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# Melanoma growth is reduced in fat-1 transgenic mice: Impact of omega-6/omega-3 essential fatty acids

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An important nutritional question as to whether the ratio of omega-6 (n-6) to omega-3 (n-3) fatty acids plays a role in tumorigenesis remains to be clarified in well qualified experimental models. The recently engineered fat-1 mice, which can convert n-6 to n-3 fatty acids and have a balanced ratio of n-6 to n-3 fatty acids in their tissues and organs independent of diet, allow carefully controlled studies to be performed in the absence of potential confounding factors of diet and therefore are a useful model for elucidating the role of n-6/n-3 fatty acid ratio in tumorigenesis. We implanted mouse melanoma B16 cells into transgenic and WT littermates and examined the incidence of tumor formation and tumor growth rate. The results showed a dramatic reduction of melanoma formation and growth in fat-1 transgenic mice. The level of n-3 fatty acids and their metabolite prostaglandin E<sub>3</sub> (PGE<sub>3</sub>) were much higher (but the n-6/n-3 ratio is much lower) in the tumor and surrounding tissues of fat-1 mice than that of WT animals. The phosphatase and tensin homologue deleted on the chromosome 10 (PTEN) gene was significantly up-regulated in the fat-1 mice. *In vitro* experiments showed that addition of the n-3 fatty acid eicosapentaenoic acid or PGE<sub>3</sub> inhibited the growth of B16 cell line and increased the expression of PTEN, which could be partially attenuated by inhibition of PGE<sub>3</sub> production, suggesting that PGE<sub>3</sub> may act as an antitumor mediator. These data demonstrate an anticancer (antimelanoma) effect of n-3 fatty acids through, at least in part, activation of PTEN pathway mediated by PGE<sub>3</sub>.

prostaglandin E<sub>3</sub> | eicosapentaenoic acid | tumorigenesis

Polyunsaturated fatty acids (PUFAs) are important as structural components of membrane phospholipids and as precursors of families of signaling molecules (eicosanoids), including prostaglandins (PGs), thromboxanes, leukotrienes, and lipoxins (1–3). The principal eicosanoid precursors are arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3). The eicosanoids derived from omega-6 (n-6) and omega-3 (n-3) PUFAs are functionally distinct, and some have important opposing physiological functions (3, 4). For example, AA-derived eicosanoids such as PGE<sub>2</sub> and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) have been shown to promote cancer formation, growth, and metastasis, whereas EPA-derived eicosanoids have suppressive effects (4–9). Because eicosanoid precursors (i.e., AA and EPA) compete for the same metabolic enzymes [cyclooxygenase (COX) and lipoxygenase], the availability of AA and EPA and their ratio in cellular lipids determine the cell eicosanoid profile. Thus, it has been suggested that a balanced n-6/n-3 ratio of body lipids is essential for normal growth and development and plays an important role in prevention as well as treatment of many clinical problems, including cancer (10–13).

The role for the ratio of n-6 to n-3 fatty acids in tumorigenesis has recently become the focus of omega-3 research. Emerging evidence suggests that the n-6/n-3 fatty acid ratio, rather than the absolute levels of the two classes of PUFAs, is the principal factor in the antitumor effects of n-3 PUFAs (10–12). Experimental data show that the efficacy of n-3 PUFAs in suppressing cancer growth

depends not only on the amount of n-3 PUFAs but also on background levels of n-6 PUFAs (14). It has also been shown that the therapeutic benefit of dietary n-3 PUFAs is greatest when its proportion greatly exceeds that of n-6 PUFAs (15). According to recent findings (16, 17), the ratio of n-6 to n-3 fatty acids in today's diet is  $\approx 10$ –30:1, indicating that Western diets are deficient in n-3 PUFAs compared with the diet on which humans evolved and their genetic patterns were established (n-6/n-3 = 1:1). The excess n-6 PUFAs and the very high n-6/n-3 ratios may result in excessive and unbalanced production of n-6-derived eicosanoids. This may contribute to the increased incidence of modern diseases, including cancers (16, 17). Thus, balancing the tissue ratio of n-6 to n-3 fatty acids may be a feasible approach to the control of cancer. However, whether a high n-6/n-3 ratio ( $>15$ ), as found in most Westerners, promotes tumorigenesis, or whether a balanced n-6/n-3 fatty acid ratio can reduce cancer development remains to be established in well qualified experimental *in vivo* models.

