

Warning Concerning Copyright Restrictions

The Copyright Law of the United States (Title 17, United States Code) governs the making of photocopies or other reproductions of copyrighted materials.

Under certain conditions specified in the law, libraries and archives are authorized to furnish a photocopy or other reproduction. One of these specified conditions is that the photocopy or reproduction is not to be used for any purpose other than private study, scholarship, or research. If electronic transmission of reserve material is used for purposes in excess of what constitutes "fair use," that user may be liable for copyright infringement.

University of Nevada, Reno

Response to docosahexaenoic acid treatment in genetically matched malignant and nontransformed lung epithelial cells

A thesis submitted in partial fulfillment of the
requirements for the degree of
Bachelor of Science in Biochemistry and Molecular Biology and the Honors Program

by
Kristen L. Spencer
Dr. Ronald S. Pardini, Thesis Advisor
May, 2011

**UNIVERSITY
OF NEVADA
RENO**

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

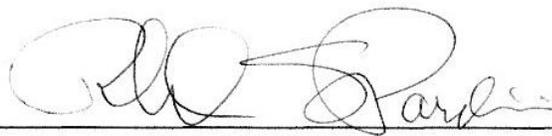
KRISTEN LINA SPENCER

entitled

**Response to docosahexaenoic acid treatment in genetically matched malignant and
nontransformed lung epithelial cells**

be accepted in partial fulfillment of the
requirements for the degree of

BACHELOR OF SCIENCE



Ronald S. Pardini, Ph.D., Faculty Mentor

Tamara Valentine, Ph.D., Director, **Honors Program**

Abstract

DHA is an omega-3 polyunsaturated fatty acid established to have anti-cancer effects in a variety of models of cancer. The consequences of DHA supplementation on malignant cells has been widely studied, though little has been done on nontransformed cells. In the current study, an evaluation of DHA's effect on two genetically matched pairs of malignant and nontransformed lung epithelial cell lines is proposed. Current data suggests the two pairs of cell lines show significant differences in how well they respond to DHA treatment. Cell signaling analysis showed a differential response between the neoplastic and nonneoplastic cell lines. Preliminary data showed the nontransformed and transformed cell lines to have a decrease in proliferation when treated with high doses of DHA. In addition, lipid peroxidation as an indicator of oxidative stress was confirmed upon treatment with docosahexaenoic acid. Further identifying the mechanisms of action is critical to understanding how response to DHA can be enhanced in tumors, while improving the health of normal tissues.

Acknowledgement

Gratitude must be paid to Dr. Ronald S. Pardini for permitting me to conduct research under his tutelage, to Dr. Keith D. Kikawa for his valuable advice as a mentor, and to my loved ones and family for their infinite support. Generous financial contributions to fund this research were made by the Idea Network of Biomedical Research Excellence and the Office of Undergraduate and Interdisciplinary Research through the Honors Program.

Table of Content

Abstract	i
Acknowledgements	ii
Table of Contents	iii
List of Figures	iv
Introduction	1
Methods	3
Results	6
Discussion	9
Figures	12
References	14

List of Figures

Cellular Proliferation	12
Lipid Peroxidation with Manual Cell Counts	12
Fatty Acid Analysis	13
Protein Analysis	13

Introduction:

Epidemiological studies from the 1970's initially spurred interest in diets rich in omega-3 polyunsaturated fatty acids (PUFAs), because populations consuming high amounts of these PUFAs demonstrated lower incidence of both cancer and cardiovascular disease [1]. Since then, a variety of research has been done on omega-3 PUFAs, most notably docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), to determine how they exert their anti-cancer effects. These outcomes have been attributed to a variety of mechanisms, including alterations in signal transduction, eicosanoid synthesis, membrane composition, and increases in reactive oxygen species [2]. DHA is particularly interesting, as it is the longest and most unsaturated fatty acid found normally in biological membranes having 22 carbons with 6 double bonds [3].

While a large amount of research has been done into how DHA affects malignant tumor models, very little work has been done on DHA's impact on normal tissues. Interestingly, DHA has been implicated in protecting normal cells from traditional anti-cancer treatments clinically, including radiation therapy and certain drugs [4]. Understanding how DHA may be exerting these very different roles in tumors and normal tissue could provide valuable insight into how DHA might be effectively used as a combination therapeutic to enhance clinical outcomes with traditional treatments.

