Endogenously synthesized n-3 fatty acids in fat-1 transgenic mice prevent melanoma progression by increasing E-cadherin expression and inhibiting β-catenin signaling

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Abstract. Malignant melanoma is the most lethal form of skin cancer. Although preclinical studies have shown that n-3 polyunsaturated fatty acids (PUFAs) are beneficial for prevention of melanoma, the molecular mechanisms underlying the protective effects of n-3 PUFAs on melanoma remain largely unknown. In the present study, endogenously increased levels of n-3 PUFAs in the tumor tissues of omega-3 fatty acid desaturase (fat-1) transgenic mice was associated with a reduction in the growth rate of melanoma xenografts. This reduction in tumor growth in fat-1 mice compared with wild-type controls may have been associated, in part, to the: i) Increased expression of E-cadherin and the reduced expression of its transcriptional repressors, the zinc finger E-box binding homeobox 1 and snail family transcriptional repressor 1; ii) significant repression of the epidermal growth factor receptor/Akt/β-catenin signaling pathway; and iii) formation of significant levels of n-3 PUFA-derived lipid mediators, particularly resolvin D2 and E1, maresin 1 and 15-hydroxyeicosapentaenoic acid. In addition, vitamin E administration counteracted n-3 PUFA-induced lipid peroxidation and enhanced the antitumor effect of n-3 PUFAs, which suggests that the protective role of n-3 PUFAs against melanoma is not mediated by n-3 PUFAs-induced lipid peroxidation. These results highlight a potential role of n-3 PUFAs supplementation for the chemoprevention of melanoma in high-risk individuals, and as a putative adjuvant agent in the treatment of malignant melanoma.

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Introduction

Malignant melanoma is the most aggressive form of cutaneous malignancy, and accounts for ~3% of all cases of malignant tumor (1). The global incidence of melanoma has been rising, including an annual increase of between 3 and 7% for Caucasians in developed countries (2). Although melanoma accounts for only 4% of all skin cancer cases, it is responsible for ~80% of all skin cancer-related cases of mortality due to its extreme aggressiveness and resistance to current therapeutic agents (3). To date, a number of promising targeted agents, including the B-Raf inhibitors vemurafenib and dabrafenib, are undergoing or have completed phase III clinical trials; however, the majority of these have been demonstrated to be ineffective and the 5-year survival rate of the vast majority of patients diagnosed with metastatic melanoma is <5% (4). Therefore, novel treatment strategies are required for patients with metastatic melanoma.

Malignant transformation is often characterized by major changes in the organization of the cytoskeleton, decreased cell-to-cell adhesion and increased cell motility. The loss of the cellular adhesion molecule and single-span transmembrane glycoprotein, E-cadherin, is positively associated with tumor invasiveness, metastatic dissemination and poor patient prognosis (5). E-cadherin is expressed in epithelial cells, and intercellular homophilic interactions with E-cadherin expressed by neighboring cells, leads to the formation of cell-to-cell adherens junctions (AJs) and a tight polarized cell layer (6). Loss of E-cadherin expression triggers epithelial-to-mesenchymal transition (EMT), which provides cancer cells with increased motility and invasiveness (7). Clinical studies have shown that the majority of patients with melanoma treated with targeted B-Raf proto-oncogene,

serine/threonine kinase inhibitors, such as vemurafenib and dabrafenib, develop resistance to these therapies after only a several months due to decreased E-cadherin expression in the tumor tissues of patients (8). β-Catenin, the principal effector of the Wnt signaling cascade, serves a crucial role in morphogenesis and human cancer through its dual function in mediating cell-to-cell AJs, and as a signaling molecule in numerous signaling pathways (9). β-catenin forms an AJ complex with E-cadherin through binding to the intracellular domain of E-cadherin, which sequesters it at the plasma membrane and hinders its entry into the nucleus (10). Numerous studies have demonstrated that β-catenin signaling is implicated in melanoma progression and metastasis (11,12). In recent years, a number of plant-derived natural products have been described as potential alternative therapies for metastatic melanoma, that function by increasing E-cadherin expression and by disrupting the β-catenin signaling pathway (13). For instance, catechins, a class of flavonoids, exhibit anti-melanoma activity through the restoration of E-cadherin and the suppression of N-cadherin expression levels (14). In addition, the flavonoid complex silymarin, blocks the invasion of melanoma cells by inhibiting β-catenin translocation (15). Therefore, inhibiting the proliferative, migratory and invasive capacity of cancer cells with chemopreventative dietary factors, may provide a potential strategy for melanoma prevention and/or treatment.

Among the dietary factors, n-3 polyunsaturated fatty acids (PUFAs) have gained attention as potential preventative and/or adjuvant agents in the treatment of malignant melanoma (16). A number of preclinical *in vitro* and *in vivo* studies have attempted to clarify the possible mechanisms underlying the proposed anticancer effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are the two principal n-3 PUFAs found in fish and seafood (17,18). A number of published preclinical and epidemiological studies provide evidence to suggest that dietary or exogenously derived fatty acids may serve a vital role in the development and progression of malignant melanoma (19,20); however, conflicting results and discrepancies between studies preclude definitive conclusions (21,22). For instance, Kirkpatrick *et al* (23) reported no association between the consumption of PUFAs and melanoma risk, whereas, Salem *et al* (24) demonstrated that fish oil supplementation of B16 melanoma-bearing mice enhanced melanoma cell growth and metastases and decreased mouse survival rates. These conflicting results reflect numerous confounding dietary elements. Indeed, fish and corn oil, which are generally used to investigate the effects of the ratio of n-3/n-6 PUFAs on disease development and progression, consist not only of n-3 and n-6 PUFAs, but also additional fatty acids and lipid soluble vitamins (25).

