Published in final edited form as:

Prostaglandins Leukot Essent Fatty Acids. 2014 April ; 90(4): 105–115. doi:10.1016/j.plefa.2013.12.001.

Key roles for GRB2-associated-binding protein 1, phosphatidylinositol-3-kinase, cyclooxygenase 2, prostaglandin E2 and transforming growth factor alpha in linoleic acid-induced upregulation of lung and breast cancer cell growth

M. Mouradian^{#*}, K.D. Kikawa[#], E.D. Johnson, K.L. Beck, and R.S. Pardini

Department of Biochemistry and Molecular Biology, University of Nevada, Reno, 1664 N. Virginia St, MS0330, Reno, NV 89557

[#] These authors contributed equally to this work.

SUMMARY

The omega-6 polyunsaturated fatty acid linoleic acid (LA; C18:2 n-6) is prevalent in Western diets and has been shown to enhance tumorigenesis of several cancer models. However, the modes by which LA affects carcinogenesis have not been fully elucidated. In this study, a mechanism for LA-induced upregulation of cancer cell growth is defined. Cellular proliferation was enhanced with LA treatment in BT-474 human breast ductal carcinoma and A549 human lung adenocarcinoma cell lines. Enrichment of LA increased COX activity and led to increases in PGE2 production, followed by increases in MMP and TGF- α levels, which are all key elements involved in the enhancement of cancer cell growth. Further investigation revealed that LA supplementation in both BT-474 breast and A549 lung cancer cell lines greatly increased the association between the scaffolding protein Gab1 and EGFR, while at the same time dramatically decreasing Gab1 protein levels. These changes are concomitant with increases in activated Akt (pAkt), a downstream signaling component in the PI3K signaling pathway. Moreover, inhibitors of EGFR, PI3K and Gab1-specific siRNAs were capable of reversing LA-induced upregulation of pAkt, as well as observed increases in cell proliferation for these models. These data establish Gab1 as major target in LA-induced enhancement of tumorigenesis.

INTRODUCTION

Cancer-related research on essential fatty acids (EFAs) has tended to focus on the beneficial properties and mechanisms of omega-3 polyunsaturated fatty acids (PUFAs) [1, 2].

Omega-3 PUFAs initially garnered interest due to epidemiological studies demonstrating

© 2013 Elsevier Ltd. All rights reserved

*Corresponding Author: Michael Mouradian University of Nevada, Reno 1664 N. Virginia St. MS330 Reno, NV 89557 775-784-6237 (Phone) 775-784-1419 (FAX) memouradian@gmail.com.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors have no conflicts of interest or financial relationships to disclose

that despite high levels of consumed dietary fat, Greenland Eskimo populations had much lower incidence of many types of disease, including certain types of cancer [3]. Other studies showed increases in cancer rates in Japanese populations who had migrated and adopted Western diets, rich in omega-6 PUFAs, further emphasized the importance of the type of fat being consumed [4]. While numerous studies have been done identifying the mechanisms by which omega-3 PUFAs exert their anti-cancer effects, much less work has been done in regard to how omega-6 PUFAs may be enhancing tumorigenesis.

Omega-6 PUFAs, particularly linoleic acid (LA; C18:2 n-6), are found in abundance in Western diets [5]. The role of omega-6 PUFAs in cancer, specifically LA, remains somewhat unclear. A review of animal models suggests that while LA may not have a large effect on tumor initiation for many types of cancer, it can highly influence tumor progression and growth [6]. In the case of breast cancer, high fat diets rich in omega-6 PUFAs increase carcinogenesis and enhance tumor progression [7]. Similar studies have suggested that LA enhances tumorigenesis in models of colon [8] and prostate cancer [9]. The activity of the cyclooxygenase (COX) and lipoxygenase (LOX) families of enzymes in eicosanoid synthesis has been identified as having a clear role in LA-induced tumorigenesis [10–13]. Specific eicosanoids have been shown to initiate signaling events in cancer [14]. In fact, studies have suggested that eicosanoid inhibitors could potentially play an important role in inhibiting LA-induced increases in cancer growth [15]. Prostaglandin E2 (PGE2) has been of particular interest, as it is a pro-inflammatory eicosanoid usually found at high levels in cancers [16]. Additionally, PGE2 is now understood to initiate pro-oncogenic signaling events, including the transactivation of the epidermal growth factor receptor 1 (EGFR) through a mechanism involving matrix metalloproteinases (MMPs) and transforming growth factor- α (TGF- α) [17]. However, despite knowledge of the involvement of these various eicosanoids in LA-related tumorigenesis, no specific mechanisms have been identified. Therefore, developing a better understanding of how LA upregulates tumorigenesis is essential because it is a dietary component that is so pervasive in Western cuisine. The current study has identified a series of signaling events initiated by the LA-induced upregulation of COX activity and PGE2, characterizing key proteins and signaling events involved in its enhancement of cell growth in models of human breast and lung cancers.

MATERIALS AND METHODS

Cell Lines, Antibodies & Reagents

A549 human lung adenocarcinoma and BT-474 human breast ductal carcinoma cell lines were acquired from ATCC (Manassas, VA). The E10, E9, C10 and A5 cell lines were obtained from Dr. Lucy Anderson at NCI Frederick. Fatty acids (Sigma, St. Louis, MO) were dissolved in ethanol (EtOH), flushed with nitrogen gas, protected from light and stored at -20°C for no more than 60 days. Antibodies for Akt, pAkt, EGFR, Gab1, MMP-2, MMP-9, STAT3 and pSTAT3 were purchased from Cell Signaling Technologies (Boston, MA), while an antibody specific to β -actin was bought from Abcam (Cambridge, MA).