In the current study, we will use two pairs of genetically matched malignant and nontransformed lung epithelial cells E10/E9 and C10/A5 (with E9 and A5 being the malignant cell lines) to evaluate how DHA treatment influences their growth and signaling [5]. In order to develop these cell lines, researchers previously explanted lungs from a BALB/c mouse into culture, cloned, passaged them, and confirmed them to be

non-tumorigenic cells [6]. Upon treatment with dexamethasone, an immunosuppressant, they saw inhibited growth in most colonies but resistance in some. The resistant colonies were then isolated, cloned and characterized. Interestingly, upon subcutaneous injection to BALB/c mice these resistant clones produced tumors as determined by the cellular invasion of adjacent adipose and muscle tissue [6]. As genetic derivatives, minimal differences are present between the sibling pairs. The transformed cells have a mutated *k-Ras* allele which provides for an increase in mRNA expression. The Ras pathway is important in the transmission of growth-promoting signals from cell surface receptors, eventually travelling to the nucleus where these signals affect the production and regulation of other influential proteins [7]. Mutated p53 is also a hallmark of different cancers and is mutated in the E9, and A5 transformed lines affecting proliferative and apoptotic events [8]. Overall, these neoplastic and nonneoplastic transformants represent sibling pairs, and their genetic similarity provides for germane conclusions about the anti-cancer affects exhibited by treatment with polyunsaturated fatty acids.

Numerous investigations have identified several mechanisms of action that DHA may be using to accomplish this affect including alterations in cell signaling, differential eicosanoid production, oxidative stress mechanism, and alterations in membrane fluidity. The potential methods of DHA's anti-cancer trends have been identified in malignant human models, but more research must be completed before it can be definitively proclaimed [9]. This work will comprehensively analyze the previously identified trends using the genetically matched derivatives in order to confirm or deny the role that fatty acid treatment may have in tumorigenesis.

Methods:

Cell Lines and Reagents- E9 and E10 transformed and nontransformed murine lung epithelial cells were obtained from Dr. A. Malkinson. Fatty acid methyl esters (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.

Cell Culture- E9 and E10 cells were maintained in CRML-1066 (Mediatech, Manassas, VA) and the A5 and C10 cells were maintained in DMEM 1x (Mediatech, Manassas, VA). Both medias were supplemented with 10% FBS (Atlanta Biological, Atlanta, GA), 1% L-glutamine, and a mix of glucose, HEPES, and sodium pyruvate. Cells were grown in single layer suspension at 37°C in a humidified environment with 5% CO₂.

Cell Viability Assays- Cell proliferation kit II (XTT) was performed on E9 and E10 cells after 48h treatment of DHA or LA at varying concentrations according to manufacturer protocol (Roche, Indianapolis, IN). 24 hours after plating, cultures were supplemented with either 150 uM, 100 uM, 75 uM, 50 uM, 25uM or 10 uM FAME of docosahexaenoic acid (DHA) and linoleic acid (LA), or an equal volume of ethanol (EtOH) was added. The production of formazon dye was measured spectrophotometrically at 450nm referencing absorbance at 650nm and directly correlated with viable cells. Manual cell counting was also performed following 75uM treatment as described above. The single cell suspensions were washed twice with sterile phosphate buffered saline (PBS) and

trypsinized. An equal volume of cells and trypan blue were mixed and counted using Hemacytometer counting method with percent differences relative to EtOH control.

Lipid Peroxidation Assay- Fatty acid treatment was conducted as previously described at 75uM and 100uM concentrations to be analyzed for lipid peroxidation. Vitamin E was also incorporated at 50 uM concentration. Dimethylsulfoxide was added in equal volume to groups not receiving Vitamin E. Cell counts were obtained using Hemacytometer (Hausser Scientific) counting method described above and normalized to constant cell concentration. GTP lysis buffer containing 20uL/ml PMSF, 20uL/ml Na₃VO₄, 10uL/ml Aprotinin, 10uL/ml NaF, and 10uL/ml protease cocktail was used in addition to manual displacement of cells through 26.5 gauge needle (Becton Dickinson & Co). Equal volumes of lysate and 0.67% Thiobarbituric Acid in 20% Trichloroacetic Acid were heated at 100°C for 20 minutes and placed on ice for 10 minutes. The samples were microcentrifuged for 5 minutes at 4°C. The supernatant was removed and measured spectrophotometrically at 538nm for malondialdehyde equivalents.

