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The Evolution of Cancer Research and the Dietary Intervention that Could Save Lives

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Introduction:

Late on a Saturday night, the soft glow of a single light in the laboratory shines through the crack in the door. Eyes strained and blood shot from staring through a microscope and meticulously counting each and every cancer cell, a researcher is on the brink of a great discovery, or simply an extremely small step forward on a very long journey to try to understand the mechanisms behind the unpredictable nature of cancer.

Over the past 250 years, the advancements in cancer research have been astronomical. Every day, new discoveries are being made in laboratories and in hospitals around the world. Cancer research and treatment has exponentially advanced from the death sentence a cancer diagnosis used to be to a more manageable disease for some patients. However, there is still a significant amount of information to discover about cancer. One of the largest mysteries surrounding cancer treatment is if there is truly a cure. Even though there have been great advancements in the treatment of cancer, the American Cancer Society estimates that in 2017, in the United States alone, there will be an estimated 1,688,780 new cancer cases and approximately 600,920 cancer related deaths (Jemal, 2017). It is also estimated that 1 in 73 women are diagnosed with ovarian cancer in their lifetime. This makes ovarian cancer the fifth most common cancer in women. Of these women diagnosed with ovarian cancer, only 44% of patients survive more than five years after diagnosis and initial treatment (Holland, 2014). These staggering statistics indicate the severity of ovarian cancer and the need to conduct more research to develop an effective treatment, a valuable prevention method, an early detection technique and eventually a cure for ovarian cancer.

Stated in the 1937 article, “Cancer: The Great Darkness” in Fortune magazine, “For 3,000 years and more, this disease [cancer] has been known to the medical profession. And for 3,000 years and more, humanity has been knocking at the door of the medical profession for a “cure”.” (Cancer: The Great Darkness, 1937). With time and the valiant efforts of researchers around the world, a cure may eventually come for this devastating disease.

Background:

A Limited Biography of Cancer based on *The Emperor of All Maladies* by Suddhartha Mukherjee:

Stated as simplistically as possible, cancer is a disease caused by the uncontrollable growth and clonal division of a single cell. A single cell, from almost anywhere in the body, mutates and causes a change in the DNA that alters the regulation of cell growth and cell death, or apoptosis. Over time, the cancer evolves and mutates further from the initial mutation to spread or metastasize to other locations in the body. It also becomes resistant to both the body’s immune response and many different advanced medical treatments. Cancer cells can also take advantage of the surrounding microenvironment and use the resources from the healthy cells and blood vessels in the area (“What is Cancer?”, 2015). One of the major challenges with cancer is that the cell division which drives the growth of cancer is also critical to the growth, repair and maintenance of normal healthy cells in the body. This property makes the treatment of cancer difficult because the two processes are so highly intertwined. How do you target a pathway that is driving cancer growth when you also need that same pathway to grow and repair normal healthy cells in the body? This question has baffled scientists for decades.

It is believed that cancer was first recorded as an illness around 2500 B.C. An Egyptian papyrus (ancient paper like material) contained the accounts of Imhotep, a prominent physician of the time. This papyrus, called the Edwin Smith Surgical Papyrus, contained forty-eight different medical cases and diseases described in detail. In addition to the description, the recommended treatments of the time were also included. The forty-fifth disease listed, which accurately describes the signs and symptoms of advanced breast cancer, stated that it simply had no treatment ("The Edwin Smith Surgical Papyrus"). Signs of bone cancer, osteosarcoma, has also been uncovered through the analysis of ancient Egyptian mummies ("Early History of Cancer", 2018). Ancient accounts of cancer, however, were very rare. One reason for this is that cancer is an age-related disease, and in the past, before medical advances and sanitization techniques, most people did not live long enough to even develop cancer. Most people of that time passed away due to other diseases, illnesses or injury.

Around 400 B.C., Hippocrates began referring to the forty-fifth described disease as cancer. In the Greek language, the term cancer translates to crab. It is thought that this term was used by Hippocrates because the cancerous projections that grew from the central tumor often appeared in the shape of a crab. Claudius Galen later used the term Oncos to describe tumors. Oncos is Greek for swelling. This is where the term oncologist originates ("Early History of Cancer", 2018).

Hippocrates believed that the body was composed of four humors: blood, black bile, yellow bile and phlegm. In theory, each of these four fluids must be in perfect balance or homeostasis to remain in good health (Sudhakar, 2009). Roman doctor, Claudius Galen then took Hippocrates' theory further and described cancer as an "internal overdose" of black bile. He thought that you could not treat cancer through surgery because the black bile, which was

distributed throughout the body, would simply fill in the hole where the cancer was located. As soon as the surgery was complete the cancer would return to that same area due to the humor filling in the hole. So, a long list of cleansing “medicines” became the popular treatment for rebalancing the black bile and treating cancer. These treatments included bizarre mixtures of fox lungs, boar teeth, the paste of crab eyes, goat dung and cow feet along with many other natural remedies (Mukherjee, 2011).

Building from the work of Galen, in 1533, Andreas Vesalius began creating sketches of the human anatomy. He began by observing the bones in a grave yard and expanded his sketches from there. He soon noticed how interconnected each system was and how they seemed to be dependent on one another. He wanted to create a cohesive sketch of the entire body and its systems to support Galen’s theory of cancer and to expand the understanding of the human body as a whole. However, in the process Vesalius found no evidence of black bile and instead of supporting Galen’s theory, he disproved it. In 1543, Vesalius’ book *De humani corporis fabrica libri septem* (“The Seven Books on the Structure of the Human Body”) was printed (Florkin, 2017).

