University of Nevada, Reno

Effects of Docosahexaenoic Acid on TGF-ß and SMAD Proteins in COLO205 and WiDr Cells

A thesis submitted in partial fulfillment of the requirements of the degree of

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by

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ABSTRACT

Transforming growth factor beta (TGF-ß), a cytokine capable of altering cell growth and differentiation, as well as related transcription factors called SMADs, are among the proteins known to be modified by docosahexaenoic acid (DHA) treatment in T-cells. Unpublished data has indicated that these proteins may be modified by DHA in colon cancer cells. In the current project, a thorough investigation of the effects of DHA on TGF-ß and SMAD protein expression and activation in COLO205 and WiDr colon carcinomas, both of which show significant inhibition of cell proliferation upon DHA supplementation, will be examined using Western Blotting and ELISA. Overall, DHA does not appear to effect TGF-ß expression, but it may still play a role in down-regulating several SMAD proteins induced by TGF-ß and BMP signaling.

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INTRODUCTION

Unchecked cell growth is the essence of cancer. So much basic research has focused on how cancer cells escape normal growth controls. Scientists have turned their focus to a family of secreted growth regulatory proteins, known collectively as transforming growth factor-beta (TGF-ß), which are one of the few known classes of proteins that can inhibit cell growth.[1,2] The TGF-ß system is a basic pathway that provides a simple route for signals to pass from the extracellular environment to the nucleus and controls a plethora of cellular responses and figures prominently in animal development. In its normal state, the TGF-ß pathway restricts cell growth, differentiation, and cell death. When a healthy cell becomes cancerous, various components of the TGF-ß signaling pathway become mutated, which makes the newly cancerous cell resistant to the effects of normally functioning TGF-ß.[3,4] These resistant cells then grow without regulation. Previous research has shown that in colon cancer, one third of tumors have mutated TGF-ß receptors, and the remainder of the tumors have mutations in the signaling pathway activated by TGF-ß, both conditions that lead to unchecked proliferation.[4,5]

Recent cellular, biochemical, and structural studies have revealed significant insight into the mechanisms of the activation of TGF-ß receptors through the activation of SMAD proteins through phosphorylation, the transcriptional regulation of target gene expression, and the control of SMAD protein activity and degradation.[6] TGF-ß may induce apoptosis, programmed cell death, in numerous ways including the SMAD pathway.[6] The SMAD pathway is the canonical signaling pathway that TGF-β family members signal through. In this pathway, TGF-β dimers bind to a type II receptor which

recruits and phosphorylates a type I receptor. The type I receptor then recruits and phosphorylates a receptor regulated SMAD (R-SMAD), which consists of SMAD2 and SMAD3. The R-SMAD then binds to SMAD4 and forms a heterodimeric complex. This complex then enters the cell nucleus where it acts as a transcription factor for various genes.[7] Elements involved in cell proliferation regulation, which have been clearly shown to be controlled in part by TGF-ß, include: "the cyclin-associated proteins cyclin D1, cyclin-dependent kinase 4, p21, p27, p15, c-myc, Rb, p130, and p107."[4] In addition to the cyclin associated proteins, the extracellular matrix proteins and regulators of extracellular matrix proteins: fibronectin, tenascin, plasminogen activator inhibitor 1, and a variety of other genes that affect apoptosis, differentiation, and other cell behaviors, appear to be transcriptionally regulated by TGF-ß.[4]

Another pathway that parallels the TGF-β/SMAD pathway is the BMP (bone morphogenetic protein)/SMAD pathway. Like TGF-β, BMPs constitute a large family of signaling molecules that regulate a variety of important cellular processes including: "morphogenesis, cell-fate determination, proliferation, differentiation, and apoptosis."[8] The receptor for BMP is a member of the TGF-β Ser/Thr kinase receptors. Therefore, binding a BMP ligand will induce multimerization, autophosphorylation, and activation of the receptor.[8] The BMP receptor subsequently phosphorylates R-SMAD, which constitutes SMAD1, SMAD5, and SMAD8. These phosphorylated SMADs then dimerize with SMAD4 and translocates to the nucleus where they target a variety of DNA binding proteins. While TGF-β and BMP induce an independent set of SMAD proteins, both pathways eventually have their R-SMADs form a heteromeric complex with SMAD4 to stimulate transcription of target genes involved with proliferation.[8] It is undoubtedly

possible that mutations anywhere along the SMAD pathway may lead to unchecked growth in cancer.

In the current research, certain dietary fatty acid supplementations on cancer cells have been known to induce apoptosis. Specifically, docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (PUFA), has demonstrated anti-cancer properties in numerous *in vitro* and *in vivo* models of cancer.[9] Tumors enriched with long chained omega-3 polyunsaturated fatty acids, such as DHA, possess membranes with increased fluidity, an elevated unsaturation index, enhanced transport capabilities that results in accumulation of selective anti-cancer agents, increased activity of selected drug activating enzymes, and alteration of signaling pathways important for cancer progression.[7,10] Studies have found that the effect of DHA on various cancer cell lines, including the human breast cancer cell line MDA-MB-231, show that DHA incorporation has: an anti-proliferative effect, induces apoptosis, and reduces the invasive potential of cancer cells.[9] Past research has also determined that DHA can inhibit growth of human colon carcinoma cells, COLO205 and WiDr, both of which will be used in this study.[11] Provided that apoptosis can be affected by DHA, an investigation will be made to correlate its possible effect on the TGF-ß system and/or the BMP system as well as the SMAD signaling pathway.