Protein Analysis- Cells cultured for Western blot analysis were scraped using GTP lysis buffer as described above. Cell lysates were mixed with 1x Laemmli sample buffer and loaded at the protein concentrations indicated in the figure legends on Tris-Glycine gels (Invitrogen): 6% gels for growth factor receptor; 10% gels for β -actin and electrophoresed at 100V. After transfer to nitrocellulose or PVDF (BioRad Laboratories) membranes, the blots were blocked in 5% dried milk for 2h at room temperature and probed overnight with antibodies at 1:1000 or 1:2000 dilution for growth factors and

HIF-1 α respectively. The appropriate secondary antibody was applied and an ECL kit (Amersham) was used for the detection of the bound antibody. For reprobing with another antibody, blots were stripped in buffer containing 100 mM β -mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 62.5 mM Tris for 30 minutes at 50°C and washed 3 x 10 minutes with Tris buffered saline with 0.05% Tween-20.

Fatty Acid Analysis- C10 and A5 cells were supplemented with docosahexaenoic acid, linoleic acid, or ethanol control at 100 μ M for 48h and treated with 150ul freshly completed GTP-lysis buffer as previously described along with 0.02% butylated hydroxytoulene (BHT). Cell homogenate was fatty acid extracted after addition of a 19:0 standard. Aliquots of organic solvents methanol (500ul) and methanolic hydrochloric acid (1,500ul) were applied to the samples and followed by a nitrogen flush. The samples incubated for 2h at 95°C and cooled to room temperature while drying with nitrogen. 0.88% sodium chloride and hexane were added to the samples. Nitrogen flushed the test tube and the samples were centrifuged at 1,000 x g for 5 min.. The hexane layer was removed to a clean test tube and another hexane layer was added, centrifuged as described and removed. The pooled hexane fractions were stored at -20°C until further use. An Agilent Technologies 6890N Chromatograph, containing an SP2340 capillary column (Supelco, St. Louis, MO), was used. A temperature program of a total time of 20 min. with a starting temperature of 120°C was formulated. The injector was set at 245°C and the flame ionization detector at 250°C. The program was modified so that Rate 1 was set at 5°C/min to 160°C and Rate 2 was set at 10°C/min to 240°C and held at 240°C for 4 min. Values of each fatty acid are reported as percentage of total fatty acids.

Results:

Two methods were used to determine DHA's effect on cellular proliferation: Trypan blue staining for viable cells and XTT Assay for metabolically active cells. Interestingly, some variance was observed between the two experiment types while determining proliferation. A measurable decrease in cellular proliferation was observed in the nontransformed and transformed pair E10/E9 after treatment with docosahexaenoic acid (DHA 22:6) at high concentrations using both methods (Fig. 1A, 2C). This effect was observed at varying PUFA concentrations in both cell lines with the transformed component exhibiting an IC₅₀ at 100 μ M DHA supplementation. The nontransformed cell line, E10, shows a higher decrease in proliferation at the same concentration of fatty acid. The C10/A5 nontransformed and transformed pair also showed a response to PUFA treatment. Supplementation with linoleic acid (LA 18:2) and DHA at 100 μ M were applied as well as an equal volume of ethanol control. The nontransformed-C10 cell line showed a 53.05% decrease in proliferation versus ethanol control when treated with DHA. Treatment with linoleic acid showed an increase of 122.18% in proliferation in the A5 neoplastic line. The A5 cell line also showed an increase in proliferation upon DHA treatment using the XTT method (Fig. 1B). This data contradicts the measured cell viability through Trypan blue staining with manual cell counts. The secondary method showed a decrease in the number of viable A5 cells when treated with DHA (Fig. 2D). Despite contradictions in preliminary data, this information may provide insight to the validity of the murine lung epithelial response to fatty acid treatment and its correlation with previously published data on immortalized human carcinoma cell lines.