We recently engineered a transgenic mouse that carries a fat-1 gene from the roundworm *Caenorhabditis elegans* (18). This gene encodes an n-3 fatty acid desaturase that catalyzes conversion of n-6 to n-3 fatty acids and that is absent in most animals, including mammals. There is a remarkable difference in tissue n-6/n-3 fatty acid ratio between WT and fat-1 transgenic mice. When both WT and fat-1 transgenic mice are maintained on an identical diet high in n-6 but low in n-3 fatty acids, the WT mice exhibit very high contents of n-6 fatty acids but very little n-3 fatty acids, with a n-6/n-3 ratio of 20–50 in their tissues, whereas the fat-1 transgenic mice are rich in n-3 fatty acids and have a lower n-6/n-3 ratio in their tissues. Thus, this allows us to produce two different fatty acid profiles (high vs. low n-6/n-3 ratios) in the animals by using just a single diet and therefore eliminates the need of two different diets for a comparative study, so that the potential variations in the impurities and caloric and other components (potential confounding factors) in the supplemented oils between two diets can be avoided. Hence, the fat-1 transgenic mouse is a useful *in vivo* system for elucidating the role of n-6/n-3 fatty acid ratio in carcinogenesis.

The B16 melanoma cell line was derived from melanoma of C57BL/6 mice and can be grown to form tumors in fat-1 transgenic mice. Thus, in this study, we chose to implant these cells in the fat-1 and WT mice and examined their tumorigenicities.

## Results

**Tumorigenicity of B16 Melanoma Cells in fat-1 Transgenic and WT Mice.** To test our hypothesis that the fat-1 transgenic mice, which have a balanced n-6/n-3 fatty acid ratio, may have a lower risk of

Conflict of interest statement: No conflicts declared.

Abbreviations: PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; AA, arachidonic acid; PG, prostaglandin; COX, cyclooxygenase; n-6, omega-6; n-3, omega-3; PTEN, phosphatase and tensin homologue deleted on chromosome 10; LC, liquid chromatography; MS/MS, tandem MS.

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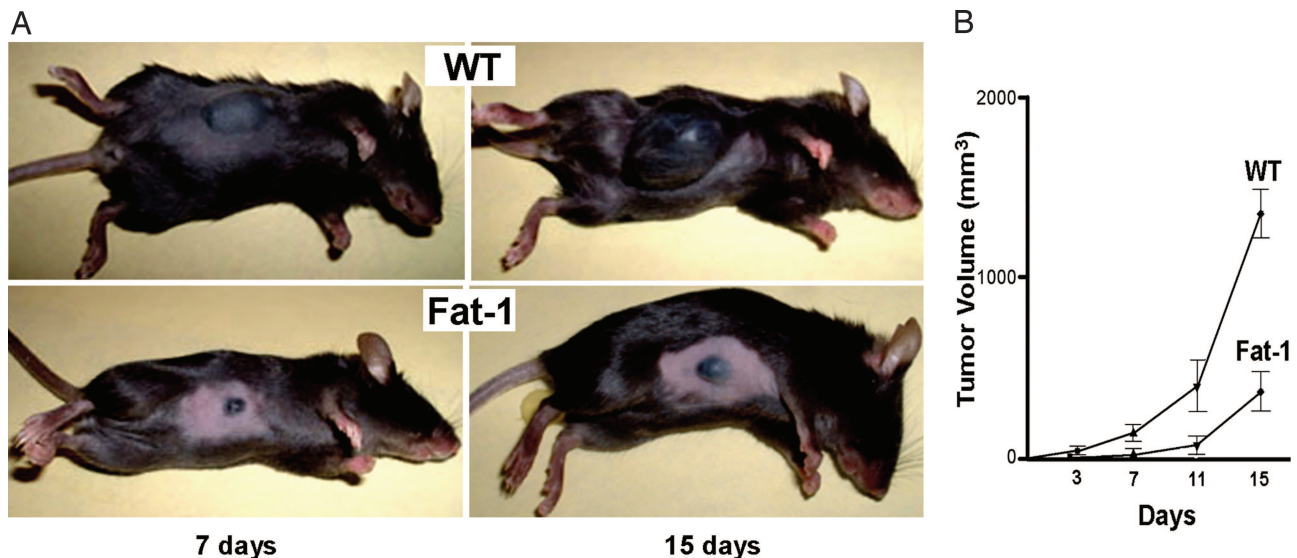