METHODS

Cell Culture

WiDr cells were maintained in MEM Eagle (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1.5 g/L sodium bicarbonate, and 1.0 mM sodium pyruvate. COLO205 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, 0.15% D (+)glucose, 0.3 mM sodium pyruvate, and 5.0 mM4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid mixture. Both cell lines were grown in flasks as monolayers at 37◦C in a saturated humidified environment consisting of 5% CO_2.[12]

Fatty Acid Treatments

Fatty acid methyl esters (LA and DHA) were purchased from Sigma (St. Louis, MO) and diluted in ethanol to a final concentration of 10 mg/mL stock solution. Fatty acid stock solutions were flushed with nitrogen gas and stored in the dark at –20◦C. Cultured cells were plated with approximately 50% confluency on a 96 well micropipette plate. Following 24 hour incubation, cells were treated with 125 μM LA and DHA respectively. Cells treated with ethanol were used as controls for each experiment. [12]

Cell Proliferation Assay

XTT Cell Proliferation Assay Kit was used to measure cell proliferation (Roche, Indianapolis, IN). The cells were plated in a 96-well format and treated with fatty acid and/or siRNA as described. After fatty acid treatment, the cells were incubated for 24- 48 hours incubation at 37◦C in a saturated humidified environment consisting of 5% CO_2.

At the end of incubation, XTT reagent was added 4-24 hours before the experiment end point and was read on a spectrophotometer at 450 nm with a reference point at 650 nm.

TGF-ß siRNA Treatment

The cancer cells were transfected with siRNA using OptiMem following manufacture protocol 24 hours of incubation after cell culturing (Invitrogen, Carlsbad, CA). The cells were treated at a 5 nM concentration of siRNA using HiPerFect reagent following manufacture protocol (Qiagen, Valencia, Ca). If the experiment involved FA treatment, the cells were treated 24 hours after transfection with siRNA.

TGF-ß ELISA Kit

Conditioned media was collected for analysis after 48 hour incubation of FA treated cells. TGF-ß levels were quantified using the TGF beta ELISA kit following manufacture protocol (eBioscience, San Diego, Ca). The plates were read on a spectrophotometer at 450 nm without a reference wavelength.

Western Blot

Cells were washed with ice-cold PBS and lysed using GTP-lysis buffer [50 mM HEPES (pH 7.5), 15 mM NaCl, 6 mM sodium deoxycholate, 1% NP-40, 10% gylercol, 10 mM $MgCl_2$, 1 mM EDTA] containing freshly added protease and phosphatase inhibitors. Supernatants were analyzed for protein concentration using Bio-Rad's DC assay (Hercules, CA). Samples were resolved by SDS-PAGE, transferred to a PVDF (Polyvinylidene Fluoride) membrane (Bio-Rad, Hercules, Ca), blocked in 5% milk protein with TBS-T (Tris Buffered Saline with 0.05% Tween), and probed with the appropriate primary antibody in 3% milk protein with TBS-T. Detection was performed

using HRP-conjugated secondary antibody and visualized with ECL reagent (GE Healthcare, Buckinghamshire, UK).

RESULTS

Previous data in the lab has shown that the cell proliferation of COLO205 and WiDr have a dosage response to DHA treatments. In order to determine an inhibitory working concentration of which 50% of the tissue cultures show inhibition of growth, IC50; both colon carcinoma cell lines were treated with varying concentrations of DHA, using linoleic acid (LA) as a fatty acid control. See Figure 1 and Figure 2.