In the present study, the potential of high n-3 PUFAs supplementation in the prevention of melanoma cell growth was investigated using omega-3 fatty acid desaturase (fat-1) transgenic mouse xenografts, which carry the fat-1 gene from the *Caenorhabditis elegans* roundworm (26). This gene, which is not present in mammals, encodes an n-3 PUFA desaturase that catalyzes the conversion of n-6 to n-3 PUFAs. Therefore, the fat-1 transgenic mice were endogenously enriched with n-3 PUFAs and were characterized by a lower ratio of n-6/n-3 PUFAs compared with their wild-type (WT) littermates, by using a single diet high in n-6 PUFAs. The need for dietary n-3 PUFAs supplementation was eliminated and corresponding confounding factors of diet were removed. Therefore, the fat-1 transgenic mouse model is ideal for addressing the effects of the tissue n-6/n-3 fatty acid ratio in melanoma tumorigenesis.

Endogenous n-3 PUFAs have been previously reported to exert their antitumor effects in melanoma through the prostaglandin (PG) E3-mediated activation of the phosphatase and tensin homologue deleted on chromosome 10 signaling pathway in fat-1 transgenic mice injected subcutaneously with melanoma B16-F0 cells (27). However, whether n-3 PUFA-derived lipid mediators and the E-cadherin/β-catenin signaling pathway are associated with n-3 PUFA-mediated antitumor effects in malignant melanoma remains largely unknown. In the present study, B16-F10 mouse melanoma cells, which have a higher metastatic potential compared with B16-F0 melanoma cells, were injected into fat-1 transgenic and WT mice in order to evaluate its tumorigenicity. The results demonstrated that increased E-cadherin expression, inhibition of the β-catenin signaling pathway and biosynthesized n-3 PUFA-derived bioactive mediators, are involved in the antitumor effects of n-3 PUFAs in melanoma.

Materials and methods

Cells and reagents. The murine B16-F10 melanoma cell line (American Type Culture Collection, Manassas, VA, USA) was maintained in a humidified incubator at 37˚C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum. The primary antibodies, mouse anti-E-cadherin (catalog no. 14472), mouse anti-N-cadherin (catalog no. 14215), rabbit anti-β-catenin (catalog no. 9582), rabbit anti-zinc finger E-box 1 (ZEB1; catalog no. 3396), rabbit anti-Snail (catalog no. 3879), rabbit anti-Slug (catalog no. 9585), rabbit anti-c-Myc (catalog no. 13987), rabbit anti-Akt (pan; catalog no. 4685), rabbit anti-phosphorylated (p)-Akt (Thr308; catalog no. 13038), rabbit anti-nuclear factor κB p65 (NF-κB p65; catalog no. 2765), rabbit anti-p-NF-κB p65 (Ser536; catalog no. 3033), rabbit anti-glycogen synthase kinase-3β (GSK-3β; catalog no. 9315), rabbit anti-p-GSK-3β (Ser9; cataolog no. 9322), rabbit anti-signal transducer and activator of transcription 3 (STAT3; catalog no. 12640), rabbit anti-p-STAT3 (Tyr705; catalog no. 9145), rabbit anti-epidermal growth factor receptor (EGFR; catalog no. 4267), rabbit anti-GAPDH (catalog no. 5174) and the horseradish peroxidase-linked secondary antibodies, anti-rabbit IgG (catalog no. 7074) and anti-mouse IgG (catalog no. 7076) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The primary mouse anti-twist (catalog no. ab175430) and rabbit anti-smad interacing protein 1 (ZEB2; catalog no. ab138222) antibodies were obtained from Abcam (Cambridge, MA, USA). Primary and secondary antibodies were used at dilutions of 1:1,000 and 1:4,000, respectively. Vitamin E (Vit E) powder was purchased from Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China). Chloral hydrate was purchased from Shanghai Meilian Biotechnology Co., Ltd. (Shanghai, China).

Animals and diet. The fat-1 transgenic mice were donated by Dr Jing X. Kang at Massachusetts General Hospital and

Harvard Medical School (Boston, MA, USA). A total of 13 male heterozygous fat-1 mice were bred with 26 WT C57BL/6 female mice (26). Each mouse was genotyped and phenotyped by polymerase chain reaction and gas chromatography using isolated DNA and total lipids from mice tails, respectively. Specific pathogen-free transgenic and WT animals were fed a 10% safflower oil diet, and housed in standard cages in temperature under humidity-controlled conditions with a 12-h light/dark cycle. At 10-12 weeks of age, heterozygous fat-1 male mice and nontransgenic littermate controls were used for the purposes of the present study. Their diets (per 100 g) consisted of 4.5 g sucrose, 18.6 g casein, 8.6 g cellulose, 50 g wheat starch, 0.3 g DL-methionine, 7 g mineral mix, 1 g vitamin mix and 10 g safflower oil. A total of 10 fat-1 and 10 WT mice were assigned to Vit E groups and another 10 fat-1 and 10 WT mice were assigned to negative control groups. The Vit E-treatment group was administered with 100 IU/kg Vit E (dissolved in sterile water) via oral gavage for 3 weeks. Sterile water was administered in the same manner to the control group. All animal experiments were conducted in accordance with the guidelines for the use and care of laboratory animals, and approved by the Ethical Committee of Ningbo University (Ningbo, China).