Cell Culture

As described previously [18], A549 cells were maintained in RPMI-1640 (Mediatech Inc., Manassas, VA) supplemented with 10% FBS (Hyclone, Logan, UT). BT-474 cells were cultured in HybriCare (ATCC, Manassas, VA) with 10% FBS. E10 and E9 cells were cultured in CMRL 1066 (Mediatech Inc., Manassas, VA) with 10% FBS, while C10 and A5 were maintained in DMEM (Mediatech Inc., Manassas, VA) with 10% FBS. Cells were grown as monolayers at 37°C in a humidified environment with 5% CO₂. 24 hours after plating, cultures were supplemented with specific concentrations of docosahexaenoic (DHA; C22:6 n-3), linoleic acid (LA; C18:2 n-6), or an equal control volume of ethanol (EtOH).

Cell Viability Assay

Cells were trypsinized and counted using trypan blue staining and a hemocytometer. Unstained cells were counted as viable.

XTT Assay

Cell Proliferation Kit II from Roche (Indianapolis, IN) was used following the manufacture's protocol. Briefly, an equal number of cells were seeded in 96-well plates for 24 hours prior to specific treatments. At the end of the incubation times, XTT reagents were mixed and added to the wells for 4–24 hours before being read at 450 nM (with a 650 nM reference wavelength) on a SpectraMax M5 machine (Molecular Devices, Sunnyvale, CA).

Immunoblotting and Immunoprecipitation

As described previously [19], cells were washed with ice-cold PBS and lysed using GTP-lysis buffer [50mM HEPES (pH 7.5), 15mM NaCl, 6mM sodium deoxycholate, 1% NP-40, 10% glycerol, 10mM MgCl₂, 1 mM EDTA] containing freshly added protease and phosphatase inhibitors. Tumors were homogenized in the same buffer. Samples were centrifuged at 16,000 × g for 10 minutes at 4°C. Supernatants were analyzed for protein concentration using DC assay (BioRad, Hercules, CA). Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (BioRad, Hercules, CA) and probed with specific antibodies. Detection was performed using HRP-conjugated secondary antibodies and visualized with ECL (GE Healthcare, Buckinghamshire, UK). For immunoprecipitations, defined amounts of protein were incubated overnight with specific antibodies before the addition of protein G-agarose followed by washing.

qRT-PCR

Total RNA was isolated and purified using RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. cDNA was then synthesized using cDNA Synthesis VILO kit (Invitrogen, Carlsbad, CA). Quantities of cDNA were measured by quantitative real-time PCR on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA). Reactions used SYBR green FAST master mix and conditions followed manufacture's recommendations (Applied Biosystems, Carlsbad, CA). Gab1 primer set was designed using Primer3 Input [20] (Sense: TGGCTGGGATTTTTCATTTC; Antisense: AGCCTCACCTACCCAAACT). A published primer set was used for beta-Actin (Sense: CGTCTTCCCCTCCATCG; Anti-sense: CTCCTTAATGTCACGCAC) [21]. The relative

abundance of mRNA was determined by comparative Ct method [22]. Data represents at least n=3 independent experiments $\pm$ SD.

COX Activity, PGE2 and TGF- α Kits

COX Activity Assay Kit (Item # 760151), which includes isozyme-specific inhibitors of COX-1 and COX-2, and the Prostaglandin E2 Express EIA Kit (Item # 500141) (Cayman Chemical Company, Ann Arbor, MI) were performed according to the manufacturer's instructions. The TGF alpha Human ELISA Kit (Item # ab100646) (Abcam, Cambridge, MA) was also carried out according to the manufacturer's protocol.

Fatty Acid Analysis

Lipids from BT-474 and A549 cells were extracted as described previously [19]. Briefly, cells were lysed in 1.0 mL of GTP-lysis buffer containing 0.02% butylated hydroxytoluene (BHT) [50mM HEPES (pH 7.5), 15mM NaCl, 6mM sodium deoxycholate, 1% NP-40, 10% glycerol, 10mM MgCl₂, 1 mM EDTA, 0.02% BHT] containing freshly added protease and phosphatase inhibitors. Samples were analyzed for protein concentration using DC assay (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Whole cell homogenate was fatty acid extracted based on the Folch method [47], using a 2:1 chloroform:methanol mixture. The samples were vortexed and centrifuged at 1,000 $\times$ g for 5 minutes. The chloroform layer was taken and a C19:0 internal standard was added and dried under nitrogen. 14% boron trifluoride (BF₃) in methanol was added to the dried sample and incubated at 110°C for 15 minutes. Petroleum ether containing 0.02% BHT was added to the samples. The petroleum ether fatty acid containing fraction was taken and dried in anhydrous Na₂SO₄ / NaHCO₃ (2:1, w,w). The samples were flushed with nitrogen and stored at -20°C until use. The FAMES were resolved by gas chromatography to determine the fatty acid content using conditions similar to those described previously [18]. The composition for individual fatty acids is reported as percentage of total fatty acids present in the sample.

Xenograft Tissue

The feeding study was performed as described previously [19] with adult male NCr homozygous (nu/nu) athymic nude mice maintained and bred under aseptic conditions with constant temperature and humidity. Animals were implanted with A549 xenografts in the right flank and randomly assigned to experimental treatment groups, which were fed diets composed of the American Institute of Nutrition (AIN)-93M casein based diet containing defined amounts of essential fatty acids starting the day of implantation (Table 1). Three experimental dietary groups, each containing 14 mice, consisted of (1) a low fat omega-6, 8% corn oil, (2) a high fat omega-6, 24% corn oil, and (3) a high fat omega-3, 8% corn oil and 16% Dhasco™ oil (Martek, Columbia, MD). Food intake was monitored daily and isocalorically controlled. Tumor growth was monitored biweekly by caliper measurement, with a volume of 3.38 cm³ or signs of ulceration defined as the experimental endpoint. The study was ended 23 days after implantation because xenografts in the 24% corn oil group reached volumes defined as an endpoint for the study. Xenograft tissue was collected and processed as described in the Immunoblotting and Immunoprecipitation section.