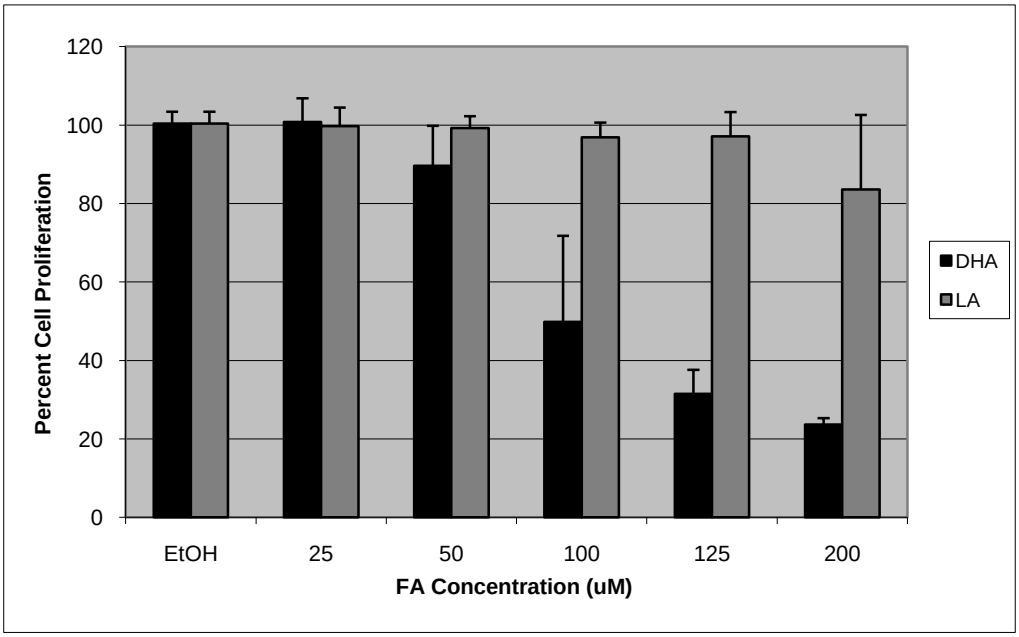

Figure 1. Fatty Acid Titration on COLO205
(n=3) COLO205 cells were plated on a 96-well plate format and treated with their respective FA concentrations. After 48 hour incubation, percent cell proliferation with respect to the ethanol (EtOH) control treatment was measured using the XTT assay kit.

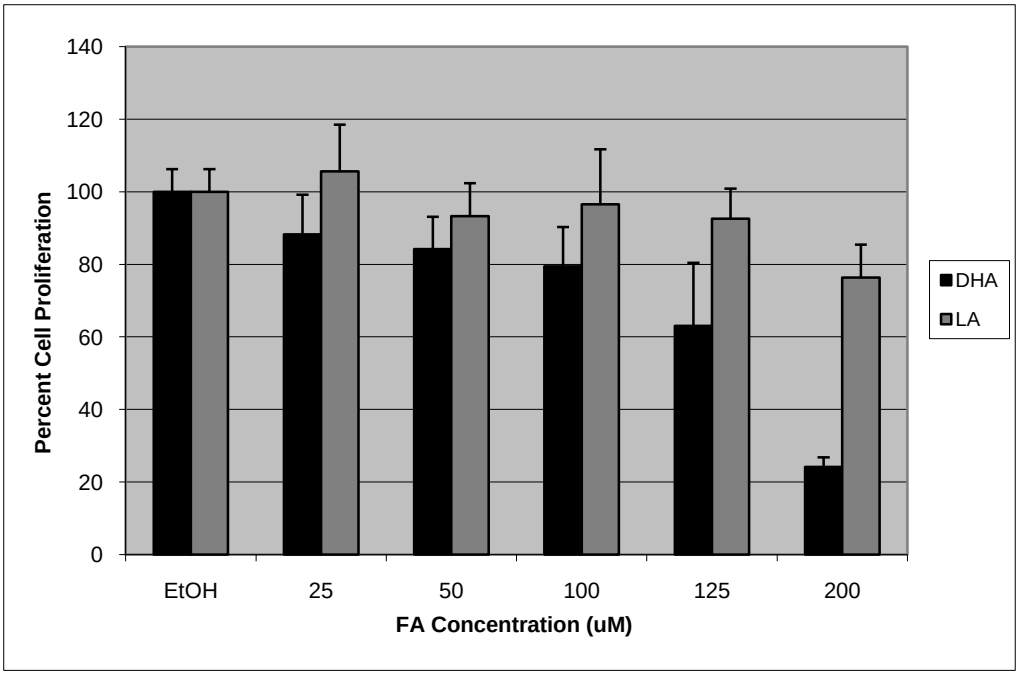

Figure 2. Fatty Acid Titration on WiDr
(n=3) WiDr cells were plated on a 96-well plate format and treated with their respective FA concentrations. After 48 hour incubation, percent cell proliferation with respect to the ethanol (EtOH) control treatment was measured using the XTT assay kit.

The results of Figure 1 and Figure 2 showed with statistical significance that both colon carcinoma cell lines have a dosage response to FA treatment. With respect to LA, DHA was more effective in inhibiting cell growth. Overall it could be determined that both cell lines have a DHA IC50 concentration of around 100-150 μM. For future experiments involving DHA and LA treatment, both cell lines were treated at a 150 μM concentrations.

Knowing that DHA significantly inhibited cell growth, the next approach was to examine the mechanism of how it affects cell signaling. The signaling molecule, TGF-ß, is a cytokine found in the extracellular environment that is known to induce apoptosis. TGF-ß levels in the conditioned media were analyzed following DHA treatments. See Figure 3.