Lipid peroxidation, an indicator of oxidative stress, was confirmed upon treatment with DHA in the genetically matched transformants (Fig. 2A, B). The nontransformed E10 cell line exhibited the highest amount of lipid peroxidation at 212.38% malondialdehyde (MDA) formed relative to EtOH control (Fig. 2A). Increases in MDA were still observed in the other cell lines in lesser amounts after treatment with DHA at 100 μ M (Fig. 2A, B). Vitamin E, or α -tocopherol, an essential nutrient, was used in this experiment for its function in counteracting lipid peroxidation by removing lipid radicals thus protecting the lipid membranes from damaging oxidation. It was shown to decrease the lipid peroxidation when combination treatment of DHA and Vitamin E was used (Fig. 2A, B). Manual cell counts were completed to verify previously reported cellular proliferation data. Hemacytometer counting method after Trypan blue staining was used. In the transformed and nontransformed E10/E9 sibling pair, a decrease in viable cells was observed in a dose dependent correlation based on increasing concentration of DHA treatment (Fig. 2C). The experiment was repeated in the C10/A5 cell lines, and the dose dependent decrease in viable cells was confirmed (Fig. 2D). In addition, upon treatment with linoleic acid an increase in viable cells relative to ethanol control was observed in both C10/A5 transformants (Fig. 2D). It has been observed when cellular proliferation is decreased by DHA treatment lipid peroxidation is increased; this may provide insight to one aspect of the anti-cancer mechanism of action that DHA takes.

To confirm fatty acid incorporation into the membrane, a hexane fatty acid extraction was completed as well as analysis with gas chromatography. The presence of DHA was confirmed to incorporate at high levels in the cell upon 100 μ M treatment in

the A5 and C10 cell lines (Fig. 3). In addition, linoleic acid was confirmed to incorporate at higher levels into the membrane (Fig. 3). Data must be repeated before the fatty acid composition trends can be definitively described. The presence of arachidonic acid was approximately three fold higher in the nontransformed C10 cell line compared to the transformed A5 cell line when treated with docosahexaenoic acid (Fig. 3). As arachidonic acid is a key inflammatory intermediate, it may be inferred that the nontransformed cell line is altering eicosanoid production in response to the fatty acid supplementation.

Epidermal growth factor receptor (EGFR) is a key membrane bound protein known previously to be affected by DHA supplementation through alterations of the membrane microdomain excluding EGFR from lipid rafts [10]. In a variety of immortalized human lines such as A549 lung adenocarcinoma, WiDr colon carcinoma, and MDA-MB-231 breast carcinoma, there has been demonstrated promise for combination therapy between EGFR inhibitors and DHA. Its downstream signals can alter cellular proliferation, apoptosis, and necrosis along with translocation of molecules to the nucleus affecting DNA replication specifically through the Ras/MAPK signaling cascade. EGFR is overexpressed in a variety of cancers and is a current target in numerous clinical therapeutics. We have used Western blot techniques to probe for activation of EGFR as shown by phosphorylation at the Y1068 residue. In the C10/A5 sibling pair, there is a marked decrease in signal upon treatment with DHA when compared to EtOH or linoleic acid (Fig. 4). In addition, there is an absence of expression of total EGFR in the DHA treated cells. In the nontransformed C10 cell line, EGFR

appears to have an increase in expression when treated with 100 μ M linoleic acid (Fig. 4). Hypoxia inducible factor (HIF-1 α) is a protein found in mammalian cells under reduced oxygen environments which plays an essential role in maintaining cellular and systemic homeostatic responses. Intratumoral hypoxia and genetic alterations can lead to HIF-1 α overexpression, which has been correlated with increased patient mortality in a variety of cancers [11]. Treatment with DHA resulted in a downregulation of HIF-1 α expression (Fig. 4). While these trends may provide insight to the alterations in cellular signaling, more probing must be completed in order to definitively explain the molecular interactions.

Discussion:

This study demonstrates the differential effect that DHA supplementation has *in vitro* on two genetically matched pairs of murine lung epithelial cells. DHA supplementation has not previously been investigated in these cell lines. The conclusions drawn provide to be specifically germane as there are minimal genetic differences between the sibling pairs apart from p53 and k-Ras mutations influencing metastasis. The data presented here establishes treatment with docosahexaenoic acid to result in decreases in proliferation in the nontransformed and transformed E10/E9 cell lines as well as the transformed C10 cell line (Fig. 1). Furthermore, lipid peroxidation was confirmed to correlate with a decrease in viable cells upon treatment with DHA (Fig. 2). This inhibition can be attributed to the increase of oxidative stress observed. As DHA is the longest, unsaturated fatty acid normally found in biological membranes, its high

