. Polymorphisms in human soluble epoxide hydrolase. *Mol Pharmacol.* 2003;64:482–90. [PubMed: 12869654]

- [43]. Simopoulos AP. Genetic variants in the metabolism of omega-6 and omega-3 fatty acids: their role in the determination of nutritional requirements and chronic disease risk. *Exp Biol Med* (Maywood). 2010;235:785–95. [PubMed: 20558833]
- [44]. Zeisel SH, Waterland RA, Ordovas JM, Muoio DM, Jia W, Fodor A. Highlights of the 2012 Research Workshop: Using nutrigenomics and metabolomics in clinical nutrition research. *JPEN J Parenter Enteral Nutr.* 2013;37:190–200. [PubMed: 23042849]

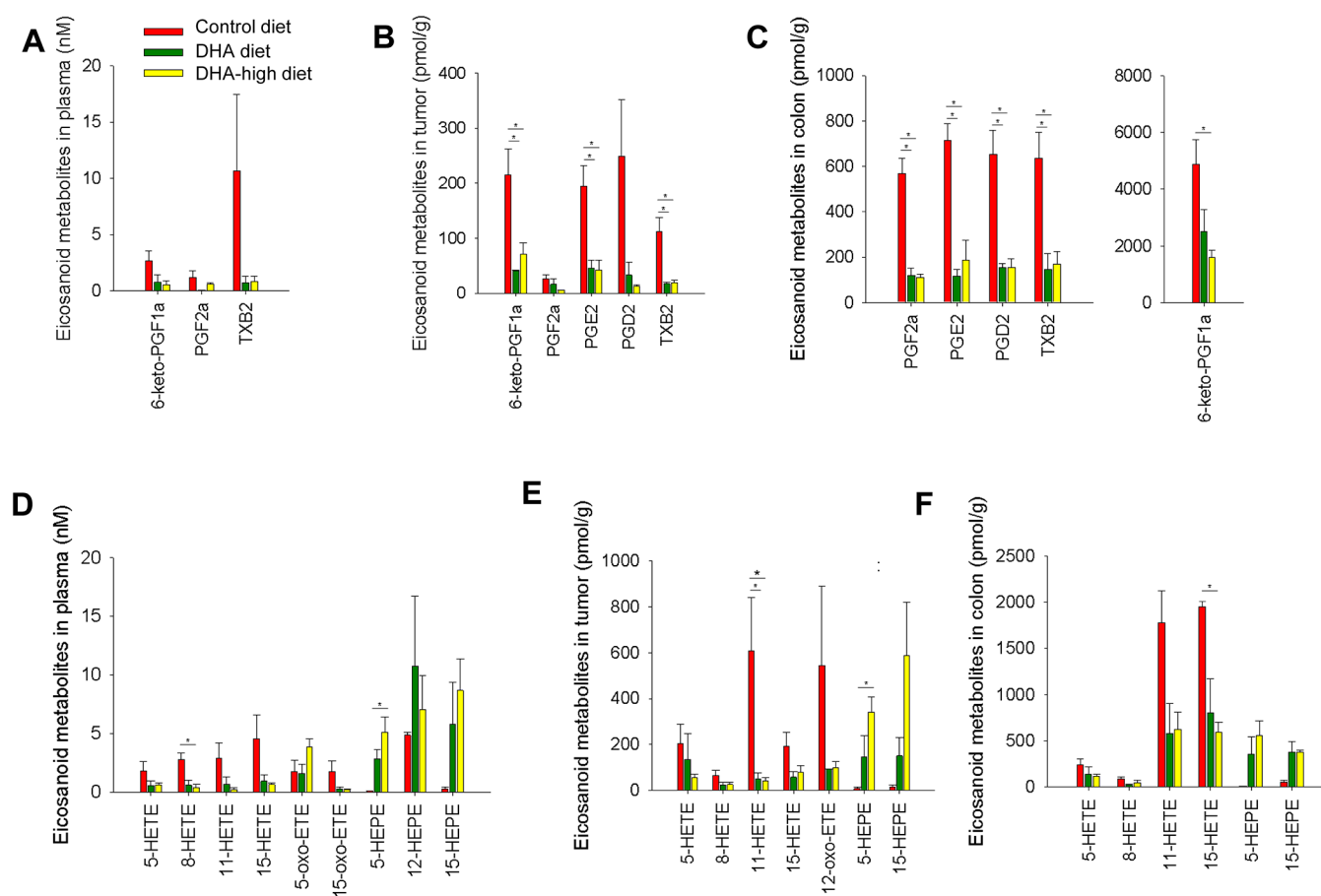
**Fig. 1.**

Dietary feeding of  $\omega$ -3 PUFAs-rich diets suppressed growth of MC38 colorectal tumor in mice. (A) scheme of animal experiment, (B) time-course of MC38 tumor sizing, (C) quantification of tumor weight, (D) representative image of dissected MC38 tumors, (E) flow cytometry quantification of endothelial cells in MC38 tumors.  $n = 8$  mice per group, the results are expressed as mean  $\pm$  SEM, \*  $P < 0.05$ .