In 1846, William Morton, a Boston dentist, performed the first surgery using anesthesia that was neither alcohol or opioids. He used a mask with ether to anesthetize the patient. After the surgery, the patient claimed that he felt no pain during the surgery even though he was aware that the surgery was being conducted ("History of Anesthesia"). This critical advancement made the practice of surgery a more viable option for disease and injury treatment. Another critical advancement was the use of antiseptics. In 1867, Joseph Lister applied the ideas of Louis Pasture, the germ theory (the idea that microorganisms cause disease) and his pasteurization process to surgery. Upon completion of a surgery, Lister used a sewage cleaner, carbolic acid, to

clean the wound in order to prevent the spread of infection. After his successful prevention of infection post-surgery, he began to perform more surgeries using this strategy. This proved to be highly effective and he is now known as the Father of Modern Surgery (Pitt, 2012). The surgeries performed using these new techniques included the removal cancer, since the additional threat of post-surgery sepsis was considerably reduced.

The surgeries performed to treat cancer began in a relatively conservative manner, removing as little tissue as possible. As time progressed, the surgeries became more and more extreme. For example, a patient with breast cancer may undergo a radical mastectomy that removes the breast, the pectoralis major, pectoralis minor, axillary lymph nodes, lymph nodes under the clavicle, glands under the clavicle and glands in the neck. These surgeries were extreme and left the patient highly disfigured and often unable to move their arm or shoulder on the side of the surgery. Although the survival rate was higher than the previous, less radical surgical procedures, this was not a cure and depending on the stage of the cancer, the cancer almost always returned within three years of the surgery.

In 1895, Wilhelm Rontgen of Germany discovered x-rays and this radically changed the face of cancer treatment. Within the next year, many experiments were conducted to obtain more information on the newly discovered x-rays. These experiments included determining what elements produced radioactivity. It was found that radiation attacks the genetic material (DNA) of the cells causing the affected cells to die. The x-rays seemed to specifically target only the most rapidly dividing cells of the body such as, hair, skin and nails. It was then thought that this could be a new treatment for cancer which could potentially target the rapidly dividing cancer cells. Wilhelm Rontgen was honored with The Nobel Prize in Physics in 1901 for his contributions to science ("Wilhelm Conrad Röntgen - Biographical", 2014). Just one year after

the discovery of x-rays, in Chicago, Emil Grubbe attempted to treat a case of breast cancer with radiation. After eighteen harsh treatments, the tumor shrank despite the painful regimen. Similar to the success of surgery, radiation was very effective for localized tumors, but not cancer that had metastasized throughout the body (Markel, 2015). It was later found that the x-rays could also potentially cause more cancer to form because it initiated mutations in the DNA. Because of this discovery, the treatment unfortunately lost much of its support and most of the ongoing research was abruptly stopped.

In the 1940s, medicine and the life expectancies of Americans greatly improved with significant medical discoveries. Different antibiotics including penicillin, tetracycline, chloramphenicol and streptomycin were among the antibiotics discovered. In addition, public health and sanitization became a priority for the nation. However, with all of these advancements, very little progress was being made in the world of cancer. If the cancer was confined to a single location, it could often be removed though extirpations (an advancement made in the 19th century). In the 1890s, Dr. William Halsted at Johns Hopkins pioneered the mastectomy to "cure" breast cancer. In the early 1900s, radiation was used to kill local tumors after the discovery of x-rays. However, there was still not a cure for this aggressive, deadly disease (Mukherjee, 2011).

As other advancements in medicine continued to improve, the treatment for cancer remained stagnant. By 1926, cancer became the nation's second most common cause of death behind heart disease. With cancer research at a standstill due to the lack of funding, senator Matthew Neely from West Virginia began the effort to obtain federal funding for cancer research after a failed proposal for funding from President Taft. Finally, with the help of senator Homer Bone and Representative Warren Magnuson, a bill passed in the House of Representatives, and

in 1937, President Roosevelt signed the National Cancer Institute Act ("National Cancer Act of 1937", 2016). The National Cancer Institute (NCI) was created and was designed to coordinate cancer research using an advisory council and providing substantial laboratory space for scientists to conduct cutting edge research.

In 1924, British physician, Geoffrey Keynes took a different approach from radical mastectomies and extreme radiation for the treatment of breast cancer. He had many successes performing a local, and relatively simple surgery to remove the hard, granular, localized tumor followed by radiation treatment in low doses to ensure the margins of the surgical site were clear of breast cancer. Although he had success, many American physicians did not take him seriously and mocked his procedure by calling it a "lumpectomy". However, his work was noticed by George Barney Crile, an American surgeon. Crile began to realize that cancer did not spread radially out from a central tumor. It often seemed to jump to distant locations from the initial tumor. This observation would indicate that if the cancer was going to spread, performing a radical mastectomy would not improve the outcome; it would simply be a more disfiguring surgery with little to no benefit. Crile began performing standard mastectomies with radiation and the results were similar to those of the radical, disfiguring mastectomies. The medical field was slow to accept this change in procedure and significant data needed to be presented to prove and convince other physicians that the radical mastectomy was no better than the standard mastectomy or lumpectomy procedure. It took many years to obtain this data due to the lack of support, but by 1981 the data was presented. It showed that there was, in fact, no significant difference in outcomes between the radical mastectomy, standard mastectomy and a standard mastectomy with radiation treatments after surgery (Saxon, 1992).

Sidney Farber, the father of modern chemotherapy and a pediatric pathologist at Boston Children's Hospital devoted his life to studying leukemia. After many years of research, he had his first breakthrough in 1947. An old friend and chemist, Yellapragada Subbarao, created a foliate antagonist. Folic acid had previously been discovered to treat a type of anemia and was also found to increase the severity of leukemia. It was believed that if the folate antagonist were given to leukemia patients, it would stop or reduce the production of the cancerous leukemia cells. The first folate antagonist, pteroylaspartic acid or PAA was ineffective. However, a variation of the drug, aminopterin, served to be effective in the first patient and many subsequent patients despite the lack of support from the Boston Children's Hospital. In many cases, it reduced the number of cancerous cells in the blood, and in some patients completely killed all of the cancerous cells. Ultimately, after a few months in remission, the cancer came back with a vengeance and the patients suddenly passed without time for additional treatment. Farber published a paper with these findings in 1948 (Miller, 2006).