**Fig. 1.** Tumorigenicity of B16 melanoma cells in fat-1 transgenic and WT mice. (A) Different sizes of melanomas in WT and fat-1 transgenic (FAT-1) mice at two different time points. A number of  $5 \times 10^6$  viable cells in 50  $\mu$ l of PBS were injected s.c. into each of 10 transgenic and 10 WT littermates (2-month-old female). On days 7 and 15 after cell implantation, animals were anesthetized briefly with isoflurane, and tumors were examined and photographed by using a digital camera. (B) Growth rates of melanomas in WT and transgenic mice. Tumor growth was monitored at the indicated time points by measuring the length,  $L$ , and width,  $w$ , of the tumor with a caliper and calculating tumor volume on the basis of the following formula: volume =  $(1/2)Lw^2$ . The points are mean values  $\pm$  SD of 10 tumors ( $n = 10$ ) for the WT group or of 7 tumors ( $n = 7$ ) for the fat-1 transgenic group (fat-1).

tumorigenesis than WT mice, which have a high-tissue n-6/n-3 fatty acid ratio ( $>20$ ), we implanted mouse melanoma B16 cells into the transgenic and WT mice and examined the incidence of tumor formation and tumor growth rate. As shown in Fig. 1, there is a marked difference in the incidence of tumor formation and tumor growth rate between fat-1 transgenic and WT mice. Over an observation period of 15 days, all ( $n = 10$ ) WT mice developed a palpable tumor by day 3, whereas only 7 of 10 transgenic mice developed a minor tumor palpable by day 7 or even day 10 (Fig. 1A). The tumor growth rate (mean tumor volume over time) was much slower in the fat-1 transgenic mice when compared to the WT mice (Fig. 1B). The smallest tumor in the WT group was still bigger than the biggest one in the transgenic group.

**Fatty Acid Profiles of B16 Melanoma and Stromal Tissues of fat-1 Transgenic and WT Mice.** Analysis of the total lipids extracted from the tumor cells and surrounding tissues showed distinct lipid profiles between fat-1 and WT mice (Table 1). There are significantly higher levels of n-6 fatty acids [18:2n-6, 20:4n-6 (AA) and 22:4n-6 and 22:5n-6] and much lower concentrations of n-3 fatty acids [18:3n-3, 20:5n-3 (EPA), 22:5n-3 [docosapentaenoic acid (DPA)], and 22:6n-3 [docosahexaenoic acid (DHA)]] in the tumor and, particularly, stromal tissues from WT mice than those in the tumors from fat-1 transgenic mice. The ratios of the n-6 fatty acids (20:4n-6, 22:4n-6, and 22:5n-6) to the n-3 fatty acids (EPA, DPA, and DHA) in stromal tissues were 9:1 and 0.7:1 in WT and fat-1 transgenic mice, respectively. Similarly, the n-6/n-3 ratios in tumors were 27:1 and 4.6:1 in WT and fat-1 transgenic mice, respectively.

**Differential Profiles of Eicosanoids in B16 Melanoma and Stromal Tissues of fat-1 Transgenic and WT Mice.** Metabolism of the n-6 AA (20:4n-6) and the n-3 EPA (20:5n-3) by the COX pathway produces PGs of two (e.g., PGE<sub>2</sub>) and three series (e.g., PGE<sub>3</sub>), respectively. These metabolites, PGE<sub>2</sub> and PGE<sub>3</sub>, have been shown to differently affect cancer growth (5–9). To determine the impact of the change in tissue ratio of n-6/n-3 fatty acids as a result of fat-1 expression on the generation of the cancer-related eicosanoids, we chose to measure the contents of PGE<sub>2</sub> and PGE<sub>3</sub> in melanoma and stromal tissues of fat-1 transgenic

and WT mice by using lipid mediator lipidomics [liquid chromatography (LC)-UV-tandem MS (MS/MS)]. As shown in Fig. 2, there is a marked difference in both PGE<sub>2</sub> and PGE<sub>3</sub> contents between the fat-1 and WT mice. In WT mice, both the tumor and surrounding tissues have a large amount of PGE<sub>2</sub> but little PGE<sub>3</sub>. In contrast, the amounts of PGE<sub>2</sub> in the tumor and surrounding tissues of fat-1 transgenic mice are lower than those of WT mice, whereas PGE<sub>3</sub> are highly abundant in both the tumor and stromal tissues of fat-1 transgenic mice (Fig. 2).