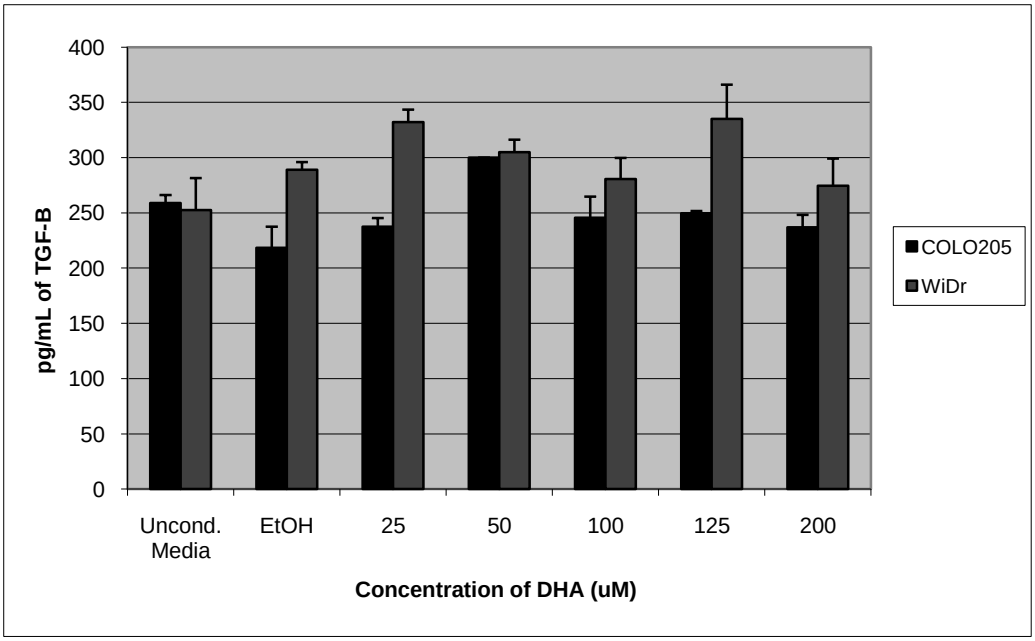

Figure 3. Effects of DHA on TGF-ß levels
(n=2) The conditioned media of both cell lines were analyzed 48 hours after FA treatment. TGF-ß levels were quantified using the TGF-ß ELISA kit. Unconditioned media controls were RPMI for COLO205 and MEM for WiDr.

According to Figure 3, TGF-ß levels did not appear to be significantly affected by DHA treatment on the cells. It is important to note that TGF-ß is present in the control unconditioned media possibly due to the fetal bovine serum ingredient. Variation could be seen in the figure so the experiment must be repeated to attain statistical significance. In addition, the amount of TGF-ß quantified must be normalized to cell count when this experiment will be repeated since it is important to account for the fewer number of cells producing TGF-ß in the DHA treatment group.

Although DHA did not appear to have any effect on the expression of TGF-ß, the next approach was to knock down the expression of TGF-ß, but still quantify any changes in expression in response to fatty acid treatment. The knockdown of this gene was performed using TGF-ß siRNA. See Figure 4 and Figure 5.

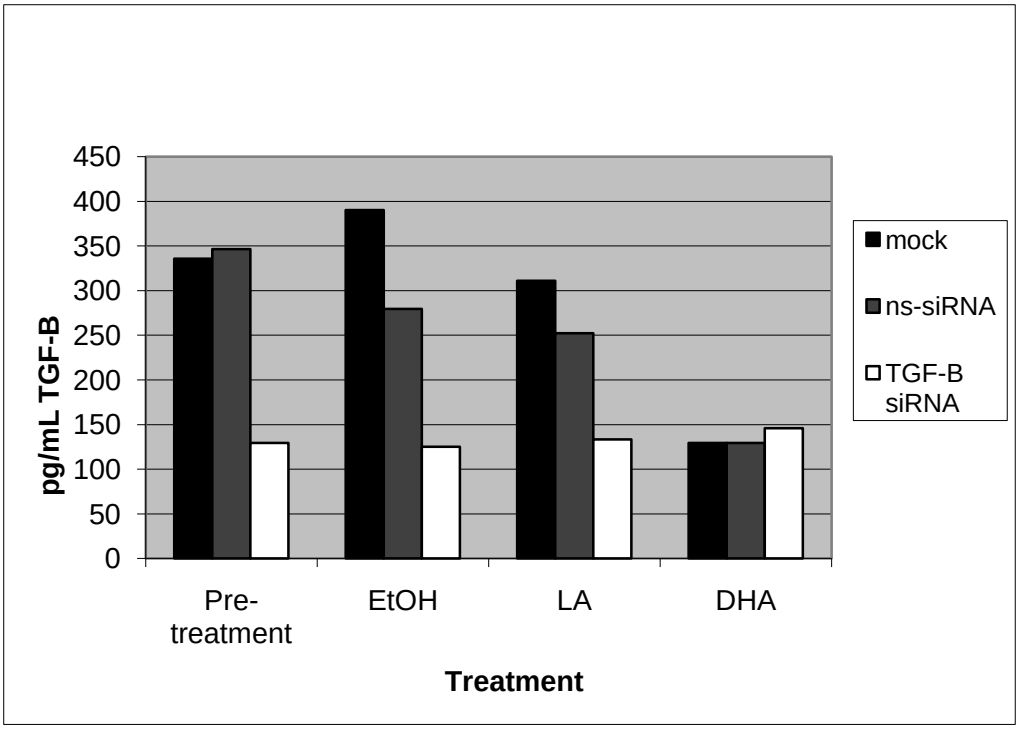

Figure 4. Knockdown of TGF-ß in COLO205
(n=1) TGF-ß levels were quantified using the TGF-ß ELISA kit. Conditioned media was collected 48 hours after siRNA (2 nM) and FA treatment (125 uM). Conditioned media for the pre-treatment group was collected 24 hours after treatment with siRNA, but prior to FA treatment.