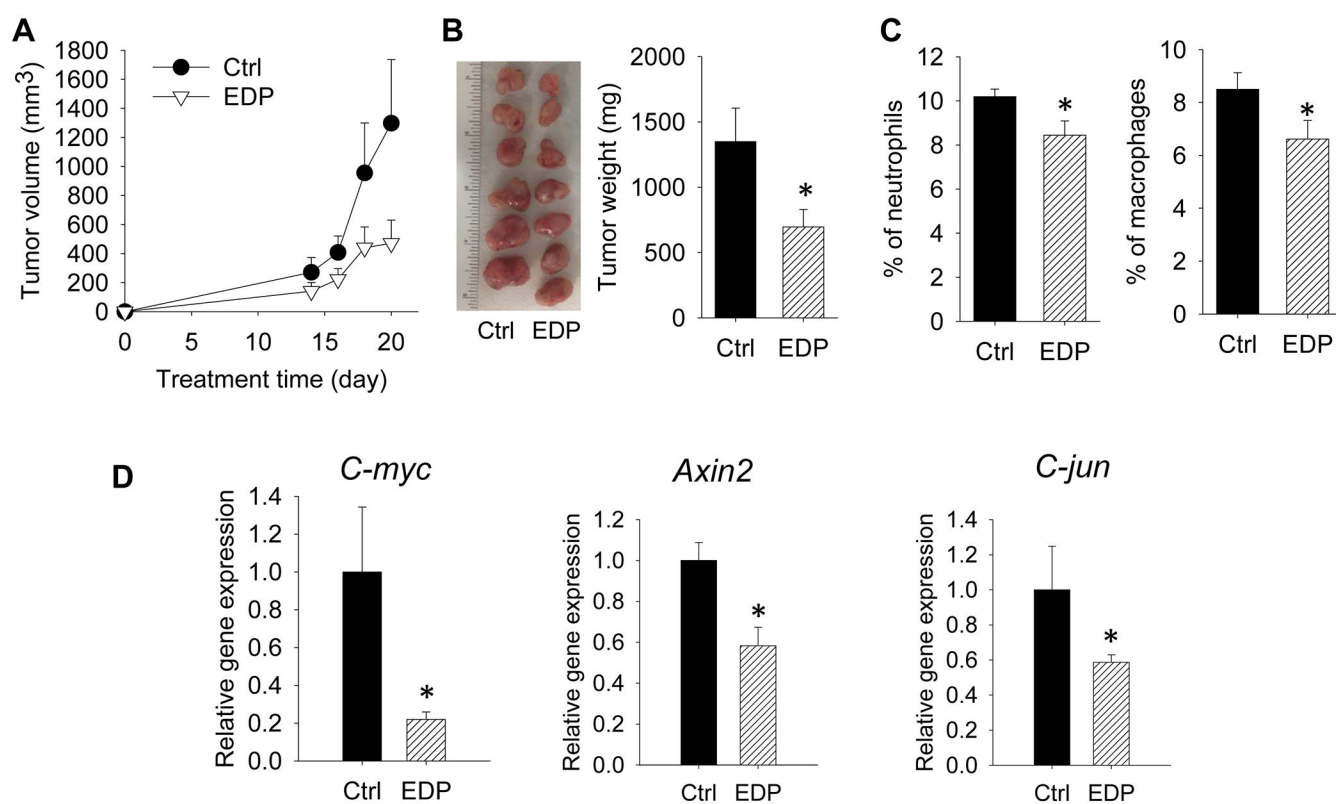
**Fig. 2.**

Dietary feeding of  $\omega$ -3 PUFAs-rich diets modulated profiles of eicosanoid metabolites from CYP pathway in mice. (A) scheme of CYP pathway. (B) plasma, (C) MC38 tumor, and (D) colon profiles of CYP-derived fatty acid epoxide and diols.  $n = 4$  mice per group for control and DHA-high groups,  $n = 3$  mice per group for DHA group, the results are expressed as mean  $\pm$  SEM, \*  $P < 0.05$ .