**Differential Expression of Phosphatase and Tensin Homologue Deleted on Chromosome 10 (PTEN) in Melanoma Cells Grown in the fat-1 Transgenic and WT Mice.** PTEN is a critical tumor suppressor in melanoma tumorigenesis (19–21). To determine whether PTEN

**Table 1. Profiles of polyunsaturated n-6 and n-3 fatty acids in stromal and tumor tissues from WT or fat-1 transgenic (fat-1) mice**

| PUFAs                           | Stroma         |                | Tumor          |                |
|---------------------------------|----------------|----------------|----------------|----------------|
|                                 | WT             | fat-1          | WT             | fat-1          |
| 18:2n-6                         | 27.5 $\pm$ 1.5 | 25.1 $\pm$ 1.0 | 12.5 $\pm$ 0.8 | 11.5 $\pm$ 0.6 |
| 18:3n-3                         | 0.1 $\pm$ 0.1  | 0.3 $\pm$ 0.1* | ND             | ND             |
| 20:4n-6                         | 2.0 $\pm$ 0.2  | 1.5 $\pm$ 0.1* | 12.8 $\pm$ 0.6 | 9.1 $\pm$ 0.3* |
| 20:5n-3                         | ND             | 0.5 $\pm$ 0.1* | ND             | 0.3 $\pm$ 0.1* |
| 22:4n-6                         | 0.5 $\pm$ 0.1  | 0.3 $\pm$ 0.1  | 1.7 $\pm$ 0.3  | 1.0 $\pm$ 0.2* |
| 22:5n-6                         | 1.0 $\pm$ 0.2  | 0.4 $\pm$ 0.1* | 1.8 $\pm$ 0.2  | 0.5 $\pm$ 0.2* |
| 22:5n-3                         | ND             | 0.5 $\pm$ 0.2* | ND             | 0.7 $\pm$ 0.1* |
| 22:6n-3                         | 0.4 $\pm$ 0.1  | 2.2 $\pm$ 0.4* | 0.6 $\pm$ 0.1  | 1.3 $\pm$ 0.3* |
| n-6/n-3 (total)                 | 62             | 7.6            | 48             | 9.5            |
| n-6/n-3 ( $>$ C20) <sup>†</sup> | 9              | 0.7            | 27             | 4.6            |

Total lipids of tumor or surrounding tissues were extracted and subjected to analysis of gas chromatography. The values (% of total fatty acids) are means of four separate measurements  $\pm$  SD.

\*Significant difference ( $P < 0.05$ ) between WT and fat-1.

<sup>†</sup>Ratio of those n-6/n-3 fatty acids with 20 or more carbons in length, i.e., (20:4n-6 + 22:4n-6 + 22:5n-6)/(20:5n-3 + 22:5n-3 + 22:6n-3).