Statistical Analyses

All experimental results were independently repeated at least three times. All quantitative data shown represent the compiled data as percentages versus control treatments with error bars representing standard deviation, and statistical analyses were performed using the Student's *t* test and/or ANOVA with the Tukey method for pairwise comparison on SAS® software, with values of at least $P = 0.05$ being considered significant.

RESULTS

When supplemented with increasing concentrations of linoleic acid (LA; C18:2 n-6), both the BT-474 human breast ductal carcinoma and the A549 human lung adenocarcinoma cell lines showed increases in cellular proliferation rates in a dose-dependent manner (Figure 1A). For BT-474 cells, this increase was statistically significant at 50 μM concentrations, while for A549 cells, statistical significance was not achieved until 100 μM concentrations. Although toxic levels were never reached and 500 μM concentrations, we continued to see cell proliferation trending upward. However, these treatments were not statistically significant from 150 μM concentrations for either cell line. Alternatively, docosahexaenoic acid (DHA; C22:6 n-3)-induced decreases in cell proliferation were observed and reached statistical significance at 50 μM concentrations in both BT-474 and A549 cell lines (Figure 1B). LA and DHA fatty acid methyl ester (FAME) incorporation lipid composition was evaluated to determine if the changes in cell proliferation were due to the incorporation of the fatty acids. Indeed, a high percentage of incorporation of either LA or DHA was observed in both cell lines with their respective 100 μM treatments (Table 2).

Cyclooxygenase (COX) activity has been previously shown to play a role in LA-induced upregulation of tumorigenesis [23]. Consistent with these observations both BT-474 and A549 cell lines showed a dramatic upregulation of COX activity when treated with LA (Figure 2A). However, these LA-induced increases were reversed to levels no longer significantly different from control ethanol (EtOH) treatments by the addition of a COX-2 specific inhibitor. COX-1 inhibition did not have the same trend with LA supplementation and did not significantly decrease COX activity levels, suggesting that LA-induced COX activity is specific to a COX-2 mechanism. Furthermore, combination of the COX-1 inhibitor with DHA decreased COX activity in both cell lines and for the EtOH treatment in BT-474 cells, which confirms data previously published, showing that DHA and eicosapentaenoic acid (EPA; C20:5 n-3) can decrease COX activity through the activation of NF- κ B [24].

Prostaglandin E2 (PGE2) synthesis from arachidonic acid (AA; C20:4 n-6) relies on the COX family of enzymes [25] and LA-induced upregulation of AA and PGE2 synthesis has been previously described [42, 45, 46]. In addition to the large increases noted in regard to COX activity, further examination revealed that LA also greatly increased the levels of PGE2 in conditioned media collected from cell cultures of BT-474 and A549 cells (Figure 2B), whereas DHA treatment showed a significant decrease in PGE2 levels for BT-474, but not for A549 cells.

To further investigate the effect of COX activity and PGE2 on cell growth rates, a COX-2 specific inhibitor and exogenous PGE2 were employed and cell proliferation was measured. The addition of exogenous PGE2 was capable of significantly increasing cell proliferation levels of control treatments and LA-treated cells (Figure 2C). However, no significant effect was found with the addition of exogenous PGE2 in the LA group for either cell line. Moreover, when an inhibitor specific to COX-2 was added, the proliferation rates of LA-treated cells were brought down to levels similar to what was seen in the control group treated with the same inhibitor (Figure 2C).

PGE2 increases, induced by LA have been shown to be associated with increased levels of the transforming growth factor- α (TGF- α) and upregulated expression/secretion of the matrix metalloproteinase (MMP) family members, MMP-2 and MMP-9 [26]. Indeed, when levels of TGF- α were examined after polyunsaturated fatty acid (PUFA) supplementation, significant increases were noted in the LA treatment groups for both cell lines (Figure 3A). In addition, LA enrichment induced large increases in the protein expression of both MMP-2 and MMP-9 (Figure 3B), which are secreted MMPs associated with increased migration and invasion in cancer [27].

Among the proteins altered downstream of the signaling events initiated by the TGF- α growth factor increases brought about by LA supplementation is GRB2-associated-binding protein 1 (Gab1). Gab1 is a scaffolding protein that allows epidermal growth factor receptor (EGFR) dimers to signal directly to phosphatidylinositol-3-kinase (PI3K) [26]. PI3K is often dysregulated in cancer and is responsible for potent oncogenic signals allowing for cell survival and proliferation [29]. Enrichment with LA decreased Gab1 protein levels, but unexpectedly increased association of the scaffolding protein with EGFR that resulted in the upregulation of activated Akt (pAkt), a downstream component of the PI3K signaling cascade [29] (Figure 4A). While Gab1 protein levels were dramatically decreased, there was not a concomitant decrease in Gab1 mRNA levels, though DHA groups showed increases that are not reflected in protein expression (Figure 4B).