**Fig. 3.**

Dietary feeding of  $\omega$ -3 PUFAs-rich diets modulated profiles of eicosanoid metabolites from COX and LOX pathway in mice. (A) plasma, (B) MC38 tumor, and (C) colon profiles of COX-derived prostaglandins and thromboxanes. (B) plasma, (C) MC38 tumor, and (D) colon profiles of LOX-derived metabolites.  $n = 4$  mice per group for control and DHA-high groups,  $n = 3$  mice per group for DHA group, the results are expressed as mean  $\pm$  SEM, \*  $P < 0.05$ .

**Fig. 4.**

Treatment with synthetic EDPs suppressed growth of MC38 tumor in C57BL/6 mice. (A) time-course of MC38 tumor sizing, (B) representative images of dissected MC38 tumors, and quantification of tumor weight, (C) flow cytometry analysis of immune cells in MC38 colon tumors, and (D) RT-PCR analysis of pro-oncogenic genes in MC38 tumors. n = 9–10 mice per group, the results are expressed as mean  $\pm$  SEM, \*  $P < 0.05$ .

**Table 1:**

Composition and PUFA profiles of the experimental diets used in the study

Ingredients (gm/kg)	Control diet	DHA diet	DHA-high diet
Casein	200	200	200
L-Cystine	3	3	3
Sucrose	100	100	100
Dyetrose	132	132	132
Cornstarch	397.5	397.5	397.5
Cellulose	50	50	50
Mineral mix #210025	35	35	35
Vitamin mix #310025	10	10	10
Choline Bitartrate	2.5	2.5	2.5
Corn oil	100	38	20.7
Algae oil	0	62	79.3
<b>PUFAs (% to total fatty acids)</b>			
C10:0	0	1.0	1.3
C12:0	0	4.3	5.5
C14:0	0	9.6	12.3
C16:0	12.9	13.3	13.5
C16:1	0	2.3	2.9
C18:0	1.6	1.1	1.0
C18:1	27.7	28.1	28.3
C20:0	0.3	0.1	0.1
LA (18:2 $\omega$ -6)	56.1	22	12.5
ALA (18:3 $\omega$ -3)	0.81	0.31	0.17
DHA (22:6 $\omega$ -3)	0	17.2	21.9
Ratio of $\omega$ -6-to- $\omega$ -3 PUFA	69.3:1	1.26:1	0.56:1

**Table 2:**

Profiles of fatty acids in MC38 colorectal tumors maintained on control diet and DHA diet (n = 4 mice per group).

Fatty acid	Control diet		DHA diet		P value
	Mean	SEM	Mean	SEM	
C10:0	0.055	0.005	0.053	0.005	0.73036
C12:0	0.333	0.204	0.238	0.077	0.67888
C14:0	2.838	0.882	2.885	0.269	0.96059
C15:0	0.093	0.005	0.095	0.005	0.73036
C16:0	22.420	0.428	25.748	1.027	0.02426
C16:1	1.358	0.085	1.733	0.053	0.00968
C16:1	4.303	0.774	4.405	0.598	0.91998
C17:0	0.145	0.013	0.118	0.010	0.15216
C18:0	13.718	1.783	14.080	1.555	0.88324
C18:1 $\omega$ -9	22.203	1.969	22.725	1.221	0.82907
C18:1 $\omega$ -7	3.863	0.253	3.785	0.267	0.84002
C18:2 $\omega$ -6 (LA)	11.230	2.573	7.418	0.672	0.20168
C18:3 $\omega$ -3 (ALA)	0.095	0.030	0.038	0.019	0.15702
C20:0	0.235	0.027	0.200	0.020	0.34343
C20:1	0.145	0.022	0.110	0.011	0.20023
C20:1	0.358	0.027	0.325	0.030	0.44725
C20:1	0.125	0.010	0.118	0.010	0.62695
C20:2 $\omega$ -6	0.345	0.050	0.158	0.027	0.01592
C20:3 $\omega$ -6	0.545	0.040	0.445	0.062	0.22434
C20:4 $\omega$ -6 (ARA)	9.958	1.706	2.888	0.385	0.00679
C20:5 $\omega$ -3 (EPA)	0.000	0.000	1.308	0.205	0.00071
C22:0	0.218	0.043	0.000	0.000	0.00234
C24:0	0.520	0.051	0.000	0.000	0.00005
C22:6 $\omega$ -3 (DHA)	0.908	0.160	8.333	1.001	0.00033