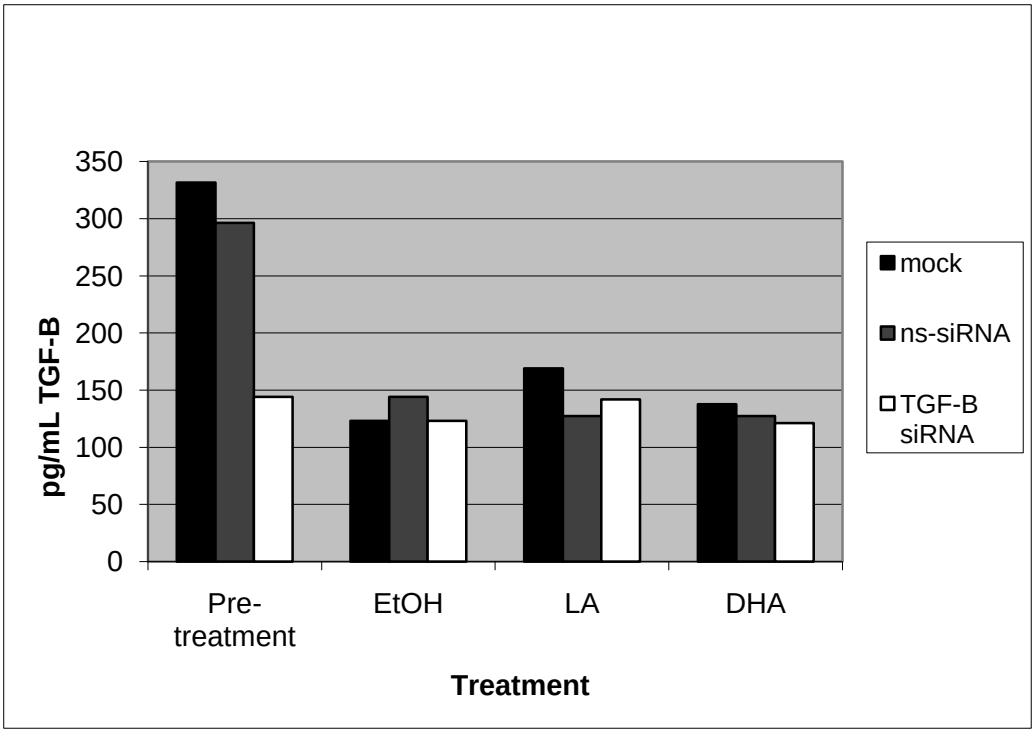

Figure 5. Knockdown of TGF-ß in WiDr
(n=1) TGF-ß levels were quantified using the TGF-ß ELISA kit. Conditioned media was collected 48 hours after siRNA (2 nM) and FA treatment (125 uM). Conditioned media for the pre-treatment group was collected 24 hours after treatment with siRNA, but prior to FA treatment.

According to Figure 4 and Figure 5, the knockdown of TGF-ß using siRNA was successful for both cell lines with respect to their control mock treatment and non-silencing siRNA (ns-siRNA) treatment groups. In Figure 4, there appeared to be no effect of LA on TGF-ß levels with respect to the EtOH control. However, treatment with the DHA on COLO205 showed that TGF-ß levels decreased to the level of TGF-ß siRNA treatment in the pre-treatment group. On the other hand, this shows some discrepancy with respect to Figure 3, where the conclusion was drawn that DHA had no effect on TGF-ß levels with respect to the EtOH control. As for Figure 5, treatment of WiDr with EtOH, DHA, and LA appeared to have reduced overall expression of TGF-ß to the level of TGF-ß siRNA treatment in the pre-treatment group. Unlike COLO205, the WiDr cells

showed no significant change in TGF-ß expression between the DHA treatment and EtOH control group. A definitive conclusion cannot be drawn on these results so the experiments must be repeated. Similarly to Figure 3, the amount of TGF-ß quantified must be normalized to cell count for any definitive conclusions. Overall, these results indicated the effectiveness of treating the two cell lines with TGF-ß siRNA.

Although treatment of both cell lines with DHA did not appear to affect TGF-ß levels, cell proliferation was still measured to see if knockdown of this gene would have any effect. The results were seen in Figure 6 and Figure 7.