The use of an inhibitor specific to EGFR, but not PI3K, partially reversed the noted decrease in Gab1 protein levels and inhibited the association of EGFR and Gab1 (Figure 5A). Both EGFR and PI3K inhibitors also reversed the LA-induced upregulation of pAkt (Figure 5A). While the EGFR inhibitors completely reversed the increase in cell proliferation generated by LA supplementation to those statistically insignificant from the level seen in the control group with the same inhibitor, only a partial reversal is noted upon PI3K inhibition (Figure 5B).

When examining lysates from A549 xenografts, similar increases in EGFR/Gab1 association were noted in the corn oil diets that were rich in LA (Figure 6A). Moreover, while the 8% corn oil group did not show as strong an increase in the EGFR/Gab1 interaction, it also did not demonstrate the same degree of downregulation in total Gab1 protein levels, or upregulation of Akt activation. Analyzing the sera from animals fed the various diets (Table 1), increases in PGE2 and TGF- α similar to what was noted in vitro were observed (Figure 6B and 6C). Although Gab1 protein levels were largely depressed by LA treatment, inhibiting Gab1 to test the extent of its involvement in LA-induced changes in signaling and

cell proliferation was critical. Gab1 siRNAs were employed to specifically target the protein. Treatment with Gab1 siRNA pools was capable of reducing Gab1 protein expression in the control and DHA groups by >70%, and despite already reduced Gab1 levels in LA groups, the siRNA still reversed increases in pAkt generated by LA treatments (Figure 7A). Moreover, when cell viability was examined, treatment with the Gab1 siRNA pools reversed LA-induced growth increases to levels that were not statistically different from control treatments (Figure 7B).

To further define that Gab1 may be a specific target of LA-induced tumorigenesis, Gab1 expressing cell models were used. The E9/E10 and A5/C10 models are two sets of paired mouse cell lines isolated from peripheral lung epithelium of the same genetic origin, and both E10 and C10 express high levels of Gab1, whereas their partners E9 and A5 express very little or no Gab1 protein [30]. When the cells were supplemented with increasing concentrations of LA, both E10 and C10 demonstrated significant increases in cell proliferation at 50 μ M and 100 μ M, respectively (Figure 8). However, the corresponding sister cell lines did not show any significant growth increases up to 500 μ M in the case of the E9 cell line, or just barely achieve significance at this very high level of LA treatment.

DISCUSSION

The anti-cancer effects of long-chain omega-3 polyunsaturated fatty acids (PUFAs), specifically eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3), have been the focus of study over several decades of dietary cancer research. However, dietary-related disease caused from the over-abundance of omega-6 fatty acids in the Western world has recently come to attention. Although linoleic acid (LA; C18:2 n-6) is as an essential fatty acid (EFA), it has been related to increased cancer incidence and tumor progression in several models [7–9]. Numerous mechanisms have been proposed for LA-induced tumorigenesis [42–44], however it still remains unclear. The results of this study define a clear and complete mechanism for how LA promotes cancer cell survival and proliferation in tumors that express high levels of GRB2-associated-binding protein 1 (Gab1) protein. While there has been interest in developing therapeutics targeting Gab1 because of its involvement in dysregulated c-Met, epidermal growth factor receptor (EGFR) and phosphatidylinositol-3-kinase (PI3K) signaling in cancer [31], the current data make it an even more attractive target in cancer treatment for cancer cells expressing Gab1.

Gab1 plays important roles in embryonic development, including proper cell migration [32]. Gab1 is also a critical component in the interaction between focal adhesion kinase (FAK) and Met in cancer cell invasion [33], which has been recently reported that LA can induce FAK activation and induce epithelial to mesenchymal transition (EMT) process [26]. Additionally, LA has been reported to increase metastasis [34, 35], and given the involvement of Gab1 in the FAK/Met interaction, it is likely playing a role in this study as well (Figure 9).

One of the most dramatic observations made was the large increase in EGFR/Gab1 association, despite the concomitant decrease in Gab1 protein levels (Figure 4A). Although the upregulated EGFR/Gab1 interaction was not surprising given the downstream increases

in pAkt, the striking decreases in Gab1 protein levels was surprising. A recent publication demonstrated that Gab1 is targeted in a Cbl-dependent manner for proteasomal degradation through its association with Met after long term exposure to hepatocyte growth factor/scatter factor (HGF/SF) [36]. While the Gab1 relationship with Met is not examined in the context of this paper, it is possible a similar mechanism is downregulating Gab1 protein levels due to prolonged association with EGFR.

Other reports have shown that LA can induce tumorigenesis by increasing inflammatory responses without increasing arachidonic acid (AA; C20:4 n-6) levels, the direct precursor of the PGE2 eicosanoid pathway [45, 46], exerting its effect in the upregulation of cancer growth through eicosanoids. In the current work, a specific signaling pathway being activated downstream of the increase in COX activity and PGE2 synthesis is identified. Transforming growth factor alpha (TGF- α) is an important growth factor capable of inducing EGFR dimerization and initiating pro-survival and pro-proliferative signals in cancer, and has shown to induce transformation in non-transformed mammary epithelial cells [37]. Levels of TGF- α in conditioned media have also been shown to be dependent of extracellular matrix (ECM), and increased upon its disruption [37]. Given the dramatic LA-induced upregulation of the gelatinases MMP-2 and MMP-9, which are capable of cleaving ECM and inducing signaling changes [38], this may serve as an explanation for increases noted in TGF- α levels in the current study (Figure 3B). Moreover, while LA is noted to increase levels of MMP-2 and MMP-9, the long chain omega-3 PUFAs DHA and EPA have been found to decrease levels of the same proteins [39].