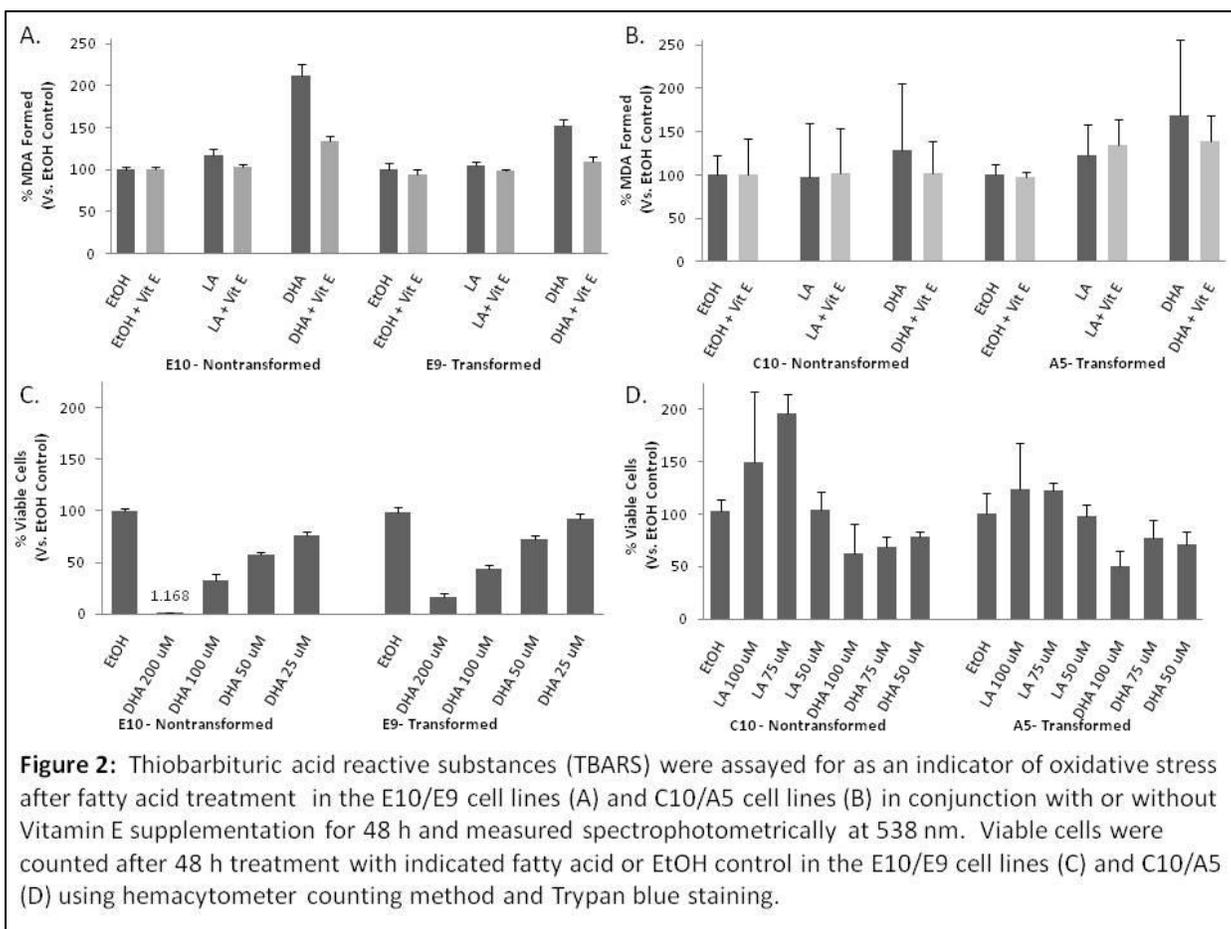
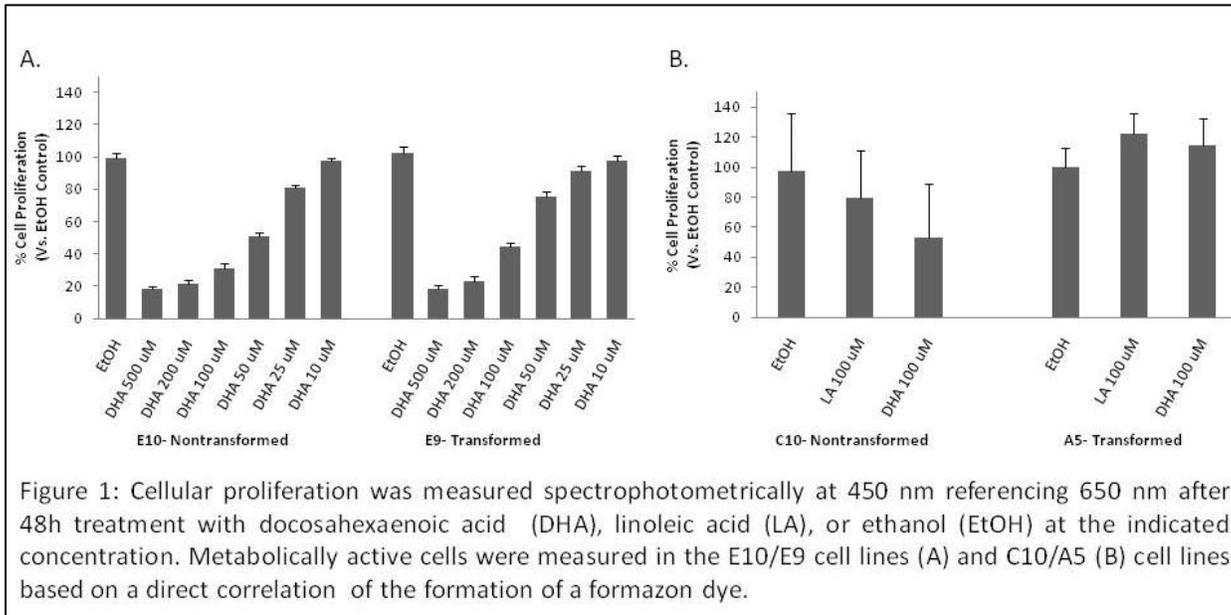
number of conjugations presents itself as a target of peroxidation. Vitamin E demonstrated its known antioxidant properties by counteracting the oxidative stress presented in DHA treatment and decreasing lipid peroxidation (Fig. 2). Interestingly, lipid peroxidation in the matched E10/E9 pair provided to be one of the few differential responses elicited between the sibling pairs. The E10 cell line produced increased amounts of malondialdehyde and seemingly had less antioxidant sensitivity. It will be of the best interest to complete the Oxygen Radical Absorbance Capacity (ORAC) Assay to directly analyze at the antioxidant effects present in relation to DHA treatment.