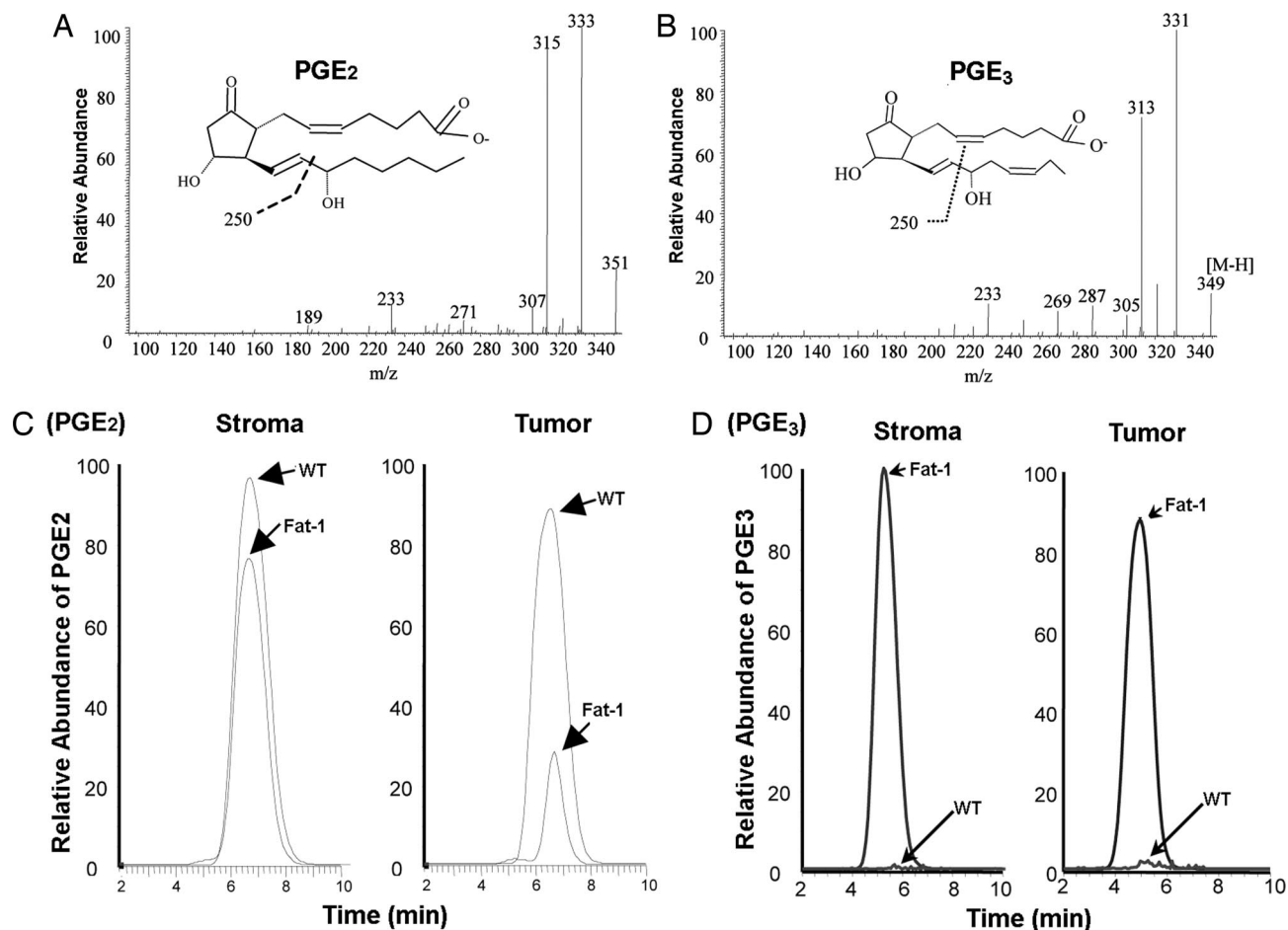


**Fig. 2.** Identification of PGE<sub>2</sub> (A; *m/z* 351) and PGE<sub>3</sub> (B; *m/z* 349) by MS/MS in the tumor samples of fat-1 transgenic mice. LC-MS chromatograms showing the relative contents of PGE<sub>2</sub> (C) and PGE<sub>3</sub> (D) in melanoma and surrounding tissues of fat-1 transgenic and WT mice. Combined samples of three aliquots of tissues (tumor or stroma) from three different animals (fat-1 or WT) were extracted and analyzed for PGE<sub>2</sub> and PGE<sub>3</sub> by LC-MS/MS.

plays a role in the anticancer effect observed in fat-1 mice, we measured the expression of this gene in the tumor cells by both Western blotting and RT-PCR. The results showed that PTEN (barely detectable in the B16 melanoma cells) was dramatically up-regulated (16- to 32-fold increase) in the melanoma tumors of fat-1 transgenic mice (Fig. 3 and Table 2). PTEN have been known to regulate cell death and cell cycle by three pathways: Akt-caspase, Akt-cyclin D, and p53/PTEN-p21 (19). To investigate whether the downstream genes of PTEN pathways could also be affected, Western blotting and quantitative real-time RT-PCR were used to assay the major downstream genes (Akt, caspase-3, p21, and cyclin D1). As shown in Fig. 3, Akt expression is almost absent in the tumors of transgenic mice but highly abundant in tumors grown in WT mice; caspase-3 expression is slightly higher in tumors from transgenic mice than in those of WT mice, suggesting the involvement of the PTEN-Akt-caspase pathway. No significant change in the expression of p21 and cyclin D1 was found (Table 2), suggesting that the PTEN-cyclin D and PTEN-p21 pathways may not be involved.

**In Vitro Validation of the Relation of PG Production and PTEN Expression to Tumor Cell Growth.** To validate the potential link of the observed differences in PGE<sub>2</sub> and PGE<sub>3</sub> to the differential tumorigenicities of B16 melanoma cells in the mice, we examined the effects of PGE<sub>2</sub> and PGE<sub>3</sub> on the growth of B16 melanoma cell line and the expression of PTEN in these cells in culture. As shown in Fig. 4A, exposure of B16 melanoma cells to 1  $\mu$ M PGE<sub>3</sub>

for 48 h inhibited cell proliferation by 30–40%; treatment with PGE<sub>2</sub>, however, had no significant effect. Flow cytometry analysis showed that PGE<sub>3</sub> induced cell apoptosis in a dose-dependent manner (Fig. 4B). Western blotting analysis showed that PGE<sub>3</sub> treatment dramatically increased PTEN expression; no change of PTEN was found in cells treated with PGE<sub>2</sub> (Fig. 4C). These results are consistent with the *in vivo* data and suggest that a PGE<sub>3</sub>-PTEN pathway may, at least in part, mediate the antitumor effect observed in fat-1 transgenic mice.