A model for the signaling cascade outlined by the data in this report is presented (Figure 9). As mentioned previously, further involvement for Gab1 in association with Met, FAK and its associated phosphatase, SHP-2, in LA supplemented cells, warrant future investigations to elucidate a more complete mechanism. However, it is interesting to note that LA supplementation is upregulating pathways involving EGFR and PI3K that are already major targets in the design of therapeutics in cancer [29, 40, 41]. This lends further to the idea that nutrition, specifically dietary modifications of PUFAs, is an area that should be considered in the treatment of cancer. With Western diets containing so much LA [5], modifying the ratio of omega-6:omega-3 PUFAs could be of critical importance in successfully treating the disease.

Acknowledgments

We would like to thank Eastern Star, the Women's Auxiliary of the Veterans of Foreign Wars and the Stout Foundation for their generous support of this work. Contributions from David Hall also helped fund this research. Finally, the hard work and expertise of Lani Pardini on the animal studies was greatly appreciated as well as the technical help of Clarissa Martins.

The project described was also funded in part by grants from the National Center for Research Resources (5P20RR016464) and the National Institute of General Medical Sciences (8P20GM103440).

REFERENCES

- [1]. Pardini RS. Nutritional intervention with omega-3 fatty acids enhances tumor response to anti-neoplastic agents. *Chem Biol Interact.* 2006; 162:89–105. [PubMed: 16846596]

- [2]. Chapkin RS, Seo J, McMurray DN, Lupton JR. Mechanisms by which docosahexaenoic acid and related fatty acids reduce colon cancer risk and inflammatory disorders of the intestine. *Chem Phys Lipids*. 2008; 153:14–23. [PubMed: 18346463]
- [3]. Bang HO, Dyeberg J, Hjoorne N. The composition of food consumed by Greenland Eskimos. *Acta Med Scand*. 1976; 200:69–73. [PubMed: 961471]
- [4]. Dunn JE. Cancer epidemiology in populations of the United States-with emphasis on Hawaii and California-and Japan. *Cancer Res*. 1975; 35:3240–3245. [PubMed: 1192400]
- [5]. Willett WC. Specific fatty acids and risks of breast and prostate cancer: dietary intake. *Am J Clin Nutr*. 1997; 66:1557S–15563S. [PubMed: 9394715]
- [6]. Welsch CW. Relationship between dietary fat and experimental mammary tumorigenesis: a review and critique. *Cancer Res*. 1992; 52:2040S–2048S. [PubMed: 1544139]
- [7]. Rose DP. Dietary fat, fatty acids and breast cancer. *Breast Cancer*. 1997; 4:7–16. [PubMed: 11091570]
- [8]. Hudson EA, Beck SA, Tisdale MJ. Kinetics of the inhibition of tumour growth in mice by eicosapentaenoic acid-reversal by linoleic acid. *Biochem Pharmacol*. 1993; 45:2189–2194. [PubMed: 8517859]
- [9]. Connolly JM, Coleman M, Rose DP. Effects of dietary fatty acids on DU145 human prostate cancer cell growth in athymic nude mice. *Nutr Cancer*. 1997; 29:114–119. [PubMed: 9427973]
- [10]. Hillyard LA, Abraham S. Effect of dietary polyunsaturated fatty acids on growth of mammary adenocarcinomas in mice and rats. *Cancer Res*. 1979; 34:4430–4437. [PubMed: 498074]
- [11]. Carter CA, Millholland RJ, Shea W, Ip MM. Effect of the prostaglandin synthesis inhibitor indomethacin on 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis in rats fed different levels of fat. *Cancer Res*. 1983; 43:3559–3562. [PubMed: 6407750]
- [12]. Abou-El-Ela SH, Prasse KW, Carroll R, Wade AE, Dharwadkar S, Bruce OR. Eicosanoid syntheses in 7,12-dimethylbenz(a)anthracene-induced mammary carcinomas in Sprague-Dawley rats fed primrose oil, menhaden oil or corn oil. *Lipids*. 1988; 23:948–954. [PubMed: 3143882]
- [13]. Kitigawa H, Noguchi M. Comparative effects of piroxicam and esculetin on incidence, proliferation and cell kinetics of mammary carcinomas induced by 7,12-dimethylbenz(a)anthracene on rats on high and low fat diets. *Oncology*. 1995; 51:407–410.
- [14]. Wymann MP, Schneider R. Lipid signaling in disease. *Nat Rev Mol Cell Biol*. 2008; 9:162–176. [PubMed: 18216772]
- [15]. Noguchi M, Rose DP, Earashi M, Miyazaki I. The role of fatty acids and eicosanoid synthesis inhibitor in breast carcinoma. *Oncology*. 1995; 52:265–271. [PubMed: 7777237]
- [16]. Schrey MP, Patel KV. Prostaglandin E2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. *Br J Cancer*. 1995; 72:1412–1419. [PubMed: 8519653]
- [17]. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med*. 2002; 8:289–293. [PubMed: 11875501]
- [18]. Kikawa KD, Herrick JS, Tateo RE, Mouradian M, Tay JS, Pardini RS. Induced oxidative stress and cell death in the A549 lung adenocarcinoma cell line is enhanced by supplementation with docosahexaenoic acid. *Nutr Cancer*. 2010; 62:1017–1024. [PubMed: 21058189]
- [19]. Rogers KR, Kikawa KD, Mouradian M, Hernandez K, McKinnon KM, Ahwah SM, Pardini RS. Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association. *Carcinogenesis*. 2010; 31:1523–1530. [PubMed: 20525880]
- [20]. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol*. 2000; 132:365–386. [PubMed: 10547847]
- [21]. de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW, Span PN. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Lab Invest*. 2005; 85:154–159. [PubMed: 15543203]
- [22]. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]