Cell injections and tumor measurement. Cultured B16-F10 melanoma cells were collected by trypsin digestion (0.05% trypsin-EDTA) for 2 min and washed twice with DMEM before the cell number was ascertained. Each male mouse was injected subcutaneously with $2.5x10^6$ cells suspended in 100 μl DMEM into the area overlying the abdomen. The day of the injection of B16-F10 cells was designated day 0. Tumor volume was assessed by conducting caliper measurements every 2 days, and calculated according to the following formula: Tumor volume = Shortest diameter^2 x the largest diameter x 0.5. Following 15 days of cell injection, the mice were anesthetized with an intraperitoneal injection of 350 mg/kg chloral hydrate and sacrificed by exsanguination. The tumors were removed and stored at -80˚C for downstream analyses.

Analysis of fatty acids and lipid mediators. Fatty acid composition was determined using gas chromatography (GC) as described previously (28). Briefly, tissues were ground to a powder form using liquid nitrogen, and the methyl esters of the fatty acids from the lipid extract were transesterified with H_2SO_4 in methanol (5%, v/v), together with toluene, in sealed tubes at 70˚C for 2 h. Fatty acid methyl esters were analyzed using the Shimadzu GC-14C gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a flame-ionization detector and a 60 m x 0.25 mm (internal diameter) x0.25 μm (film thickness) fused silica bonded phase column (DB-23; Agilent Technologies, Inc., Santa Clara, CA, USA). The fatty acid methyl esters were identified by comparing sample mixtures with standard mixtures of fatty acid methyl esters (Nu-Chek Prep, Inc., Elysian, MN, USA). A 1 μl aliquot of each sample was injected onto the column. Nitrogen was the carrier gas and the flow rate was 4 ml/min. The injector and detector temperature was 270˚C. The column temperature was 180˚C and held for 10 min; the temperature was then increased to 200˚C at a rate of 20˚C/min and held for 10.5 min; the temperature was further increased to 215˚C at a rate of 20˚C/min and held for 5 min; finally, it was increased to 215˚C at a rate of 20˚C/min and held for 4.75 min. Quantification of sample fatty acid compositions was achieved by comparing peak areas with the nonadecanoic acid internal standard (Sigma-Aldrich, St. Louis, MO, USA), which was added to the samples (1 mg internal standard/500 mg sample) prior to extraction. The composition of fatty acids was expressed as the relative percentage of the total fatty acids according to their peak areas.

Lipid mediators from n-3 and n-6 fatty acids were identified using liquid chromatography (LC)-tandem mass spectrometry (MS) methods as described previously (29). In brief, each tumor sample was homogenized in 2 ml 15% v/v ice-cold methanol. The internal standards PGB2-d_4, PGD2-d_4, 15-HETE-d_8 and resolvin (RV) E1-d_4 (20 ng per sample each) were added to each sample and n-3 and n-6 fatty acid mediators were extracted using Strata-X reversed-phase SPE columns (8B-S100-UBJ; Phenomenex, Torrance, CA, USA). An Acquity ultra performance (UP) LC system (Waters Corporation, Milford, MA, USA) was used. Reversed-phase separation was performed on an Acquity UPLC BEH shield RP18 column [2.1x100 mm (internal diameter); 1.7 μm (film thickness); Waters Corporation]. The mobile phase consisted of (A) acetonitrile/water/acetic acid (60/40/0.02% v/v) and (B) acetonitrile/isopropenyl acetate (50/50% v/v). Gradient elution was carried out for 5 min at a flow rate of 0.5 ml/min. Gradient conditions were as follows: 0-4.0 min, 0.1-55% B; 4.0-4.5 min, 55-99% B; 4.5-5.0 min, 99% B. A 10 μl aliquot of each sample was injected onto the column. The column temperature was maintained at 40˚C. Mass spectrometry was performed on an ABI/Sciex 6500 QTRAP hybrid, triple quadrupole, linear ion trap mass spectrometer (AB Sciex, Framingham, MA, USA) equipped with a Turbo V ion source. Lipid mediators were detected in negative electrospray ion mode. Curtain gas, nebulizer gas and turbo-gas were set at 10, 30 and 30 psi, respectively. The electrospray voltage was -4.5 kV, and the turbo ion spray source temperature was 525˚C, and a methanol:water:acetate (60:40:0.02%) mobile phase was used with a 0.5 ml/min flow rate. The following multiple reaction monitoring transitions were used: PGE2 *m/z* 351>271, PGE3 *m/z* 349>269, 12-hydroxyeicosapentaenoic acid (HEPE) *m/z* 317>179, 15-HEPE *m/z* 317>219, RVD2 *m/z* 375>175, RVE1 *m/z* 349>195 and Maresin1 *m/z* 359>177.

Western blot analysis. Tumor tissues (30 mg) were homogenized in Triton-X protein lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 140 mM NaCl, 1% NonidetP-40, 1% aprotinin, 1 mM phenylmethylsulfonyfluoride and 1 mM sodium vanadate; pH 7.6). The sample protein concentration was determined using a bicinchoninic acid protein assay. Aliquots of protein (50 μg) were fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After nonspecific binding sites were blocked with 5% nonfat milk in Tris-buffered saline, blots were incubated overnight at 4˚C with primary antibodies, and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. Reactive protein bands were analyzed using enhanced chemiluminescence .

Lipid peroxidation analysis. Lipid peroxidation in urine samples was determined using an malondialdehyde (MDA)

assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. Thiobarbituric acid (TBA; 200 μl) reagent was added to 100 μl urine sample. The reaction mixture was subsequently incubated in a water bath at 100˚C for 15 min. After cooling to room temperature, the mixture was centrifuged at 1,000 x g for 10 min at room temperature and the supernatant was separated, before the absorbance was read at 532 nm. The concentration of MDA was determined using a standard curve.