Next, we tested the effects of AA and EPA (precursors to PGE<sub>2</sub> and PGE<sub>3</sub>, respectively) alone or in combination with the nonselective COX inhibitor indomethacin on the growth of B16 cells. As shown in Fig. 5A, AA (50  $\mu$ M) did not affect cell growth significantly, whereas EPA (50  $\mu$ M) exhibited a marked inhibitory effect on the growth of B16 melanoma cells. Interestingly, the growth-inhibitory effect of EPA could be blocked by the presence of 50  $\mu$ M indomethacin (Fig. 5A). Lipidomic analysis of PGE<sub>2</sub> and PGE<sub>3</sub> in the cultured cells showed that addition of AA and EPA to the cells dramatically increased the production of PGE<sub>2</sub> and PGE<sub>3</sub>, respectively, which could be suppressed by the addition of indomethacin (Fig. 5B). These changes are correlated well with the effect of the fatty acids on cell growth. Thus, these results suggest that PGE<sub>3</sub>, derived from the n-3 fatty acid EPA, may mediate the antitumor effect observed in fat-1 transgenic mice.

## Discussion

This study examines the anticancer effect of n-3 fatty acids using fat-1 transgenic mice without the need of supplementation with

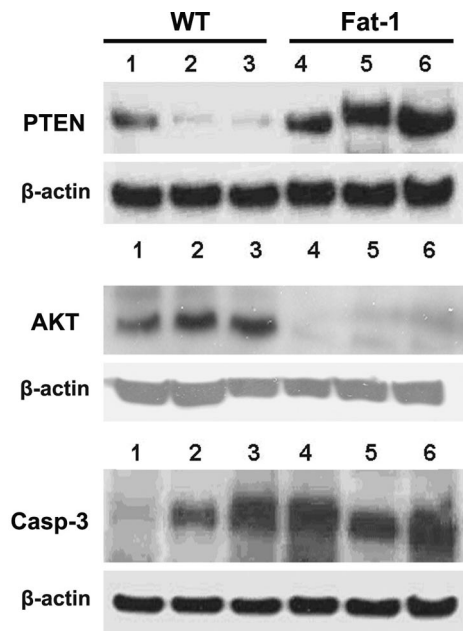


**Fig. 3.** Western blotting of PTEN (Top), Akt (Middle), and caspase-3 (Bottom) in melanoma tumors from three WT (lanes 1–3) and three fat-1 transgenic (lanes 4–6) mice.

exogenous n-3 fats. This genetic approach to modifying fatty acid composition by converting n-6 to n-3 fatty acids not only effectively increases the absolute amount of n-3 fatty acids but also significantly decreases the level of n-6 fatty acids, leading to a balanced ratio of n-6 to n-3 fatty acids in mouse tissues without alteration in the total amount of cellular lipids, which the conventional approach by dietary intervention cannot achieve. More importantly, this animal model enables us to generate two different fatty acid profiles in a litter of mice (i.e., high n-6/n-3 in WT vs. low n-6/n-3 in fat-1 transgenic littermates) by feeding them just a single diet (i.e., a high-n-6 diet). Conventionally, two different diets varying in fat composition must be used to feed the animals to establish the different fatty acid profiles. However, feeding two different diets for months make it impossible to keep everything identical with just a difference in n-6/n-3 ratio between two experimental groups of animals, because many variables may arise from the diets and the feeding procedure, including the impurities or unwanted components of the oils used (e.g., fish oil vs. corn oil), flavor, sensitivity to oxidation, diet storage, duration of diet change, etc. In fact, the quality (freshness and purity) of fish oils can vary greatly among animal diet preparations. These factors can potentially impose confounding effects on the experimental results. In the present study, the use of fat-1 transgenic mice capable of endogenously

**Table 2.** Quantitative RT-PCR assays of PTEN, p21, and cyclin D1 expression in WT and fat-1 transgenic mice

| Gene names | C/t values |            | $\Delta C/t$ | Folds changed |
|------------|------------|------------|--------------|---------------|
|            | WT*        | fat-1      |              |               |
| GAPDH      | 35.2 ± 0.4 | 33.1 ± 0.3 | 2.0 ± 0.7    | —             |
| PTEN       | 31.3 ± 0.3 | 24.1 ± 0.6 | 7.2 ± 0.9    | ≈32*          |
| P21        | 32.0 ± 0.4 | 30.5 ± 0.5 | 1.5 ± 1.0    | NS            |
| Cyclin D1  | 35.0 ± 0.4 | 35.5 ± 0.5 | 0.6 ± 0.9    | NS            |

Values are means and standard deviations of three separate assays. NS, no significant changes.