- [23]. Connolly JM, Liu XH, Rose DP. Effects of dietary menhaden oil, soy, and a cyclooxygenase inhibitor on human breast cancer cell growth and metastasis in nude mice. *Nutr Cancer*. 1997; 29:48–54. [PubMed: 9383784]
- [24]. Lee SA, Kim HJ, Chang KC, Baek JC, Park JK, Shin JK, Choi WJ, Lee JH, Paik WY. DHA and EPA down-regulate COX-2 expression through suppression of NF-kappaB activity in LPS-treated human umbilical vein endothelial cells. *Korean J Physiol Pharmacol*. 2009; 13:301–307. [PubMed: 19885014]
- [25]. Legler DF, Bruckner M, Uetz-von Allmen E, Krause P. Prostaglandin E2 at new glance: novel insights in functional diversity offer therapeutic chances. *Int J Biochem Cell Biol*. 2010; 42:198–201. [PubMed: 19788928]
- [26]. Espinosa-Neira R, Mejia-Rangel J, Cortes-Reynosa P, Salazar EP. Linoleic acid induces an EMT-like process in mammary epithelial cells MCF10A. *Int J Biochem Cell Biol*. 2011; 43:1782–1791. [PubMed: 21945809]
- [27]. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling development and disease. *J Cell Biol*. 2006; 172:973–981. [PubMed: 16567498]
- [28]. Mattoon DR, Lamonthe B, Lax I, Schlessinger J. The docking protein Gab1 is the primary mediator of EGF-stimulated activation of the PI-3K/Akt cell survival pathway. *BMC Biol*. 2004; 18:12–24.
- [29]. Engelman JA. Targeting PI3K signaling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009; 9:550–562. [PubMed: 19629070]
- [30]. Sithanadam G, Smith GT, Fields JR, Fornwald LW, Anderson LM. Alternate paths from epidermal growth factor receptor to Akt in malignant versus nontransformed lung epithelial cells: ErbB3 versus Gab1. *Am J Respir Cell Mol Biol*. 2005; 33:390–399.
- [31]. Yart A, Mayeux P, Raynal P. Gab1, SHP-2 and other novel regulators of Ras: target for anticancer drug discovery? *Curr Cancer Drug Targets*. 2003; 3:177–192. [PubMed: 12769687]
- [32]. Scheaper U, Vogel R, Chmielowiec J, Huelsken J, Rosario M, Birchmeier W. Distinct requirements for Gab1 in Met and EGF receptor signaling in vivo. *Proc Natl Acad Sci*. 2007; 104:15376–15381. [PubMed: 17881575]
- [33]. Chen SY, Chen HC. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote growth factor-induced cell invasion. *Mol Cell Biol*. 2006; 26:5155–5167. [PubMed: 16782899]
- [34]. Rose DP, Connolly JM. Influence of dietary linoleic acid on experimental human breast cancer cell metastasis in athymic nude mice. *Int J Oncol*. 1998; 13:1179–1183. [PubMed: 9824628]
- [35]. Matsuoka T, Adair JE, Lih FB, His LC, Rubino M, Eling TE, Tomer KB, Yashiro M, Hirakawa K, Olden K, Roberts JD. Elevated dietary linoleic acid increase gastric carcinoma cell invasion and metastasis in mice. *Br J Cancer*. 2010; 103:1182–1191. [PubMed: 20842125]
- [36]. Goormachtigh G, Ji Z, Le Goff A, Fafeur V. Degradation of the Gab1 adaptor by the ubiquitin-proteasome pathway hampers HGF/SF-Met signaling. *Biochem Biophys Res Comm*. 2011; 411:780–785. [PubMed: 21782801]
- [37]. Ciardiello F, Kim N, McGeady ML, Liscia DS, Saeki T, Bianco C, Salomon DS. Expression of transforming growth factor alpha (TGF alpha) in breast cancer. *Ann Oncol*. 1991; 2:169–182. [PubMed: 2043488]
- [38]. Lin CQ, Dempsey PJ, Coffey RJ, Bissel MJ. Extracellular matrix regulates whey acidic protein gene expression by suppression of TGF-alpha in mouse mammary epithelial cells: studies in culture and in transgenic mice. *J Cell Biol*. 1995; 129:1115–1126. [PubMed: 7744960]
- [39]. Suzuki I, Iigo M, Ishikawa C, Kuhara T, Asamoto M, Kunimoto T, Moore MA, Yazawa K, Araki E, Tsuda H. Inhibitory effects of oleic and docosahexaenoic acids on lung metastasis by colon-carcinoma-26 cells are associated with reduced matrix metalloproteinase-2 and -9 activities. *Int J Cancer*. 1997; 73:607–612. [PubMed: 9389579]
- [40]. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004; 304:1497–1500. [PubMed: 15118125]

- [41]. Yan L, Beckman RA. Pharmacogenetics and pharmacogenomics in oncology therapeutic antibody development. *Biotechniques*. 2005; 39:565–368. [PubMed: 16235569]
- [42]. Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. *Lipids*. 2001; 36:1007–1024. [PubMed: 11724453]
- [43]. Harbige LS. Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. *Lipids*. 2003; 38:323–341. [PubMed: 12848277]
- [44]. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med (Maywood)*. 2008; 233:674–688. [PubMed: 18408140]
- [45]. Johnson GH, Fritsche K. Effect of dietary linoleic acid on markers of inflammation in healthy persons: a systematic review of randomized controlled trials. *J Acad Nutr Diet*. 2012; 112:1029–41. 1041.e1–15. [PubMed: 22889633]
- [46]. Fritsche KL. Too much linoleic acid promotes inflammation-doesn't it? *Prostaglandins Leukot Essent Fatty Acids*. 2008; 79:173–5. [PubMed: 18990555]
- [47]. 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]

Figure 1.