Furthermore, cell signaling analysis showed a differential response upon supplementation with DHA. EGFR Y1068, which is almost universally increased in transformed human cell lines upon DHA supplementation, is decreased in the murine normal and neoplastic cells (Fig. 4). Phosphorylation at this residue results in activation of the protein. EGFR is a crucial membrane bound protein that affects the Ras/MAPK pathway as well as PI3K affecting cellular proliferation and apoptotic events. Decreases in HIF-1 α were also observed upon supplementation with DHA (Fig. 4). Traditionally in other cancer models, HIF-1 α is consistently overexpressed. The downregulation of this crucial protein provides insight into the alterations in cell signalling due to treatment with DHA. More probing must be completed in order to identify the interactions with other proteins that affect cell death or survival. In addition, fatty acid analysis showed to DHA incorporate into the membrane, which is where it initiates its anticancer effects (Fig.4).

The mechanisms of action of DHA are many and much work is still to be done to definitively elucidate which facets of tumorigenesis are altered. DHA has been previously shown to elicit a protective effect on normal tissue when used in clinical

anticancer therapies [11]. Nutritional supplementation as a non-invasive addition to traditional anti-cancer therapies may provide an amplified decrease in tumorigenesis [12]. Further investigations must be conducted to determine if murine carcinomas are exhibiting different biochemical properties than human lines. Identifying differences in how these matched cell lines respond to DHA supplementation to understand how DHA can enhance response of tumors, while improving the health of normal tissues in the clinic, is of great importance.