\*Increase.

synthesizing n-3 fatty acids themselves could eliminate the confounding factors of diet and thereby provide reliable results for the effects of n-3 fatty acids.

By taking advantage of this model, we examined the effect of n-3 fatty acids on tumorigenicity of B16 melanoma cells *in vivo*. This cell line was chosen because: (i) it was derived from melanoma of C57BL/6 mice and can be grown to form tumors in our transgenic mice whose genetic background is C57BL/6; (ii) melanoma is a common type of cancer in U.S., and potential prevention of this cancer by dietary factors, particularly n-3 fatty acids, has not been well investigated; and (iii) a conflicting effect of n-3 fatty acids on the tumorigenicity of B16 cells was reported previously in an animal study (22), and this needs to be clarified in a well qualified experimental model. Our data presented here clearly show a dramatic inhibition of melanoma formation and growth in fat-1 transgenic mice, supporting the notion that a balanced n-6/n-3 fatty acid ratio can reduce cancer development.

One of the notable changes accompanying with the reduction of melanoma formation is the up-regulation of PTEN in the tumor and surrounding tissues of fat-1 mice, as evidenced by both quantitative RT-PCR and Western blotting. PTEN was first identified as a candidate tumor suppressor gene in 10q23 in 1997 (23). The PTEN protein has at least two biochemical functions: it has both lipid phosphatase and protein phosphatase activities. Activation of the lipid phosphatase of PTEN decreases intracellular PIP3 level and downstream Akt activity (19). Hundreds of PTEN mutations or inactivation have been found in a variety of human tumors, including brain, bladder, breast, prostate, and endometrial cancers (23–26), making PTEN the second most frequently mutated tumor suppressor gene (21). Recently, PTEN has also been shown to be a critical tumor suppressor in melanoma tumorigenesis (19–21). PTEN is known to regulate cell death and cell cycle by three pathways: Akt-caspase, Akt-cyclin D, and p53/PTEN-p21 (19). Our data showing higher levels of PTEN and caspase-3 with a lower level of Akt in fat-1 mice than WT animals (but no change in cyclin D and p21) suggest that the PTEN-Akt-caspase pathway may play a role in mediating the antitumor effect of n-3 fatty acids observed in the fat-1 transgenic mice.

Another significant finding of the present study is that the level of PGE<sub>3</sub>, derived from the n-3 fatty acid EPA, is markedly higher, whereas the level of PGE<sub>2</sub>, formed from the n-6 fatty acid AA, is significantly lower in the tumor and surrounding tissues of fat-1 mice than that of WT animals, as a result of the difference in n-6/n-3 fatty acid ratio between the transgenic and WT mice. Syntheses of PGE<sub>2</sub> and PGE<sub>3</sub> are catalyzed by the same COX, but the biological effects of PGE<sub>2</sub> and PGE<sub>3</sub> are distinct. PGE<sub>2</sub> has been shown to promote cancer development (5–7), whereas PGE<sub>3</sub> has been recently found to have anticancer effects (9). Until recently, PGE<sub>3</sub> was an underappreciated and poorly understood eicosanoid because of the lack of sensitive and convenient methods for its measurement. In the present study, the use of lipid mediator lipidomics using LC-MS/MS enabled us to determine the difference of PGE<sub>3</sub> content between WT and fat-1 transgenic mice. Interestingly, our *in vitro* experiments showed that addition of the n-3 fatty acid EPA or PGE<sub>3</sub> inhibited the growth of B16 cells in culture and increased the expression of PTEN, which could be partially attenuated by inhibition of PGE<sub>3</sub> production. These results suggest that PGE<sub>3</sub> is an anticancer mediator, and generation of PGE<sub>3</sub> from EPA may underlie the antitumor effect observed in fat-1 transgenic mice. Thus, our data demonstrate an antimelanoma effect of n-3 fatty acids, at least in part through activation of the PTEN pathway mediated by PGE<sub>3</sub>. Nevertheless, involvement of other eicosanoids and pathways cannot be excluded.

One important point we learned from these results is that simply inhibiting COX-2 may not produce an anticancer effect (rather, it may give unwanted side effects), because PGE<sub>3</sub>, an effective anticancer mediator (as we show here), is a product of COX-2, and



**Cell Proliferation Assay.** Cell proliferation was analyzed by using a MTT assay (Roche, Mannheim, Germany). At a series of time points after treatment of 1  $\mu$ M PGE<sub>2</sub> or 1  $\mu$ M PGE<sub>3</sub>, respectively, the number of viable B16 cells grown on a 96-well microtiter plate was estimated by adding 10  $\mu$ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml in PBS). After 4 hr of incubation at 37°C, the stain was diluted with 100  $\mu$ l of DMSO. The optical densities were quantified at a test wavelength of 550 nm and a reference wavelength of 630 nm on a multiwell spectrophotometer.