BT-474 and A549 cells were plated in 96-well plates before being treated with indicated concentrations of linoleic acid (LA; C18:2 n-6), docosahexaenoic acid (DHA; C22:6 n-3) or a control volume of EtOH for 48 hours. XTT assays were then performed to assess cell proliferation levels. All experiments represent at least n=3. Statistical significance (P < 0.05) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH). Redundant symbols were not shown.

Figure 2.

BT-474 and A549 cells were plated in 6-well plates before being treated with 100 μ M of LA, DHA or a control volume of EtOH for 48 hours. Cells were then counted and assessed for viability, then normalized and processed according to kit instructions to assess **A.** Cyclooxygenase (COX) activity or **B.** Prostaglandin E2 (PGE2) levels. **C.** BT-474 and A549 cells were plated in 96-well plates before being treated with 100 μ M of LA, DHA or a control volume of EtOH with or without exogenous PGE2 or a COX-2 inhibitor for 48 hours. XTT assays were then performed to assess cell proliferation levels. All experiments represent at least n=3. Statistical significance (P 0.05) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); ! (versus LA); # (versus DHA). Redundant symbols were not shown.

Figure 3.

A. BT-474 and A549 cells were plated in 6-well plates before being treated with 100 μ M of LA, DHA or a control volume of EtOH for 48 hours. Cells were then processed according to kit instructions to assess TGF- α protein levels. Statistical significance ($P < 0.05$) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); ! (versus LA). Redundant symbols were not shown. **B.** BT-474 and A549 cells were plated in 100 mm plates, then treated with 100 μ M of LA, DHA or a control volume of EtOH for 48 hours before being lysed, resolved by polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose or PVDF membranes, then probed for antibodies specific to MMP-2, MMP-9 and β -actin.

Figure 4.

A. BT-474 and A549 cells were plated, then treated with 100 μ M of LA, DHA or a control volume of EtOH for 48 hours before being lysed, resolved by PAGE transferred to nitrocellulose or PVDF membranes, then probed for antibodies specific to Gab1, EGFR, pAkt, Akt and β -actin. For immunoprecipitations, 500 μ g samples were incubated with recommended amounts of antibody overnight before the addition of protein G agarose for 2 hours. Samples were then washed 3x and resolved by PAGE, transferred to nitrocellulose or PVDF membranes, then probed for antibodies specific to Gab1 and EGFR. **B.** BT-474 and A549 cells were plated in 6-well plates, then treated with 100 μ M of LA, DHA or a control volume of EtOH for 48 hours before total RNA was isolated and purified using RNeasy mini kit. cDNA was then synthesized using cDNA Synthesis VILO kit. Quantities of cDNA were measured by quantitative real-time PCR on a CFX96 Real-Time PCR System. All experiments represent at least n=3. Statistical significance (P 0.05) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); ! (versus LA). Redundant symbols were not shown.

Figure 5.

A. BT-474 and A549 cells were plated in 100 mm plates, then treated with 100 μ M of LA, DHA or a control volume of EtOH for 24 hours before treatment with an EGFR inhibitor (PD153035), an inhibitor of PI3K (LY294002) or DMSO vehicle for an additional 24 hours. Cells were then lysed, resolved by PAGE, transferred to nitrocellulose or PVDF membranes, then probed for antibodies specific to Gab1, EGFR, pAkt, Akt and β -actin. For immunoprecipitations, 500 μ g samples were incubated with recommended amounts of antibody overnight before the addition of protein G agarose for 2 hours. Samples were then washed 3x and resolved by PAGE, transferred to nitrocellulose or PVDF membranes, then probed for antibodies specific to Gab1 and EGFR. **B.** BT-474 cells were plated in 96-well plates before being treated with 100 μ M concentrations of LA, DHA or a control volume of EtOH for 24 hours before treatment with EGFR inhibitor (PD153035), an inhibitor of PI3K (LY294002) or DMSO for an additional 24 hours. XTT assays were then performed to assess cell proliferation levels. All experiments represent at least n=3. Statistical significance (P 0.05) between treatments was determined using ANOVA and is indicated as followed: * (versus DMSO); ! (versus PD153035); # (versus EtOH); & (versus LA). Redundant symbols were not shown.

Figure 6.

Adult male NCr (nu/nu) athymic nude mice were xenografted with the A549 human lung adenocarcinoma cell line and fed the indicated experimental diets (Table 1) [19]. **A.** Three xenografts from each dietary group were homogenized, resolved by PAGE, transferred to nitrocellulose or PVDF membranes, then probed for antibodies specific to Gab1, EGFR, pAkt, Akt and β -actin. For immunoprecipitations, 500 μ g samples were incubated with recommended amounts of antibody overnight before the addition of protein G agarose for 2 hours. Samples were then washed 3x and resolved by PAGE, transferred to nitrocellulose or PVDF membranes, then probed for antibodies specific to Gab1 and EGFR. Sera was collected from the animals and analyzed for **B.** PGE2 and **C.** TGF- α levels. Statistical significance ($P < 0.05$) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); ! (versus LA). Redundant symbols were not shown.

Figure 7.