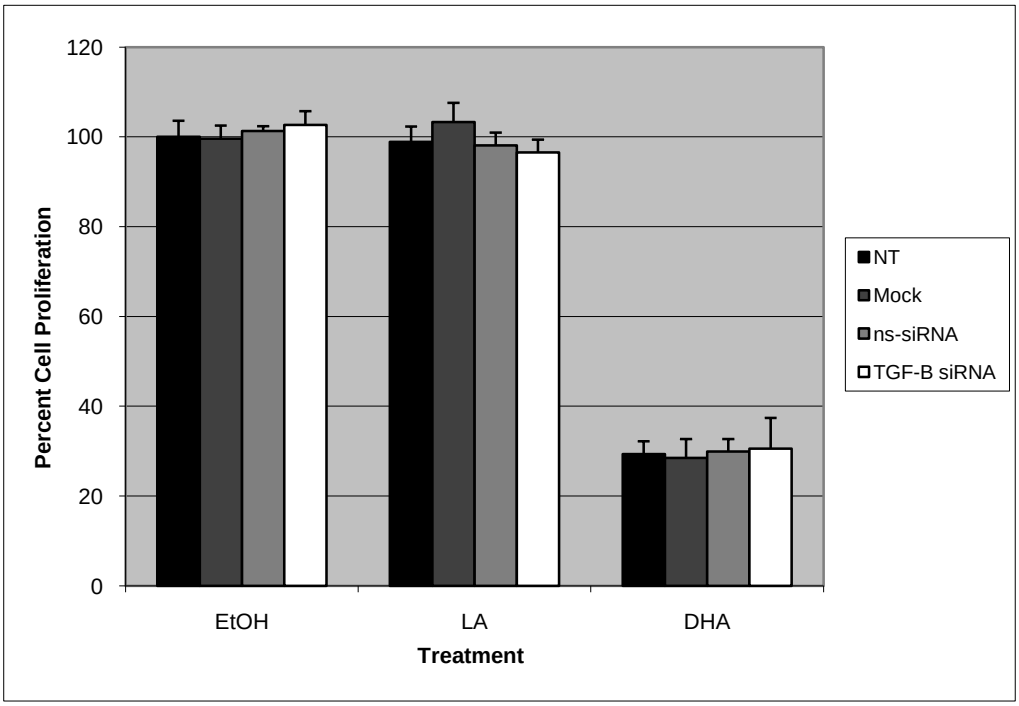

Figure 6. Fatty Acid Treatment Supplemented with TGF-ß siRNA on COLO205 (n=1) COLO205 cells were plated on a 96-well plate format and treated with FA (125 uM) and/or siRNA (2 nM). After 48 hour incubation, percent cell proliferation with respect to the ethanol (EtOH) control treatment was measured using the XTT assay kit.

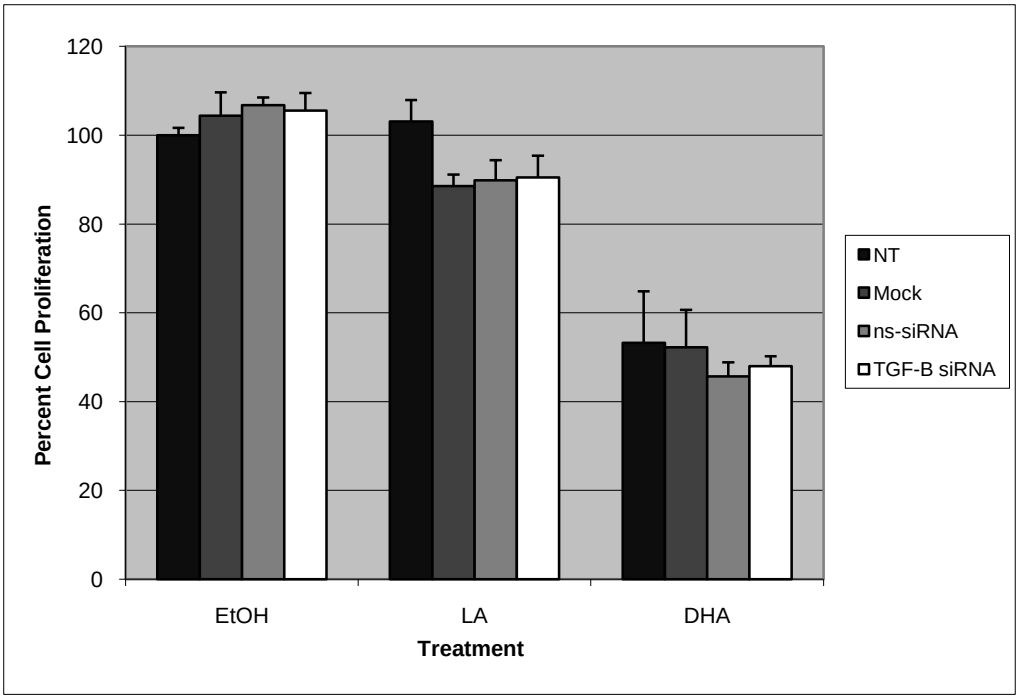

Figure 7. Fatty Acid Treatment Supplemented with TGF-ß siRNA on WiDr (n=1) WiDr cells were plated on a 96-well plate format and treated with FA (125 uM) and/or siRNA (2 nM). After 48 hour incubation, percent cell proliferation with respect to the ethanol (EtOH) control treatment was measured using the XTT assay kit.

The results indicated that both cell lines had no significant inhibition of growth in response to the knockdown of the TGF-ß. However, the experiment needs to be repeated to attain statistical significance.

After determining that TGF-ß may not appear to significantly affect both the COLO205 and WiDr cancer cell lines, an investigation was directed at the intracellular SMAD pathway. The SMAD pathway is the canonical signaling pathway that TGF-β family members signal through. Unpublished data has shown that activated p-SMAD2 is down-regulated in response to DHA treatment. The activated and total SMAD2 protein was analyzed using Western Blot in Figure 8.