Around the same time, other laboratories were also trying their hand at a type of chemical warfare within the body. In 1878, Paul Ehrlich began using synthetic chemicals used for dying cloth to treat different microbial diseases. He, however, failed to find a chemical to kill cancer because cancer cells are so similar to normal cells; the problem that has troubled scientists and physicians from the beginning. In 1919, Edward and Helen Krumbharr analyzed the effects of a World War I mustard gas bombing. They found that those individuals who survived the mustard gas bombing had bone marrow that was virtually "dried up". The chemical had specifically targeted the victim's bone marrow. However, in the midst of World War I, this information went highly unnoticed by scientists. Eventually, the effects of mustard gas on cancer was studied again

by Louis Goodman and Alfred Gilman of Yale University, but in clinical trials, the progress was minimal and the cancer returned soon after the treatment was complete (Mukherjee, 2011).

Gertrude Elion, in 1944, began working with purines. A purine is a chemical critical to the formation of DNA. Two of the four nucleotide bases that make up DNA, adenine and guanine, are purines. Elion added different chemical sidechains to the purines. After several failures, she created 6-mercaptopurine (6-MP). She conducted various tests in an animal model and it was then tested in humans. 6-MP was found to almost magically remove leukemia cells from the blood. But, much like the other treatments, the cancer quickly returned. She received the Nobel Prize in Physiology or Medicine in 1988 for her accomplishments in this field ("Gertrude B. Elion - Biographical", 2014).

Another attempt at chemotherapy came in 1954 when Sidney Farber convinced Salem Waksman to send him several antibiotics that had been isolated from various soil bacteria. Among these antibiotics, was actinomycin D. Farber found this drug to be highly effective in treating several types of cancers in mice. When he tried the antibiotic on humans, however, it was only effective in one rare form of kidney cancer. Through this study, it was also found that the combination of x-rays and chemotherapy was very effective and combining the two treatments multiplied the effect of each of the individual treatments. Farber continued to grow his studies increasing the number of doctors, scientists and patients in a wide variety of trials (Mukherjee, 2011).

In 1956, Min Chiu Li, used the same antifolates that Farber used in 1947, but used it to treat metastatic choriocarcinoma (cancer of the placenta). After the initial masses disappeared using the antifolates, he continued to administer the treatments for a lengthened period of time.

The patients did not relapse after the extended chemotherapy treatment. This discovery however, came with a cost. Because Li did not have the National Cancer Institute's (NCI) approval for the extended chemotherapy treatment, he was said to be experimenting on patients, and was fired from his position at the NCI despite his successful trial (Freireich, 2002).

In 1961, a subsequent trial was completed at the NCI by a large team of doctors. They combined four different chemotherapy drugs in an intense and aggressive treatment regimen. This treatment combined vincristine, amethopterin, mercaptopurine, and prednisone. The treatment was called VAMP and it completely wiped out the immune system and the leukemia it was meant to treat. Slowly, the body recovered and the patient went into remission. This treatment took patients to the edge of death with no immune system, extreme side effects and complete exhaustion before bringing them back. Many of the patients however, returned with their bone marrow clear of cancer, but their nervous system hosted many undetected, and unaffected cancerous cells and tumors. The cancer was able to pass the blood brain barrier, but the chemotherapy had not been able to pass this highly selective barrier meant to keep harmful substances away from the central nervous system. Soon after the symptoms of the cancer in the central nervous system first appeared, the patient fell into a coma and passed away. Even though this trial did not go as planned, there was still the successful destruction of the leukemia in the blood and bone marrow (Mukherjee, 2011).

The story of Hodgkin's Lymphoma began in 1832 when pathologist, Thomas Hodgkin, characterized an illness in cadavers that was confined to the lymph glands and caused only those glands to swell. Again, the observation went highly unnoticed. In 1898, after Hodgkin's death, Carl Sternberg realized that the disease that Hodgkin characterized was yet another form of

lymphoma. This disease was unique in that it methodically spread from one node to the next and stayed localized in the nodes. It did not seem to metastasize to other organs like most cancers.

By the 1950s, Henry Kaplan built a linear accelerator that was an extreme form of an x-ray. Through a methodical selection process including blood tests, scans, and staging of the Hodgkin's Lymphoma, Kaplan hand selected patients for a trial. It was found that through the use of the new, powerful extended field radiation, the patients did not relapse after treatment. However, the patients selected for the trial were unique, early staged patients. This theory of selecting patients and treating cancer specifically based on the type, stage and characteristics of the patient was not adopted for many years. Cancers cannot all be treated the same way, because each case is so intriguingly unique. The next step to treat Hodgkin's Lymphoma came in 1963, when Vincent DeVita decided to develop a treatment for late stage Hodgkin's disease. This treatment, called MOPP, again combined four powerful chemotherapies (Mechlorethamine Hydrochloride, vincristine (Oncovin), Procarbazine Hydrochloride and Prednisone. Like VAMP, it caused the patient's white blood cell counts to drop to nearly zero and triggered violent nausea. From the 43 patients given this treatment, 35 reached full remission. This was a huge success for DeVita and the fight against cancer. It was beginning to seem that the optimal treatment for cancer was a combination of several chemotherapies ("Hodgkin's Disease - Historical Timeline").