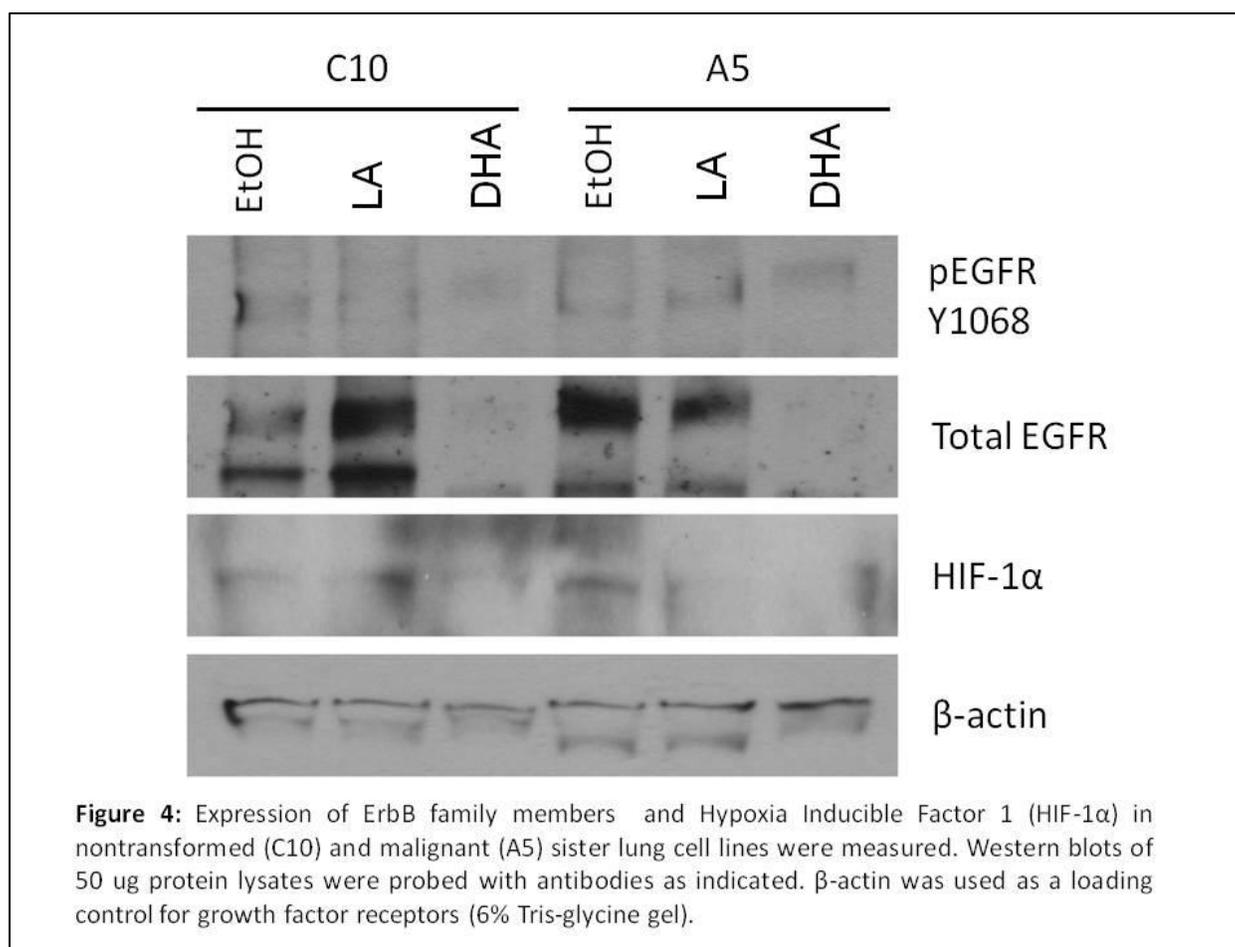
Figures:



Figures (continued):