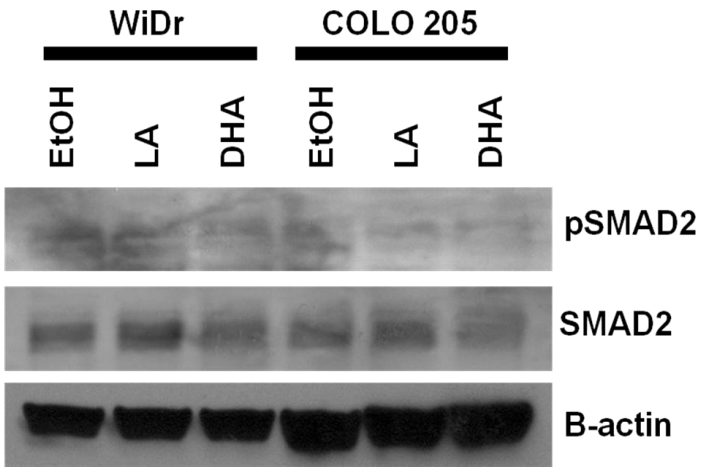

Figure 8. Effects of FA treatment on SMAD2 Protein Activation and Expression
Total cell lysates were collected from both cell lines 48 hours after FA treatment at their respective concentrations (125 uM). Supernatants were analyzed for protein concentration using the Bio-Rad's DC assay. 50 ug of each protein sample was resolved by SDS-PAGE, transferred to a PVDF membrane, probed with the appropriate primary antibody and HRP-conjugated secondary antibody, and visualized with ECL reagent.

Due to the high background signal that the pSMAD2 antibody had on the membrane, blotting for the p-SMAD2 protein was repeated multiple times. In the end, clear results could not be obtained so the experiment for Figure 8 was generously performed by Dr. Keith D. Kikawa. This figure showed pSMAD2 levels, with respect to total SMAD2 protein, in the TGF-ß/SMAD pathway was down-regulated in DHA treated WiDr cells and in both LA and DHA treated COLO205 cells. Although the blot did appear slightly dirty, a conclusion could be made that pSMAD2 levels appeared to be modified in response to DHA treatment.

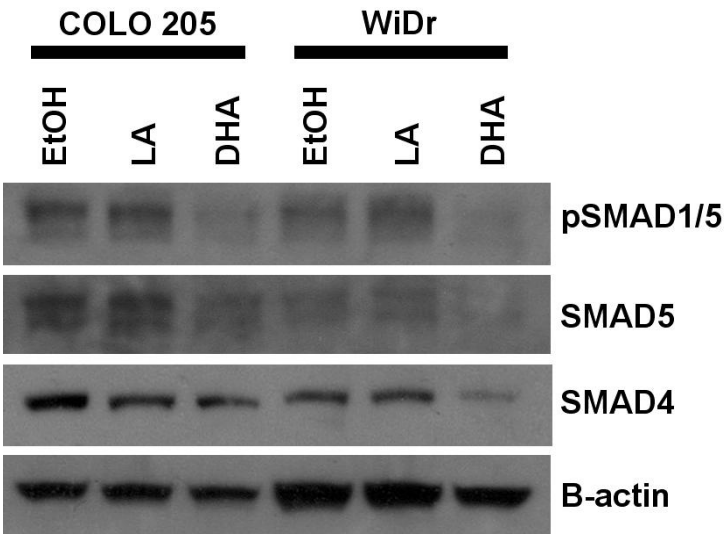

Figure 9. Effects of FA Treatment on Remaining SMAD Protein Activation and Expression
Total cell lysates were collected from both cell lines 48 hours after FA treatment at their respective concentrations (125 uM). Supernatants were analyzed for protein concentration using the Bio-Rad's DC assay. 50 ug of each protein sample was resolved by SDS-PAGE, transferred to a PVDF membrane, probed with the appropriate primary antibody and HRP-conjugated secondary antibody, and visualized with ECL reagent.

In addition to the TGF-β signaling, the intracellular SMAD pathway can be induced by BMP, bone morphogenetic protein, signaling. For Figure 9, the blot clearly indicated a change in protein levels for pSMAD1/5 in response to DHA treatment. Even though the β-actin loading control was slightly uneven for the samples, densitometry results indicated down-regulation of pSMAD1/5 from the BMP/SMAD pathway in both colon carcinoma cell lines treated with DHA. Figure 9 also showed that SMAD4, which is the protein of intersection for the TGF-ß and BMP intracellular SMAD signaling pathway, was down-regulated in DHA treated WiDr cells and in both LA and DHA treated COLO205 cells. It is important to note that SMAD4 does exhibit a similar pattern of down-regulation as pSMAD2 in the fatty acid treated cells.