Near the same time the chemotherapy war was progressing, in 1909, Peyton Rous offered a new theory that caused many researchers to change their direction of thinking. Rous was a virologist studying chickens. One of his chickens formed a tumor on its back and he gained a $200 grant to begin a cancer study on that tumor. He found that the cancer could be transferred from hen to hen by injecting tumor cells from one bird to another. He then conducted an

experiment where he filtered the tumor to remove all of the cells from the fluid and that cell free fluid was injected into the next hen. It was expected that the cancer would not grow because there were no cells, but it surprisingly did. This launched the theory that viruses were the cause of cancer since all of the cells had been filtered out. The virus he discovered was called Rous Sarcoma Virus (RSV) and he received The Nobel Prize in Physiology or Medicine in 1966 for his findings ("Peyton Rous - Biographical", 2014). In 1935, Richard Schope found a papilloma virus that caused tumors in rabbits and later a leukemia causing virus was found in rats and cats. By 1958, Denis Burkitt discovered the first known human cancer virus called Epstein Barr Virus (EBV) in a group of people from sub-Saharan Africa. Research to find other viruses consumed nearly ten percent of the NCI funding and many people believed that cancer patients should be isolated to prevent the spread of cancer (Mukherjee, 2011). A sort of panic broke out in the cancer wards of hospitals with this new theory behind the cause of cancer. Could cancer be spread like the common flu virus? If so, why didn't all of the physicians and nurses treating these patients also have cancer?

In 1976, another chemotherapy drug was discovered. This drug was cis-platinum or Cisplatin for short. Barnett Rosenberg, although not the one to discover the chemical, discovered its use when attempting to analyze the effects of an electrical current on bacterial cell division. In his experiment, he found that the cells quit dividing. However, it was later determined that the electricity was simply an accessory to the platinum electrodes that had reacted with salt during the experiment. The platinum attacked the DNA of the cells through the crosslinking between the platinum and the DNA. Once this occurred, the DNA could not be repaired and therefore caused the cell cycle to arrest and inhibited cellular division. This drug combined with bleomycin and vinblastine proved to be a very effective treatment for metastatic testicular cancer. Some of the

patients receiving this treatment never relapsed. This drug was then tested in many different combinations with many different cancers despite the fact the treatment caused debilitating and life threatening nausea ("The "Accidental" Cure—Platinum-based Treatment for Cancer: The Discovery of Cisplatin", 2014).

The NCI began testing more and more chemicals throughout the 1960s and 1970s. Drugs such as Taxol (from a Pacific Yew tree), Adriamycin, etoposide (from the fruit of poisonous mayapple), and bleomycin (from mold) were all discovered and all had serious side effects. Different combinations of drugs continued to show some promising results in various cancers. Other combinations and extreme doses proved to be too much for the body to handle causing failure and death to ensue. Between 1984 and 1985, six thousand articles were published presenting the results of the different chemotherapy trials. Despite the large quantity of studies, none produced a definite cure through chemotherapy alone (Mukherjee, 2011).

In 1927, urological surgeon, Charles Huggins studied the effects of hormones on cancer. As a urologist, he began his work with prostate cancer. He devised a way, through the use of a catheter, to collect the fluid secretions from the prostate. He then began looking at prostate cancer by studying dogs, who are the only other animal to form prostate cancer besides humans and lions. He found that if he ablated the testis, which produce the male sex hormone testosterone, the prostate would shrivel up. If he then injected the animal (with the removed testis) with testosterone, the prostate remained functioning. This indicates that the function of the prostate is dependent on the presence of the hormone testosterone. That finding indicated that prostate cancer, an extreme, aggressive version on normal prostate cells, may be highly dependent on the hormone testosterone. When he removed the testis in dogs with prostate cancer, the cancer quickly shrank due to the lack of testosterone. This was the first-time hormones had

been linked to the growth of cancer. Charles Huggins received the The Nobel Prize in Physiology or Medicine in 1966 for his contributions to medicine ("Charles B. Huggins - Biographical", 2014). In 1929, estrogen was isolated by Edward Doisy. Charles Huggins then used this isolated estrogen to feminize the male body in a successful chemical castration to treat prostate cancer. The cancer relapsed, but this theory proved that some cancers could be fought without the harsh, debilitating chemotherapies and surgeries that had been previously developed.

Taking the information produced by Huggins, his colleague Elwood Jensen, Father of Nuclear Receptors, began to study female cancers and the effects of estrogen. Previous surgeons had discovered that only about two-thirds of the patients with breast cancer responded positively to the removal of the ovaries (the major female estrogen producing organ). Jensen wanted to figure out why there was a significant variability between the women. In 1968, looking at the cellular level and using radioactively labeled estrogen, Jensen discovered that some cells had an estrogen receptor (ER). He then found that some of the breast cancers were either estrogen receptor positive or negative. He believed that this was the reason why some of the breast cancers responded to the removal of estrogen and others did not. The surgery for the removal of the ovaries to eliminate the estrogen production had been stopped due to other serious side effects. There was also little enthusiasm to study hormonal therapies for cancer due to the excitement surrounding chemotherapy treatments (O'Malley, 2013). In 1962, a group of British chemists created the drug tamoxifen by mistake. They were trying to create an estrogen stimulating drug to be used as birth control. Tamoxifen turned out to do the opposite of what they intended. Tamoxifen was an estrogen antagonist and was deemed to be useless and pushed aside. However, in 1969, after Jensen's discovery of estrogen receptors, Mary Cole tried to use tamoxifen in metastatic breast cancer patients. Ten of the forty-six women tested responded

positively to the drug. V. Craig Jordan stained breast cancer cells for ER. He found that cells with ER responded to the tamoxifen treatment. When the tamoxifen was competitively bound to the ER, it caused the extinction of the estrogen responsiveness in the cells. In turn, this would eliminate the cell's growth and further division (Jordan, 2014).