Statistical analysis. For each treatment group, data were presented as the arithmetical mean ± standard error. Statistical significance between groups was determined using the Student's *t*-test. P<0.05 and P<0.01 was considered to indicate a statistically significant difference.

Results

n-3 PUFAs inhibited the tumorigenicity of B16-F10 melanoma cells in fat-1 transgenic mice. In order to test the hypothesis that a balanced n-6/n-3 fatty acid ratio in fat-1 transgenic mice is associated with a decreased risk of melanoma, B16-F10 murine melanoma cells were injected into transgenic fat-1 and WT mice, and the tumorigenicity of inoculated tumor cells was examined. As shown in Fig. 1, a marked difference in the tumor growth rate was observed between fat-1 transgenic (n=10) and WT mice (n=10). Over an observation period of 15 days, all mice initially developed a palpable tumor by day 7. The tumors in fat-1 transgenic mice exhibited a markedly slower growth rate when compared to those in WT mice.

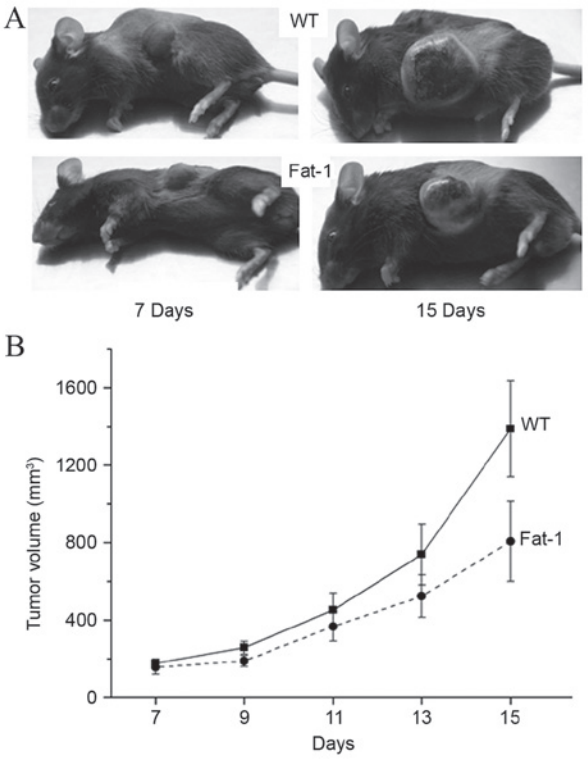

Figure 1. Effect of fat-1 gene expression on the tumorigenicity of B16-F10 melanoma cells in vivo. B16-F10 cells (2.5x106) were injected subcutaneously into 10 male, 12-week-old fat-1 transgenic and 10 WT mice of the same age. (A) Representative photographs showing tumor formation at 7 and 15 days post-injection in WT and fat-1 transgenic mice. (B) The growth rates of melanoma tumors in WT and fat-1 transgenic mice. Data points represent the mean ± standard error. Fat-1, omega-3 fatty acid desaturase; WT, wild-type.

Fatty acid profiles in the tumor tissues of fat-1 and WT mice. Analysis of the total lipids extracted from the tumor tissues of fat-1 and WT mice revealed distinct lipid profiles between the two groups (Fig. 2). Compared with WT mice, fat-1 transgenic mice displayed significantly increased levels of n-3 fatty acids, including 20:5n-3 (EPA; P=0.0441), 22:5n-3 [docosapentaenoic acid (DPA; P=0.0482)] and 22:6n-3 (DHA; P=0.0151), and exhibited decreased concentrations of n-6 fatty acids, including 18:2n-6 (linoleic acid), 20:3n-6 [dihomo-gamma-linolenic acid (DGLA)], 20:4n-6 [arachidonic acid (AA)] and 22:4n-6 (adrenic acid) in the tumor tissues (Fig. 2A). In particular, the levels of linoleic acid (P=0.0356) and AA (P=0.0122) n-6 fatty acids were significantly reduced in tumors from fat-1 mice compared with WT animals. As shown in Fig. 2B the n-6/n-3 fatty acid ratio in tumors from fat-1 mice (7.7±1.6) was significantly lower than that of WT mice (22.0±3.1; P=0.0097), despite the animals being fed the same diet. These results indicate the fat-1 transgene is functionally active *in vivo*, and endogenously catalyzes the conversion of n-6 to n-3 PUFAs.

Lipid mediators derived from AA (prostanoids, leukotrienes, lipoxins and epoxyeicosatrienoic acids), EPA (E-series resolvins) and DHA (D-series resolvins, protectins and maresins) exert their potent actions through the promotion or resolution of inflammation (30). Therefore, the levels of n-6 and n-3 PUFA-derived lipid mediators in the melanoma tumor tissues of WT and fat-1 transgenic mice were determined using UPLC-UV-tandem MS analysis, in order to determine whether the observed difference in tumor growth between these two groups was associated with differences in the levels of lipid mediators. As shown in Fig. 3, a significant increase in the levels of fatty acid metabolites derived from EPA, [12-HEPE (P=0.0187), 15-HEPE (P=0.0224), PGE3 (P=0.0320) and RVE1 (P=0.0098)] and DHA (RVD2 (P=0.0092) and Maresin1 (P=0.042)) were observed in tumors from fat-1 mice compared with WT controls. In contrast, no significant difference in the levels of proinflammatory PGE2 was observed between fat-1 and WT mice.