**Quantitative Real-Time PCR.** Total RNA from the tumors of transgenic and WT mice was extracted by using an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's suggested protocol. Concentration and purity of extracted RNA were determined by using a Shimadzu UV1600 spectrophotometer (Shimadzu, Kyoto, Japan). The quality of RNA was checked by 1% agarose gel electrophoresis. Oligonucleotide primers were designed by using Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by Invitrogen (Carlsbad, CA). Forward primers (5'-3'): TTTGTGGGTCTGCCAGCTAAAGG, GCAGATC-CACAGCGATATCCAG, TGCTGCAAATGGAAGTCTTC, and GCGATGTAGGCCAACTGCTTAG for PTEN, P21, cyclin D1, and GAPDH, respectively. Reverse primers (5'-3'): ATCAC-CACACACAGGCAATGG, CGAAGAGACAACGGCA-CACCTT, CATCCGCCTCTGGCATTCT, and GGCATG-GACTGTGGTCATGAGT for PTEN, p21, cyclin D1, and GAPDH, respectively. The fluorescent dye SYBR green was ordered from Stratagene (La Jolla, CA). GAPDH gene was used as an endogenous control to normalize the expression of these genes. Quantitative real-time RT-PCR was performed in triplicate using a 96-well optic tray on an ABI Prism 7000 sequence detection system (Applied Biosystems). The negative controls lacking template RNA were included in each experiment. PCR products were then run on a 1% agarose gel to confirm the presence of a single band with the expected size. Data collection and analysis were performed with SDS Version 1.7 software (Applied Biosystems). Data were then exported and further analyzed in Excel. Results, expressed as *N*-fold differences in target gene expression relative to the control gene, termed "*N*," were determined by the formula:  $N = 2^{\Delta\Delta C_t}$ , where the  $\Delta C_t$  value [PCR cycles (*Ct*)] of the sample was determined by subtracting the average *Ct* value of the target gene from the average *Ct* value of the control gene. All *Ct* values of the samples were normalized by human GAPDH.

**Western Blot.** Anti-Akt mouse monoclonal antibody and anti-caspase-3 mouse monoclonal antibody were purchased from Cell Signaling (Beverly, MA). Anti-PTEN goat polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Total protein from tumors (included from WT and transgenic mice) was isolated. Briefly, 100 mg of tissue was extracted with extraction buffer containing 150 mmol/liter NaCl/10 mmol/liter Tris (pH 7.2)/5 mmol/liter EDTA/0.1% Triton X-100/5% glycerol/2% SDS in addition to a mixture of protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). Aliquots of protein (20  $\mu$ g per lane) were fractionated on SDS/10% PAGE gels and transferred onto poly(vinylidene difluoride) membranes. The Western blot procedure was carried out as described (29). The level of  $\beta$ -actin expression was used as the internal control for equal loading. Reactive protein bands were developed with chemiluminescence detection reagents (Amersham Pharmacia Biosciences, Piscataway, NJ).

**Cell Viability Assay.** B16-F<sub>0</sub> cells were cultured in a 96-well plate at  $1 \times 10^4$  cells per well and treated in triplicate for the times indicated. Background control wells were treated with 0.1% saponin for 10 min. Wells were washed gently with 100  $\mu$ l of  $1 \times$  PBS and 100  $\mu$ l of  $1 \times$  calcein-AM buffer. Then 50  $\mu$ l of 1  $\mu$ M calcein-AM (Trevigen, Gaithersburg, MD) was added to the well and incubated at 37°C under 5% CO<sub>2</sub> for 30 min. Fluorescence was quantified on a Wallac 1420 VICTOR<sup>3</sup> fluorescent plate reader (Perkin-Elmer) at excitation/emission wavelengths of 490/520 nm. The percent control fluorescence was determined by using the following formula: % control fluorescence = (treated sample – background)/(control sample – background)  $\times$  100.

**Lipidomic Analysis.** Bioactive mediators derived from n-3 and n-6 fatty acids in the tumor or surrounding tissues or cultured cells were identified by using lipidomics methods, as described by Gronert *et al.* (30). Samples were extracted with 2 ml of cold methanol and analyzed by GC-MS and/or LC-UV-MS/MS.

**Statistical Analysis.** Results are means  $\pm$  SD. Statistical analysis was performed by using Student's *t* test, and *P* values of  $<0.05$  were considered significant.

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