DISCUSSION

Overall, both the COLO205 and WiDr colon cancer cell lines showed significant inhibition of growth in response to DHA treatment in a dosage response manner according to Figure 1 and Figure 2. As a result, an investigation of the TGF-ß/SMAD signaling pathway was proposed as DHA's mechanism of action because of their involvement in cellular proliferation and apoptosis. The extracellular signaling protein, TGF-ß, was analyzed first. TGF-ß levels did not appear to significantly change upon treatment of DHA with respect to the EtOH control for both cell lines in Figure 3. However, according to Figure 4, upon treatment of the cells with TGF-ß siRNA, DHA appeared to have downregulated the expression of TGF-ß in COLO205 cells. As for Figure 5, treatment of WiDr with EtOH, DHA, and LA appeared to have reduced overall expression of TGF-ß to the level of TGF-ß siRNA treatment in the pre-treatment group. There are definitely some data discrepancies, so these experiments will have to be repeated at a later date. Although the EtOH control group should inherently have no effect on TGF-ß levels, this could possibly be due to the confluency of the WiDr cells prior to treatment. High confluency of cells may permanently alter cellular signaling. Since these experiments have been replicated no more than once and the amount of TGF-ß quantified needed to be normalized to cell count, no definitive conclusion can be made until the results are consistent and amount of cells secreting the cytokine protein is accounted for.

After observing the effects that DHA had on TGF-ß levels, cell proliferation was measured to see if knockdown of TGF-ß would have any effect on the cells. According to Figure 6 and Figure 7, the results indicated that knockdown of TGF-ß did not appear to

affect cell proliferation, nor did it affect the typical response of the cells to the FA treatment. Even though the experiment will need to be repeated in the future, the conclusion could be made that TGF-ß may not necessarily be playing a role in cancer cell survivability. Since several documented papers have indicated that one third of cancer cell tumors have mutated TGF-ß receptors or SMAD proteins, it is quite possible that COLO205 and WiDr may constitute those tumor lines.[4,5] Therefore, the SMAD proteins in the SMAD pathway were the likely candidates that may contribute to cancer cells bypassing normal cellular proliferation.

After treating both cell lines with LA and DHA at their IC50 concentrations, their total protein was analyzed using Western Blotting. Figure 8 and Figure 9 revealed several important effects that DHA had on the SMAD signaling proteins. While WiDr had a down-regulation of pSMAD2 and SMAD4 in only the DHA treatment, COLO205 had a down-regulation for the same proteins in both the LA and DHA treatment. Although they are both colon cancer cell lines, they appear to have a slightly different response to fatty acid treatments. Further studies may need to be conducted, but it seems that the TGF-β/SMAD pathway in COLO205 may be more sensitive to LA treatment than WiDr.

Figure 8 and Figure 9 also looked at pSMAD1/5, which is a SMAD protein signaled through BMP signaling. Unlike pSMAD2 and SMAD4, pSMAD1/5 appeared to be down-regulated in only the DHA treated colon cancer cells. It has been implicated that the absence of BMP signaling may allow for the progression of cancer progression, specifically in many colorectal cancer cells.[13] Although the BMP signaling is primarily known for inducing bone and cartilage growth, it has not been very well studied in cancer cells.[14] In response to DHA treatment, which reduces cancer cell growth and

proliferation, the BMP signaling appeared to be suppressed. Overall, this is in contrast to its effect on promoting the progression of cancer according to the findings of the Kodach et. al paper.[13] Several factors may contribute to this contradicting finding including the type of cancer cells being studied and the conditions by which the cells were growing. In general, different cancer cell lines can express and activate proteins in different ways and in many combinations so it is often difficult to definitively state what one mechanism is applicable to all cancer cells.

Although both TGF-ß and BMP receptors signal through different SMADs in the SMAD pathway, SMAD4, which is the protein of intersection for both pathways, follows fatty acid treatment response as the upstream signaling pSMAD2 induced by TGF-ß signaling. Although other SMAD proteins and the TGF-ß and BMP receptor need to be analyzed, a conclusion could be made that intracellular TGF-ß/SMAD signaling may take precedence over the BMP/SMAD pathway in inducing the gene activation by downstream signaling of SMAD4. Again, the experiments need to be replicated and possibly be expanded to other colorectal cell lines.

Even though this project was initially focused on the TGF-ß/SMAD signaling pathway, the project had expanded to looking at the SMAD pathway induced by BMP signaling. Even though both TGF-ß and BMP signal through different a different set of SMAD proteins and intersect at SMAD4, both pathways clearly had a response to DHA treatment. As a future direction, the TGF-ß and BMP receptors, along with other SMAD proteins (SMAD6, SMAD7, and SMAD3) and their activation states, should be examined in response to fatty acid treatment. SMAD6 and SMAD7 are antagonistic or inhibitory SMADs that prevent the dissociation of R-SMAD from the receptors, whereas SMAD3

associates with SMAD2 to form the R-SMAD complex.[8] While SMAD proteins appear to be affected in response to DHA treatment, determining a mechanism on why this occurs is a potential direction to look into. It is likely that DHA may be incorporated into the lipid membrane, thereby disrupting interactions and activation signaling that would normally occur in the membrane of cancer cells. Overall, the findings of this project appear significant and merits further depth in understanding how the SMAD pathway is affected by DHA treatment.

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