Differential protein expression levels of E-cadherin and its master regulators in fat-1 transgenic and WT mice. In order to determine whether fat-1 expression, and the associated increase in n-3 PUFA levels, affects E-cadherin and N-cadherin expression levels, melanoma tumor tissues from transgenic fat-1 mice and WT controls were analyzed for the expression of E-cadherin and N-cadherin by western blotting. As shown in Fig. 4, the protein expression levels of E-cadherin and N-cadherin were markedly upregulated and downregulated in the tumor tissues of fat-1 mice compared with those of the WT controls, respectively. This implies that E-cadherin and N-cadherin expression may be modulated by n-3 PUFAs. Several transcription factors, including Snail, Slug, ZEB1, ZEB2 and Twist, function as transcriptional repressors of E-cadherin (31). Therefore, western blotting was used to investigate whether the observed increase in E-cadherin expression levels in the melanoma tissues of fat-1 mice may be mediated by alterations in the protein expression

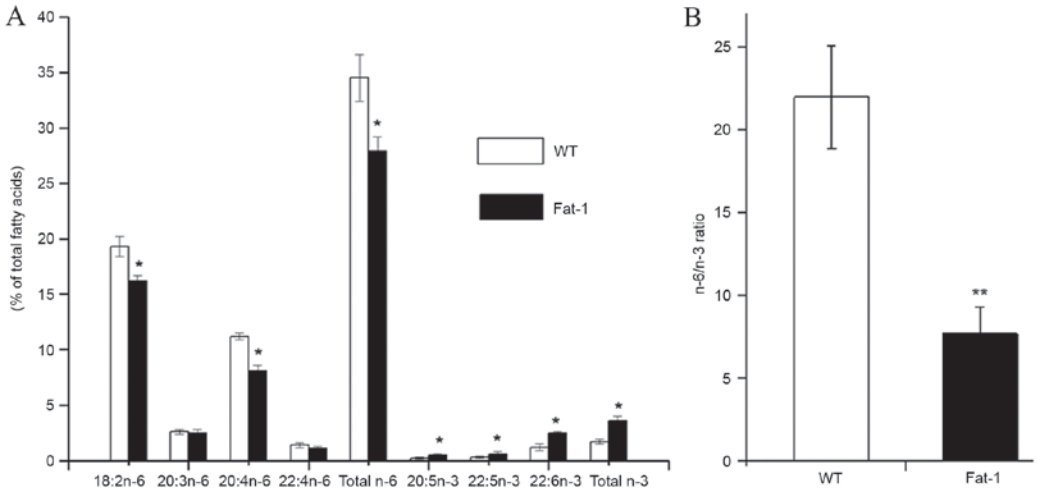

Figure 2. The n-3 and n-6 lipid profile of WT and fat-1 transgenic mouse B16-F10 melanoma tumor tissues using gas chromatography. (A) Quantitative analysis of the composition of n-3 and n-6 fatty acids and (B) the ratio of n-6/n-3 fatty acids in the melanoma tissues of WT and fat-1 mice. Values represent the mean ± standard error from three separate measurements, n=6. *P<0.05 and **P<0.01 vs. WT controls. WT, wild-type; fat-1, omega-3 fatty acid desaturase; 18:2n-6, linoleic acid; 20:3n-6, dihomo-gamma-linolenic acid; 22:4n-6, adrenic acid; 20:5n-3, eicosapentaenoic acid; 22:5n-3, docosapentaenoic acid; 22:6n-3, docosahexaenoic acid.

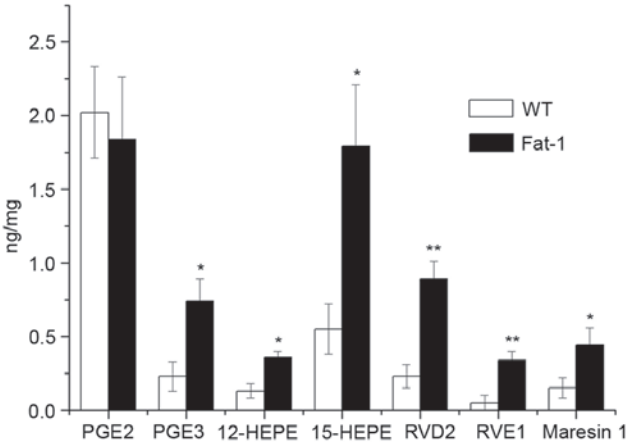

Figure 3. B16-F10 mouse melanoma tumors from transgenic fat-1 mice display altered lipid metabolite profiles. Ultra-performance liquid chromatography-ultraviolet-tandem mass spectrometry analysis of the arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid lipid metabolites in mouse melanoma tumors from transgenic fat-1 mice and WT controls. Values represent the mean ± standard error. *P<0.05 and **P<0.01 vs. WT controls. WT, wild-type; fat-1, omega-3 fatty acid desaturase; PGE, prostaglandin; HEPE, hydroxyeicosapentaenoic acid; RV, resolvin.