Percentage Total Fatty Acid Content ^a						
Fatty Acids	C10-Nontransformed			A5-Transformed		
	EtOH	LA	DHA	EtOH	LA	DHA
16:1 - Palmiticn-7	3.9 ± 0.9	4.1 ± 0.6	4.0 ± 0.8	2.8 ± 0.1	2.2 ± 0.6	0.0 ± 0.0
18:0 - Stearic	7.9 ± 2.3	7.5 ± 0.1	39.9 ± 9.8	14.1 ± 7.6	15.8 ± 6.6	27.5 ± 17.9
18:1 - Oleicn-9	45.4 ± 2.0	48.2 ± 1.1	18.0 ± 17.1	48.8 ± 1.3	43.6 ± 7.2	32.9 ± 12.8
18:2 - Linoleicn-6	30.1 ± 1.6	33.1 ± 2.0	23.0 ± 29.6	24.3 ± 9.8	27.9 ± 2.5	20.0 ± 9.3
18:3 - Linolenicn-3	3.4 ± 2.0	2.3 ± 0.4	1.5 ± 2.1	2.1 ± 1.5	2.2 ± 0.9	2.1 ± 2.9
20:1 - Eicosenoicn-9	0.7 ± 0.3	1.5 ± 0.4	1.2 ± 0.1	0.3 ± 0.5	2.2 ± 1.8	0.7 ± 0.9
20:2 - Eicosadienoicn-6	0.8 ± 0.4	0.2 ± 0.1	1.5 ± 0.5	0.2 ± 0.3	0.7 ± 0.3	2.2 ± 0.2
20:4 - Arachidonocn-6	3.0 ± 1.5	2.6 ± 0.6	3.6 ± 1.2	0.7 ± 1.0	2.3 ± 1.7	1.1 ± 1.5
22:6 - DHAn-3	4.8 ± 0.1	0.3 ± 0.3	7.9 ± 4.9	6.7 ± 4.2	3.2 ± 0.7	13.6 ± 9.3

Figure 3: ^aAbbreviations are as follows: EtOH, Ethanol; LA, Linoleic Acid; DHA, Docosahexaenoic Acid. Results are presented as percentage fatty acid content for C10-Nontransformed and A5-Transformed cell lines. All values are represented as percentage fatty acid relative to total fatty acid content ± SD (n=1).



References:

1. Armstrong, B., et. al. "Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices." *International Journal of Cancer* 15.4 (1975): 617-631.

2. Spencer, Laura, et al. "The effect of omega-3 FAs on tumour angiogenesis and their therapeutic potential." *European Journal of Cancer* 45.12 (2009): 2077-2086. *Academic Search Premier*. EBSCO. Web. 21 Feb. 2010.

3. Wassall, S.R., et. al. "A comparison of the effects of linolenic (18:3 omega 3) and docosahexaenoic (22:6 omega 3) acids on phospholipid bilayers." *Chemistry and Physics of Lipid* 54 (1990): 79-88.

4. Brand, A., et.al. "Membrane lipid modification by polyunsaturated fatty acids sensitizes oligodendroglial OLN-93 cells against oxidative stress and promotes upregulation of heme oxygenase-1 (HSP32)." *Journal of Neurochemistry* 76.3 (2010).

5. Sithanandam, Gunamani, et.al. "Alternate Paths from Epidermal Growth Factor Receptor to Akt in Malignant Versus Nontransformed Lung Epithelial Cells: ErbB3 Versus Gab1." *American Journal Respiration Cell Molecular Biology* 33 (2005): 490-499.

6. Malkinson AM, Dwyer-Nield LD, Rice PL, Dinsdale D. Mouse lung epithelial cell lines: tools for the study of differentiation and the neoplastic phenotype. *Toxicology* 1997;123:53–100.

7. Feldser DM, Kern SE. Oncogenic functionality of the dinucleotide KRAS2 mutations: G12F and GG12-13VC. *Human Mutation* 2001; 18:357.
8. Vries, A., Jacks, T. Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function. *PNAS* 2002; 99: 2948-2953
9. Semenza, Gregg. Targeting HIF-1 for Cancer therapy. *Nature Reviews Cancer* 2003; 3: 721-732 .
10. Rogers, K. R., Kikawa, K. D., Mouradian, M., Hernandez, K., McKinnon, K. M., Ahwah, S. M., & Pardini, R. S. (2010). Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association. *Carcinogenesis*, 31(9), 1523-1530.
11. Kikawa, K.D., Herrick, J.S., Tateo, R.E., Mouradian, M., Tay, J.S., Pardini, R.S. Induced oxidative stress and cell death in the A549 lung adenocarcinoma cell line by ionizing radiation is enhanced by supplementation with docosahexaenoic acid. *Nutrition and Cancer* 2010; 62; 1017-24
12. Pardini, R.S., et.al. Nutritional Intervention With Omega-3 Fatty Acids in a Case of Malignant Fibrous Histiocytoma of the Lungs. *Nutrition and Cancer* 2005; 52: 121-129.