BT-474 and A549 cells were plated and treated with pools of either nonspecific (NS) siRNA or pools specific to Gab1 for 24 hours before the addition of 100 μ M concentrations of LA, DHA or a control volume of EtOH to the culture media. **A.** Cells were then lysed, resolved by PAGE, transferred to nitrocellulose or PVDF membranes, then probed for antibodies specific to Gab1, pAkt, Akt and β -actin or **B.** harvested with trypsin and counted using a hemocytometer and trypan blue staining. All experiments represent at least $n=3$. Statistical significance ($P < 0.05$) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); ! (versus LA). Redundant symbols were not shown.

Figure 8.

E10, E9, C10 and A5 cells were plated in 96-well plates before being treated with specific concentrations of LA, DHA or a control volume of EtOH for 48 hours. XTT assays were then performed to assess cell proliferation levels. All experiments represent at least $n=3$. Statistical significance ($P < 0.05$) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH). Redundant symbols were not shown.

Figure 9.

Model for tumor promoting linoleic acid (LA; C18:2 n-6)-induced signaling cascade. LA supplementation increases cyclooxygenase-2 (COX-2) activity, prostaglandin E₂ (PGE₂) synthesis, which leads to increases in gelatinases, MMP-2 and MMP-9, and transforming growth factor alpha (TGF-α) levels. LA supplementation further induces EGFR/Gab1 association and downstream phosphatidylinositol-3-kinase (PI3K) and Akt signaling, thus increasing cell survival and proliferation.

Table 1Composition of animal diets in % component/kcal/mouse/day^a

Component	8%CO (g)	24%CO (g)	8%CO + 16%DO (g)
Com oil	29.60	73.85	24.62
Dhasco™ oil	0.00	0.00	49.23
AIN-93M mineral mix	13.65	13.69	13.69
L-cystine	0.70	0.70	0.70
choline	0.98	0.98	0.98
AIN-93 vitamin mix	3.90	3.91	3.91
casein	54.61	54.76	54.76
cellulose	19.50	19.56	19.56
comstarch	159.64	90.63	90.63
dyetrose	53.13	30.17	30.17
sucrose	34.28	19.46	19.46
Total Diet	369.99	307.71	307.71

^aCO: Corn Oil, DO: Dhasco™ Oil. Described previously [19].

Table 2

Percent total fatty acid content^a

Fatty Acids	BT-474 Breast				A549 Lung			
	EtOH	100 μ M LA	100 μ M DHA	EtOH	100 μ M LA	100 μ M DHA	100 μ M LA	100 μ M DHA
16:1 - Palmitic n-7	10.3 $\pm$ 3.2	7.5 $\pm$ 3.1	3.6 $\pm$ 0.3	4.9 $\pm$ 0.8	2.7 $\pm$ 1.1	4.8 $\pm$ 2.9		
18:0 - Stearic	24.3 $\pm$ 2.5	21.6 $\pm$ 1.4	15.7 $\pm$ 3.4	21.1 $\pm$ 3.0	13.6 $\pm$ 0.5	15.3 $\pm$ 2.3		
18:1 - Oleic n-9	42.5 $\pm$ 4.1	22.1 $\pm$ 3.9	18.1 $\pm$ 4.5	38.3 $\pm$ 1.3	22.1 $\pm$ 0.5	22.0 $\pm$ 2.9		
18:2 - Linoleic n-6	17.0 $\pm$ 1.0	46.7 $\pm$ 4.4	9.2 $\pm$ 2.8	3.8 $\pm$ 0.8	38.6 $\pm$ 1.2	4.1 $\pm$ 0.9		
18:3 - Linolenic n-3	0.3 $\pm$ 0.6	0.2 $\pm$ 0.2	1.4 $\pm$ 1.9	0.0 $\pm$ 0.0	0.2 $\pm$ 0.1	1.4 $\pm$ 2.4		
20:1 - Eicosenoic n-9	1.1 $\pm$ 1.7	0.3 $\pm$ 0.0	1.0 $\pm$ 1.6	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1	3.1 $\pm$ 1.1		
20:2 - Eicosadienoic n-6	0.9 $\pm$ 0.6	0.0 $\pm$ 0.0	0.4 $\pm$ 0.7	0.9 $\pm$ 0.1	0.9 $\pm$ 0.5	2.0 $\pm$ 1.2		
20:3 - Eicostrienoic n-3	0.8 $\pm$ 0.2	0.0 $\pm$ 0.0	0.3 $\pm$ 0.3	0.8 $\pm$ 0.3	0.4 $\pm$ 0.4	1.5 $\pm$ 0.8		
20:4 - Arachidonic n-6	2.5 $\pm$ 0.3	1.6 $\pm$ 0.2	1.8 $\pm$ 0.3	24.5 $\pm$ 1.8	17.8 $\pm$ 1.7	8.5 $\pm$ 0.5		
20:5 - EPA n-3	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0	4.3 $\pm$ 1.4	1.1 $\pm$ 0.3	0.8 $\pm$ 0.5	3.8 $\pm$ 0.6		
22:6 - DHA n-3	0.2 $\pm$ 0.1	0.0 $\pm$ 0.0	4.3 $\pm$ 1.4	1.1 $\pm$ 0.3	2.4 $\pm$ 0.1	33.5 $\pm$ 5.8		
%Total n-6	20.4	48.4	11.4	29.3	57.4	14.3		
%Total n-3	1.5	0.2	50.2	6.0	3.8	40.2		

^a Abbreviations are as follows: EtOH, ethanol; LA, linoleic acid; DHA, docosahexaenoic acid. Results are presented as percentage total fatty acid $\pm$ SD (n=3) for BT-474 human breast ductal carcinoma and A549 human lung adenocarcinoma. Cells were treated with 100 μ M PUFA FAME for 48 hours before being resolved by gas chromatography. Additionally, total n-6 and n-3 PUFA content is listed for each treatment and cell line.