After Cole's success, it was thought that treating more early staged cancers with chemotherapy may be more beneficial than just focusing on the late stage metastatic cancers. Paul Carbone, an NCI oncologist looked to treat patients with chemotherapy after the initial surgery to reduce the rate of relapse. He called this treatment "adjuvant chemotherapy". However, he had a difficult time convincing surgeons that chemotherapy after surgery would be beneficial. A pair of physicians in Italy, Gianni Bonadonna and Umberto Veronesi, were awarded the contract for the NCI trial to test if chemotherapy after surgery would improve the relapse rate of breast cancer because surgeons and chemotherapists in the United States would not agree to work together. By 1975, they presented their findings and showed that patients who received no chemotherapy after surgery had a fifty percent chance of relapse, while those treated with chemotherapy after surgery only had a thirty percent chance of relapse. In 1977, a similar trial was conducted by Bernie Fisher using tamoxifen. In this study, with 1,891 ER positive breast cancer patients, it was found that those given tamoxifen after surgery had a fifty percent reduction in relapse rate (Mukherjee, 2011).

STAMP, Solid Tumor Autologous Marrow Program, was the newest idea to radically treat cancer in the 1980s. Tom Frei, the director at Farber's institute, was convinced that a large dose of chemotherapy followed by an autologous bone marrow transplant was the solution to cancer therapy. An autologous bone marrow transplant is where marrow is harvested from one's body, frozen and then replaced at a later time, in this case after extreme chemotherapy. Joining

Frei in this study was William Peters, a young, New York doctor. In 1982, the STAMP protocol was approved and the first treatment ensued. Phase one of the trial was a success, patients could safely survive the treatment protocol. The Cancer and Leukemia Group B (CALGB) sponsored the phase three study and soon many hospitals around the world were offering this procedure both on and off trial. One trial was conducted by Werner Bezwoda, an oncologist from South Africa. His trial resulted in 60 percent of patients surviving after eight and a half years compared to the 20 percent surviving in the control group. That being said, other similar trials did not obtain the same promising results. Some trials showed high death rates due to complications and others showed no improvement. Upon further investigation, it was found that Bezwoda's whole study was a fraud and no improvements from STAMP was found (Mukherjee, 2011).

As the fight against cancer continued, researching the prevention of cancer also became an interest. Lung cancer became a target for prevention as the number of people smoking cigarettes increased along with the number of cases of lung cancer. It took a significant amount of time to fully establish the link between the smoking of cigarettes and cancer, but it was eventually established. Unfortunately, it was far too late for many people addicted to smoking. Warnings were placed on packaging and advertisements were removed but the addiction was still there. Along with the link of cigarettes to cancer, other carcinogens were discovered. These include, asbestos, DES (synthetic estrogen), x-rays, benzene compounds and many other chemicals. In addition to chemical carcinogens, viruses such as the Hepatitis B virus were found to cause cancer along with some bacteria. In addition to the identification of carcinogenic substances, preventative screenings such as pap smears, colonoscopies and mammograms were also developed to help detect early stage cancers. Early detection and prevention is the best treatment for cancer (Mukherjee, 2011).

Stepping away from clinical research that was being conducted on cancer, there was a significant amount of laboratory research also being conducted to look at cancer at a molecular and cellular level. Beginning in about 1858, Virchow began looking at cancer proliferation. He believed that cells proliferated due to inflammation. However, cancer seemed to proliferate without cause. There was potentially an unknown internal signal that triggered their proliferation. Walther Flemming, in 1879, discovered chromosomes in salamander eggs. He stained the eggs with aniline and was able to see the chromosomes and their amplification just before division (Zacharias). David Paul von Hansemann then looked at cancer cells and noticed that the chromosomes in these cells were split, frayed, disjointed, broken and multiplied (Mukherjee, 2011).

Gregor Mendel, in the 1860s, was researching normal cellular functioning and discovered the gene (unnamed at the time) and the idea of inheritance from each generation. By 1910, embryologist, Thomas Hunt Morgan rediscovered that heritable traits move through the generations in fruit flies. He also noted some mutations occurred spontaneously within the flies. He observed that genes had to be carried on chromosomes and chromosomes must be duplicated before division. This duplication of chromosomes allowed the genes to be passed on to the next generation. Building on Morgan's discoveries in fruit flies, Hermann Joseph Muller discovered that x-rays caused more mutations in flies than what naturally occurred, creating a link between cancer and mutations. In addition to these discoveries, in 1926, Oswald Avery found that genes could be transmitted laterally between bacterial cells. By 1944, it was discovered that the genes were transferred via deoxyribonucleic acid or DNA. George Beadle and Edward Tatum discovered that genes carried the map or instructions to form proteins which facilitate most of the cellular functions. In the 1950s, a group of scientists including Jacques Monod, Francois Jacob,

Sydney Brenner, Matthew Meselson, and Francis Crick discovered an intermediate between the gene and the protein called ribonucleic acid (RNA). Thus, the central dogma of DNA→RNA→Protein was created and found to be universal to most organisms (Mukherjee, 2011).

The Rous Sarcoma Virus (RSV), mentioned previously, was researched further by Howard Temin in 1956 ("Howard M. Temin – Biographical", 2014). He wanted to conduct research on how the virus converted healthy cells into cancerous cells. He wanted to research a simplistic system that did not have the interference of chickens or tumors. He tried to grow cancer from normal cells growing in a petri dish by inoculating them with the RSV. Through his research, he found that this virus did not act like other viruses by simply replicating in the host. It physically attached to the DNA of the cell altering the cell's genome. Another interesting fact discovered is that this virus did not have DNA as its genetic material, but instead it had RNA. So, the next emerging question was how did the virus then alter the DNA of the host cell. Temin concluded that there was a type of reverse transcriptase that allowed the virus to track in a retrograde fashion on the central dogma creating DNA from RNA. The DNA created from the RNA is called a provirus. Then, the provirus created new RNA to regenerate the virus. Howard Temin was awarded the Nobel Prize in 1975 for his contributions to Physiology and Medicine ("Howard Martin Temin", 2009).