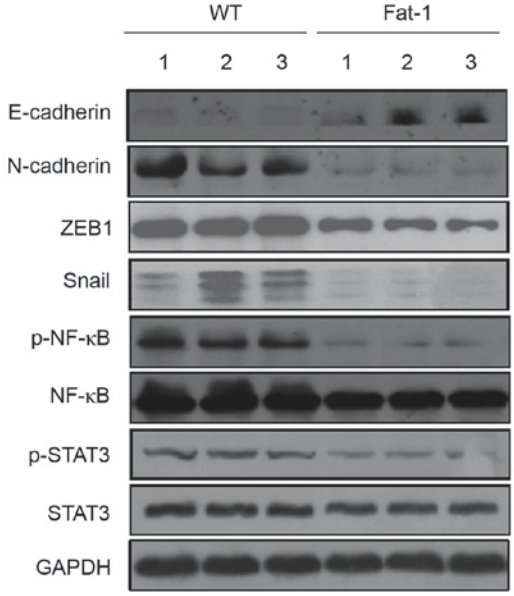

Figure 4. Endogenous n-3 PUFAs may upregulate E-cadherin expression by inhibiting the expression of its master regulators. Western blotting analysis of E-cadherin, N-cadherin, Snail, ZEB1, NF-κB, p-NF-κB, STAT3 and p-STAT3 protein expression levels in B16-F10 mouse melanoma tumor tissues from three WT (lanes 1-3) and three transgenic fat-1 mice (lanes 4-6). PUFAs, polyunsaturated fatty acids; ZEB1, zinc finger E-box 1; NF-κB, nuclear factor κB; STAT3, signal transducer and activator of transcription 3; p-NF-κB, phosphorylated nuclear factor κB; WT, wild-type; fat-1, omega-3 fatty acid desaturase.

levels of these transcriptional repressors. As shown in Fig. 4, the protein expression levels of ZEB1 and Snail were markedly decreased in the tumors of fat-1 mice compared with those of WT mice. In contrast, no significant alterations in the expression of Slug, ZEB2 and Twist proteins were observed (data not shown). The transcription factors NF-κB and STAT3 are activated in a range of human cancers and are thought to promote tumorigenesis, in part, through their ability to regulate the expression levels of E-cadherin transcriptional repressors (32). As shown in Fig. 4, fat-1 mouse tumor tissues exhibited a slight decrease in NF-κB protein expression levels, but a marked decrease in p-NF-κB (Ser536), compared to those from WT mice. Similarly, despite observing no significant alterations in the expression levels of STAT3, p-STAT3 (Tyr705) expression was substantially decreased in the tumor tissues of fat-1 mice. These results suggest that NF-κB and STAT3 may be involved in modulating E-cadherin expression.

Inhibition of EGFR/Akt/β-catenin signaling pathway by n-3 PUFAs in fat-1 transgenic mice. β-catenin functions as a transcriptional regulator in the canonical and noncanonical Wnt signaling pathways, and serves a critical role in regulating development, cell proliferation, differentiation and neoplastic

transformation (10). To determine whether the low n-6/n-3 PUFA ratio in transgenic fat-1 mice affects β-catenin signaling pathways, western blotting was used to examine the protein expression levels of β-catenin and c-Myc; a known transcriptional target of β-catenin. As shown in Fig. 5, fat-1 tumor tissues exhibited a marked decrease in the expression levels of β-catenin and c-Myc compared with those from WT mice. β-catenin is activated, not only by Wnt ligands, but also by additional signaling pathways, such as the EGFR/Akt/GSK-3β pathway (33). Cytoplasmic β-catenin is controlled by the GSK-3β-containing destruction complex. When GSK-3β is phosphorylated and inactivated, cytoplasmic β-catenin is stabilized and translocated to the nucleus (34). To determine whether β-catenin degradation may occur through inhibition of the phosphorylation of GSK-3β and upstream kinases, EGFR and Akt, the protein expression levels of GSK-3β, p-GSK-3β, EGFR, Akt and p-Akt were examined. As shown in Fig. 5 no significant differences in GSK-3β and Akt protein expression levels were observed in the melanoma tumor tissues of fat-1 and WT mice, whereas the expression levels of p-GSK-3β, p-Akt and EGFR were markedly decreased in fat-1 tissues compared with those of WT mice. This indicates that the EGFR/Akt/GSK-3β signaling pathway may be involved in controlling endogenous n-3 PUFA-induced β-catenin degradation.

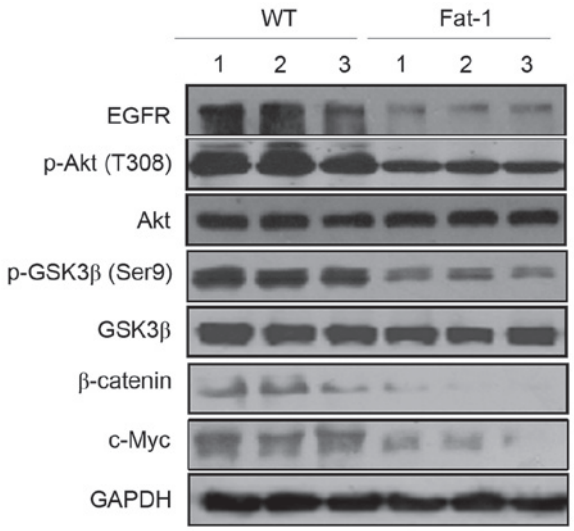

Figure 5. Endogenous n-3 PUFAs modulate β-catenin signaling. Western blotting analysis of β-catenin, c-Myc, EGFR and Akt, p-Akt, GSK-3β and p-GSK-3β protein expression levels in B16-F10 mouse melanoma tumor tissues from three WT (lanes 1-3) and three fat-1 transgenic mice (lanes 4-6). PUFAs, polyunsaturated fatty acids; EGFR, epidermal growth factor receptor; p-Akt, phosphorylated Akt; GSK-3β, glycogen synthase kinase-3β; WT, wild-type; fat-1, omega-3 fatty acid desaturase.

Tumor growth inhibition was not associated with n-3 PUFA-induced oxidative stress in fat-1 mice. A lipid peroxidation assay was performed to determine lipid peroxidation levels in fat-1 and WT mice with or without Vit E administration. As shown in Fig. 6A, the urine from fat-1 mice demonstrated an increased level of MDA compared with that of WT mice (20.3±2.1 vs. 11.7±2.7 μM; P=0.0293). Notably, no significant difference in the levels of MDA was observed between fat-1 and WT mice (12.9±3.2 vs. 8.3±1.8 μM) following the administration of Vit E. In addition, tumor growth was suppressed in fat-1 and WT mice, and fat-1 mice exhibited decreased tumor growth compared with WT-mice following Vit E supplementation (Fig. 6B).

Discussion

Malignant melanoma is one of the most common life-threatening cancers among young western populations due to its rapid progression and high metastatic potential (35). The development of new therapies for malignant melanoma, including immunotherapy and targeted-molecular therapy, faces challenges due to the occurrence of drug resistance and harmful side effects (8). Notably, there is increasing evidence that natural products, such as dietary lipids, are considered to be of crucial importance in regulating melanoma cell growth (36). However, the molecular mechanisms underlying the role of n-3 PUFAs in melanoma development, progression and prevention are not fully understood. In the present study, to evaluate the antitumor effects of n-3 PUFAs on melanoma *in vivo*, B16-F10 mouse melanoma cells, which metastasize to the lungs, were injected into fat-1 and WT mice. The fat-1 transgenic mouse is a suitable experimental model as the mice can maintain a balanced n-6/n-3 fatty acid ratio in all organs and tissues, due to the ubiquitous expression of the *Caenorhabditis elegans* desaturase gene (26). The genetic approach presented in this study enabled the production of two different fatty acid profiles in tumor tissues whilst using identical diets rich in linoleic acid (18:2, n-6) but lacking in n-3 fatty acids. Although the formation of tumors was not significantly inhibited in fat-1 transgenic mice compared with those of WT mice, endogenous production of n-3 PUFAs in fat-1 mice markedly decreased the growth rate of the xenografts. Moreover, melanoma tumor growth inhibition in fat-1 mice was correlated with the production of n-3 PUFA-antitumor derivatives, the modulation of E-cadherin expression and inhibition of β-catenin signaling.

E-cadherin is an important adhesion molecule in epithelial cells that functions to mediate cell-to-cell adhesion and is a potent invasion/tumor suppressor (5). In the present study, B16-F10 melanoma tumors from fat-1 mice displayed a significant upregulation in the protein expression levels of the E-cadherin epithelial biomarker and a marked decrease in N-cadherin expression (a marker of mesenchymal stage), compared with those of WT mice. This suggests that the reverse EMT process, known as mesenchymal-epithelial transition, may have occurred in fat-1 tumor tissues. Consistent with these results, recent studies have demonstrated that breast and colorectal cancer tissues from fat-1 mice display increased levels of E-cadherin expression, which indicates that its expression may be modulated by endogenous n-3 PUFAs (37,38). However, in the present study, the formation of metastases in the lungs or other organ sites in fat-1 and WT mice was not observed (data not shown). One possible reason for this is that the subcutaneous tumor model may not favor lung metastases of B16-F10 cells, compared with the intravenous injection tumor model. In addition, it is virtually impossible for cancer cells to disseminate from a primary tumor to a more distant tissue site within a period of 15 days (39). Therefore, further studies to investigate the anti-metastatic activities of n-3 PUFAs using the intravenous injection tumor model are

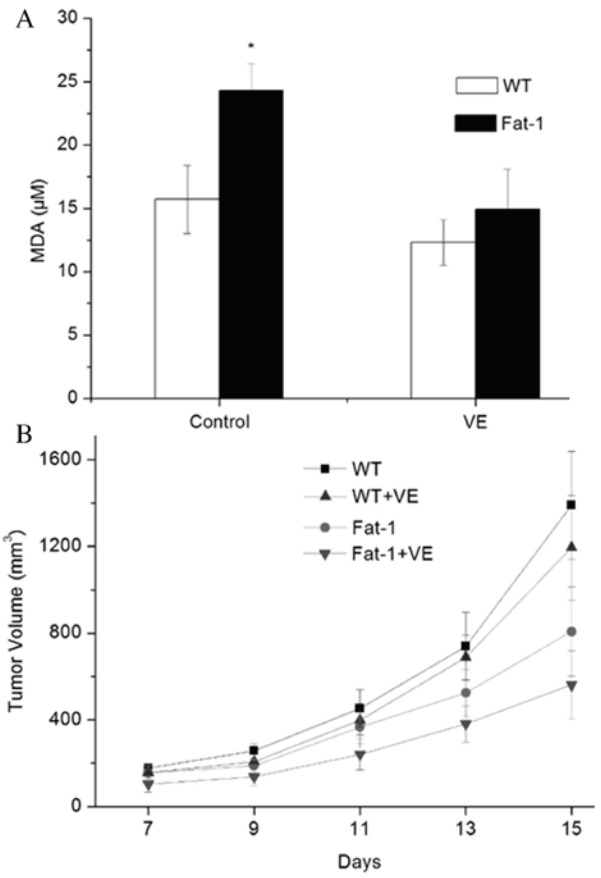

Figure 6. Vit E counteracts n-3 PUFA-induced lipid peroxidation and enhances the antitumor effects of n-3 PUFAs. (A) MDA concentrations in the urine of fat-1 and WT mice treated with or without Vit E. Results are presented as mean ± standard error. *P<0.05 vs. WT controls. (B) Mouse melanoma tumor volumes in WT and fat-1 transgenic mice following the administration of Vit E. Vit E, vitamin E; PUFA, polyunsaturated fatty acid; MDA, malondialdehyde; fat-1, omega-3 fatty acid desaturase; WT, wild-type.