With the idea that the retrovirus could cause cancer, it was now a mission to find the virus in cancer. After many years of research, a virus was found in only one type of cancer, a rare leukemia. Temin then suggested that they should look for the gene that had been released that caused the cancer in the cells he studied. Steve Martin, Peter Vogt, and Peter Duesberg discovered that gene in the 1970s and called it SRC. Ray Erikson then discovered that SRC

coded a protein (kinase) that altered the function of other proteins though phosphorylation or the addition of a phosphate group to the protein. The attachment of a phosphate to the protein acted like an on switch for that protein. The viral SRC was a hyperactive kinase that phosphorylated everything in its vicinity including regulators of the cell cycle. This in turn caused the uncontrolled growth associated with cancer. J. Michael Bishop along with Harold Varmus looked to find a gene in normal cells that was similar to the viral SRC to find the precursor to the viral SRC. Through the use of radioactivity and the nature of DNA, they found genes that were virtually identical to the viral gene in many different kinds of cells. However, it was not exact. The normal SRC found in all cells was highly regulated while the viral (cancer causing) SRC was hyperactive and unregulated. So, was cancer simply caused by this precursor that is endogenous to all cells? SRC is a proto-oncogene or a gene that can become an oncogene (cancer causing gene) if a mutation occurs (Bister, 2015).

After the discovery of SRC, Alfred Knudson wanted to study the genetic inheritance of cancer and chose to look at the retinoblastoma (Rb) cancer, which was seen as highly heritable. He found that every cell has two copies of the healthy Rb gene. However, those who have a family history of this cancer will automatically have one mutated gene. Therefore, the patient has a higher chance of the second gene mutating and forming cancer. Opposite to the SRC gene, the Rb gene is a tumor suppressor gene. This means that when the gene is properly functioning, it inhibits cell division. There are now two known types of genes: ones that activate proliferation or cell division and ones that suppress proliferation. Thad Dryja then was able to isolate the exact location of the Rb gene on chromosome 13. It was also discovered that this gene was not only present in retinoblastoma, it was also found in lung, breast, esophageal, bone and bladder cancers. It was also expressed in most proliferating cells. The normal function of Rb is to bind to

other proteins to inactivate them. When Rb is phosphorylated, it releases from the proteins where it was previously bound allowing cell division to take place (Langer, 2016).

Robert Weinberg continued the quest to find other genes that caused cancer. He thought that he could transfer the DNA of the cancerous cells to normal cells and then isolate the gene that caused the normal cells to become cancerous. He purified the DNA from the cancerous cells and used calcium phosphate the transfer the DNA to the normal cells. This method is called transfection. Other laboratories were also trying this same technique, with the same goal. Three laboratories all isolated the same cancer causing gene called RAS in 1982. Within the next ten years, many other cancer related genes were discovered including MYC, NEU, FOS, RET, AKT, p53, VHL, APC and BRCA-1 (Mukherjee, 2011).

In Weinberg's laboratory, they also isolated the HER-2 gene. This gene was later rediscovered at the Genentech pharmaceutical company by Axel Ullrich. The goal for the company was to produce an anti-cancer drug from this discovery. The Genentech company discovered how to make drugs from genes through the use of recombinant DNA. The drugs they previously produced were ones that supplemented a missing signal, for example insulin or growth hormone. They had never produced a drug to stop a signal that was over amplified. With the help of Dennis Slamon, in 1986, they found that HER-2 was highly amplified in some breast cancers. Ullrich believed that an antibody could be created to shut off the action of HER-2 and stop the growth of the cancer. By 1988, Genentech developed a mouse antibody against HER-2. When the mice were given HER-2 they quickly developed cancer and when given the antibody, the cancer disappeared. Cesar Milstein then "humanized" the antibody and by 1990 a new cancer drug was created for HER-2 amplified breast cancers. Through many studies, it was found that

this drug, Herceptin, increased the survival rate of HER-2 positive breast cancer patients by an impressive 33 percent (Mukherjee, 2011).

By 2000, enough research had been conducted to draw some conclusions about the nature of cancer. Weinberg and Douglas Hanahan published the article “The Hallmarks of Cancer”. They formulated six “rules” that cancers follow when normal cells transform into cancer cells. The rules are as follows: 1. Self-sufficiency in growth signals, 2. Insensitivity to growth-inhibitory signals, 3. Evasion of apoptosis, 4. Limitless replicative potential, 5. Sustained angiogenesis (the growth of new blood vessels), 6. Tissue invasion and metastasis (Hanahan, 2000). With some of the mechanisms and biology of cancer understood, would a cure soon follow?

Since the targeted therapy with HER-2 positive breast cancer treatments, many subsequent treatments were developed for other types of cancers. One example of this was the development of Gleevec, a targeted therapy that works to inhibit the bcr-abl tyrosine kinase found in chronic myeloid leukemia (Iqbal, 2014). This discovery and treatment significantly improved the prognosis for chronic myeloid leukemia. Dozens of other drugs were developed in addition to second and third generation drugs to account for developed resistances to the drugs (Mukherjee, 2011).

In 2003, the Human Genome Project looked to sequence all of the genes in the normal human body. The human genome contains approximately 20,000 genes. Subsequently, the Cancer Genome Atlas also began. This effort would analyze different cancers to find all of the mutated genes in different types of cancers. By 2006, about 13,000 genes in breast and colon cancers were sequenced. In 2008, brain cancers were added to the list and by 2009 ovarian,

pancreatic, melanoma, lung and several types of leukemia were also sequenced. In breast and colon cancers, fifty to eighty genes were identified as mutated. However, every case of cancer was slightly different and unique in the mutations. There are some genes that are always mutated in a particular type of cancer, while other gene mutations are variable. For example, Rb will be consistently mutated in retinoblastoma (Mukherjee, 2011).