required. In the present study, the protein expression levels of the transcriptional repressors of E-cadherin, ZEB1 and Snail, were markedly decreased, suggesting that ZEB1 and Snail may have been involved in regulating E-cadherin expression in the fat-1 tumor tissues. Consistent with these results, Galindo-Hernandez *et al* (40) reported that the expression of Snail and Twist was correlated with linoleic acid (n-6 PUFAs) -mediated downregulation of E-cadherin expression in breast cancer cells. NF-κB and STAT3 transcription factors are principle regulators of inflammation, and have been implicated in the pathogenesis of immune disorders and cancer (41). The results of the present study demonstrated that the mouse melanoma tumor tissues from fat-1 mice exhibited lower levels of p-NF-κB and p-STAT3 protein expression compared with those of WT mice. NF-κB and STAT3 have been identified as important regulators of EMT in several different types of cancer (42). Therefore, it is possible that NF-κB and STAT3 modulated E-cadherin expression in fat-1 mice, through regulating the transcriptional repressors of E-cadherin.

A notable finding of the present study was the observed inhibition of the β-catenin signaling pathway in fat-1 mice. β-catenin, a key component of the Wnt signaling pathway and the cadherin/catenin-based adhesion process, serves an important role in the development of melanoma and its evasion of the immune system (43,44). Therefore, we hypothesize that the observed disruption to the β-catenin signaling pathway may be attributed to the effective chemopreventative and antitumorigenic effects of n-3 PUFAs. Decreased tumor growth was associated with fat-1-mediated downregulation of β-catenin, p-GSK-3β and c-Myc protein expression levels. These results are consistent with those of other studies demonstrating that endogenous n-3 PUFAs were associated with a reduction in p-GSK-3β levels and a subsequent downregulation of β-catenin in prostate and pancreatic cancer tissues, which supports our hypothesis that the antitumor effects of n-3 PUFAs in melanoma may involve GSK-3β-mediated β-catenin degradation (45,46). In contrast, Castellone *et al* (47) demonstrated that PGE2, an AA (n-6 PUFA) -derived prostanoid, enhances the stability of β-catenin through promoting GSK-3β phosphorylation. n-3 PUFAs are readily incorporated into cell membranes and lipid rafts, and their incorporation may lead to changes in the levels of membrane-associated signaling proteins, including Ras, Akt, EGFR and human epidermal growth factor receptor 2 (48). In the present study and the study by Pai *et al* (49), the observed reductions in the expression levels of EGFR and p-Akt protein in fat-1 tumor tissues suggest that the EGFR/Akt signaling pathway may be involved in n-3 PUFA-mediated β-catenin degradation. In addition, increased E-cadherin expression may also account for the decreased protein expression levels of c-Myc through sequestering β-catenin in the plasma membrane, thereby blocking its translocation to the nucleus. Ultimately, the results of the present study suggest that β-catenin signaling inhibition contributes to the preventive effect of n-3 PUFAs against melanoma.

It is generally accepted that n-3 PUFAs exert their potentially beneficial anti-inflammatory effects by decreasing the levels of AA-derived mediators, such as the predominant proinflammatory prostanoids PGE2 and PGI2 (50). In the present study, no notable alterations in the levels of PGE2 between fat-1 and WT mice were observed. In contrast, marked differences in the levels of EPA metabolites (PGE3, 12-HEPE, 15-HEPE and RVE1) and DHA-derived mediators (RVD2 and Maresin1) were observed in the tumors of fat-1 mice compared with those of WT mice. Preclinical and clinical trials have provided evidence to show that EPA and DHA-derived lipid mediators exert their potential anti-inflammatory effects in a wide range of inflammatory diseases, including arthritis, lung inflammation and colorectal cancer (51). The decreased expression levels of p-NF-κB and p-STAT3 in the melanoma tumors of fat-1 mice in the present study, confirmed the anti-inflammatory effects of n-3 PUFA-derived mediators. Taking these findings into account, it is possible that the endogenously biosynthesized lipid mediators derived from n-3 PUFAs underlie the antitumor effects observed in the fat-1 transgenic mice, instead of the decrease in AA-derived mediators, such as 2-series prostaglandins (PGE2).

Oxidative stress has been reported to serve an important role in tumor initiation and progression (52). The excessive intracellular accumulation of reactive oxygen species leads to disruption of the mitochondrial membrane potential, the release of cytochrome c and ultimate cell apoptosis (53). DHA and EPA unsaturated fatty acids are susceptible to lipid peroxidation, which can lead to high levels of oxidative stress, cell growth inhibition and cell death (54). Indeed, in the present

study, a significant increase in MDA levels was observed in the urine of fat-1 mice compared with that of WT mice. However, no significant alterations in the MDA levels between fat-1 and WT mice were observed following administration of the Vit E antioxidant, which suggests that the lipid peroxidation induced by endogenous PUFAs may be counteracted by Vit E. Notably, tumor growth was suppressed in fat-1 and WT mice following Vit E administration, and tumor growth inhibition was greater in fat-1 mice supplemented with Vit E compared with WT controls. Ultimately, these data suggest that the protective role of n-3 PUFAs against melanoma progression, may not be mediated by n-3 PUFA-induced lipid peroxidation, and demonstrates that Vit E supplementation may further enhance the antitumor activity of n-3 PUFAs.

In conclusion, using an *in vivo* model involving fat-1 transgenic mice, the present study provides encouraging preclinical evidence of the molecular mechanisms by which n-3 PUFAs may regulate the malignant features of melanoma. The results presented support a protective role of n-3 PUFAs in the prevention of melanoma progression, and in the potential development of clinical interventions that combine n-3 PUFAs with conventional or innovative therapies for the treatment of patients with melanoma.

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