With these advances in genetics, chemotherapies, surgeries and preventions there are still many more uncertainties left in the world of cancer. Each day in small quiet labs, in buzzing hospitals, or at busy pharmaceutical companies, new discoveries and advancements are made in the fight against cancer. This is where my research picks up. In a laboratory in the Life Science II building at Southern Illinois University Carbondale, I have joined Dr. Dale Hales and other colleagues in the effort to find a prevention method and potentially a cure for ovarian cancer. In 2009, James Watson called researchers, like me and the Dr. Hales' Laboratory to action when he stated:

> "The National Cancer Institute, which has overseen American efforts in researching and combatting cancers since 1971, should take on an ambitious new goal for the next decade: the development of new drugs that will provide lifelong cures for many, if not all, major cancers. Beating cancer now is a realistic ambition because, at long last, we largely know its true genetic and chemical characteristics." (Watson, 2009).

Dr. Hales' Laboratory Research:

Since January 2015, I have been conducting cancer research in Dr. Dale Hales' ovarian cancer laboratory at Southern Illinois University Carbondale. To research ovarian cancer, our laboratory uses the laying hen as the organismal model. The laying hen is the only known spontaneous animal model that matches ovarian cancer with that in the human form, both pathologically and histologically.

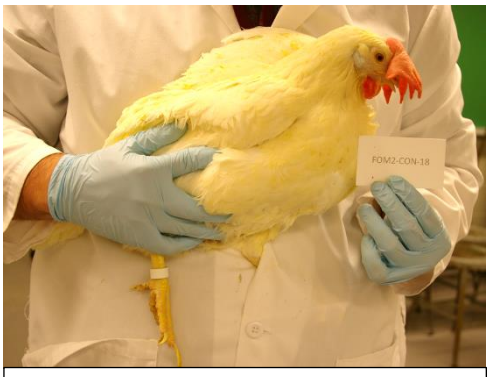


Figure 1: Control Hen 15 used in the FOM2 study that was completed in January 2015.

The hen's reproductive cycle is approximately 23 hours compared to the human 28-day cycle. The inflammatory process (a factor in the development of ovarian cancer) that coincides with ovulation is thus also more frequent in the hen model, making it a logical animal to study. Our laboratory has shown that a dietary intervention with whole flax significantly lowers both the incidence and severity of ovarian cancer in laying hens.

A whole flax diet, which is rich in omega-3 fatty acids and the phytoestrogen lignin, significantly decreases the cyclooxygenase (COX-2) expression which is the rate-limiting step in converting arachidonic acid to the prostaglandins such as PGE2 (Allaj, 2013). PGE2 is a principal regulator of inflammation. COX-2 gene (PTGS2) transcription is dependent on NFκB (nuclear factor kappa B), a principal pathway in carcinogenesis (Yamamoto, 1995). In the NFκB pathway, an inflammatory signal, such as tumor necrosis factor α (TNFα), causes IKK, a kinase enzyme complex, to phosphorylate IKB (Bastian, 2013). IKB holds NFκB inactive until it is

phosphorylated. Also, it potentially acts as a receptor for omega-3 fatty acids. It has been hypothesized that when omega-3 fatty acids are bound, IKB cannot be phosphorylated. When phosphorylation takes place and NFκB is activated, the subunits P65 and P50 enter the nucleus to transcriptionally activate a variety of target genes, such as the COX-2 gene.

The overall objective of the project was to investigate if a flax diet modulates the activity of NFκB, thus decreasing the expression of COX-2 and the production of prostaglandins. Human Embryonic Kidney (HEK-293) cells were transfected with a NFκB reporter plasmid (pNF-kB MetLuc) in order to assess the actions of inflammatory (TNFα) and anti-inflammatory (DHA) agents on the pathway. Docosahexaenoic acid (DHA) is the most biologically active of the omega-3 fatty acids derived from flax seed and is therefore a targeted interest for this project.

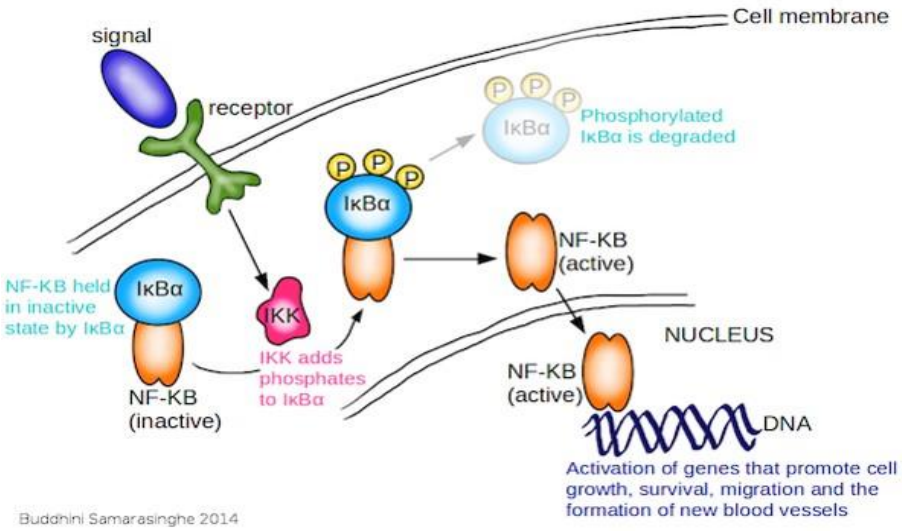


Figure 2: An inflammatory signal, such as tumor necrosis factor α (TNFα), causes IKK, a kinase enzyme complex, to phosphorylate IKB. IKB holds NFκB inactive until it is phosphorylated. When phosphorylation takes place and NFκB is activated, the subunits P65 and P50 enter the nucleus to transcriptionally activate a variety of target genes. (Image from Samarasinghe, 2014)

Transfection:

Cell transfection is the introduction of foreign DNA expression vectors into eukaryotic cells for the purpose of gene expression and experimental analysis. To transfect and optimize the transfection of the HEK293 cells, a pBabe-GFP plasmid was used. Pbabe-GFP plasmids contain a green fluorescent protein (GFP) gene which when transcribed and translated into protein can be visualized through the use of fluorescent microscopy. This property allows for easy analysis of the transfection efficiency.

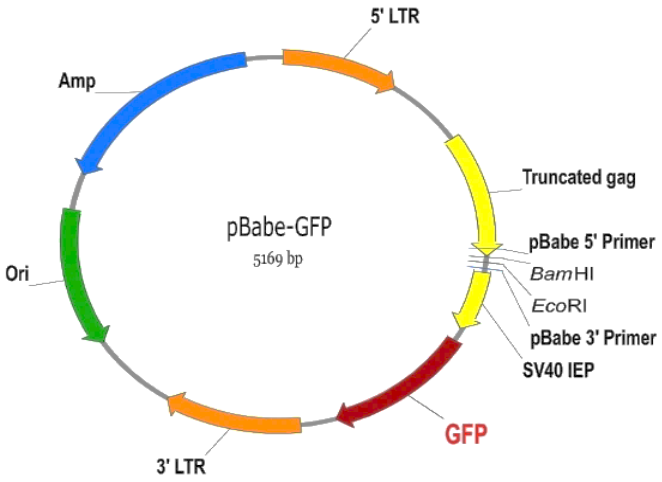


Figure 3: pBabe GFP plasmid map: This plasmid was used to optimize transfection protocol. pBABE-GFP was a gift from William Han (Addgene Plasmid #10668)

Several different transfection methods were tested to obtain a consistent and efficient transfection protocol for the HEK-293 cells. The first method used was Lipofectamine3000. This reagent is a lipid based transfection reagent that encapsulates the plasmid and allows the whole complex to pass through the cell's lipid membrane ("Lipofectamine 3000 Reagent", 2016). The second method tested was the use of polyethylenimine (PEI). PEI is a

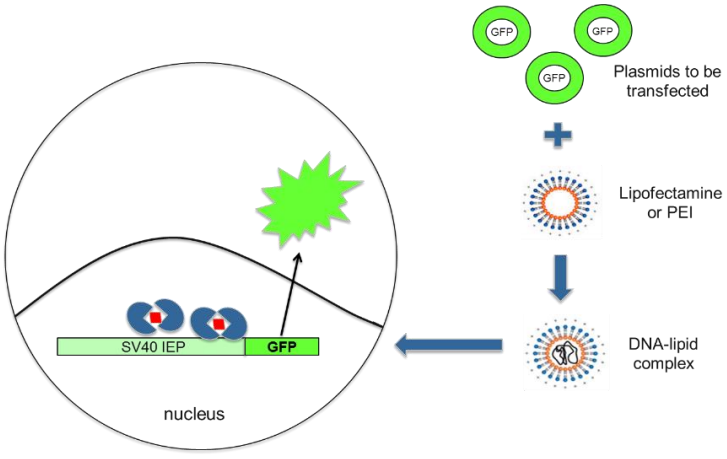


Figure 4: Model of transfection mechanism using Lipofectamine or PEI

stable cationic polymer (Boussif) that condenses DNA into a positively charged complex that can bind to cellular surface. The complex can then be endocytosed by the cells and the DNA released into the cytoplasm (Sonawane, 2003). Once in the cytoplasm it can be carried into the nucleus, transcribed and translated into proteins.

To study the NFκB pathway, a pNFκB- MetLuc reporter plasmid was transfected into HEK-293 cells. The pNFκB-MetLuc reporter has a unique feature that allows the monitoring of the NFκB activation over time by collecting the cell culture medium without lysing the cells. The plasmid includes an enhancer element that has a four-tandem repeat of the NFκB binding sequence. Then downstream, is a secreted luciferase gene from the Metridia longa. This is a marine copepod, a type of small crustacean. When a transcription factor binds to the NFκB enhancer element, the Metridia Luciferase (MetLuc) is also expressed and secreted into the medium. Because the activation can be monitored through the collection of the cell culture medium, the experiment can be conducted over a range of timepoints to see when maximum activation occurs ("pNFκB-MetLuc2-Reporter Vector Information", 2010).

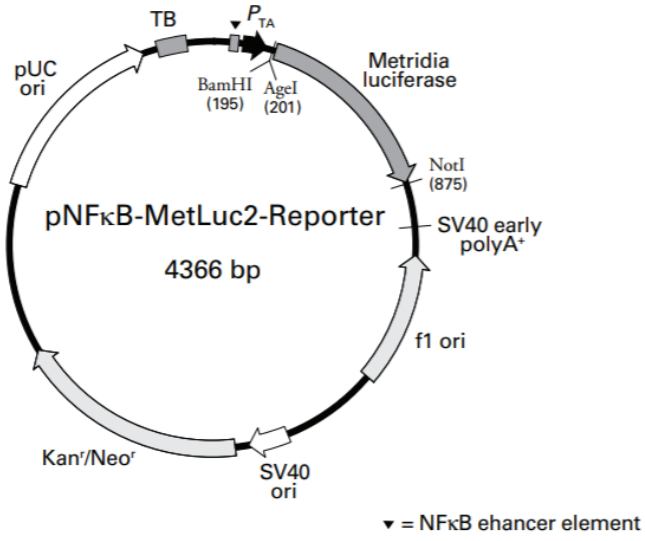


Figure 5: pNFκB-MetLuc2 Reporter Plasmid Map ("pNFκB-MetLuc2-Reporter Vector Information.", 2010).

The Renilla luciferase gene was used as a potential normalizer in these experiments. This gene is constitutively expressed meaning that it is constantly active because of the action of the

SV40 Early Enhancer/promoter. Therefore, its activity will be proportional to the overall activation of the cells. It will also take into account the transfection efficiency of the experiment. This gene has been shown to be a good normalizer (Jiwaji, 2017). The downside of this normalizer is that it is not secreted like the pNFκB- MetLuc reporter plasmid. Therefore, the cells must be lysed in order to analyze the activity.

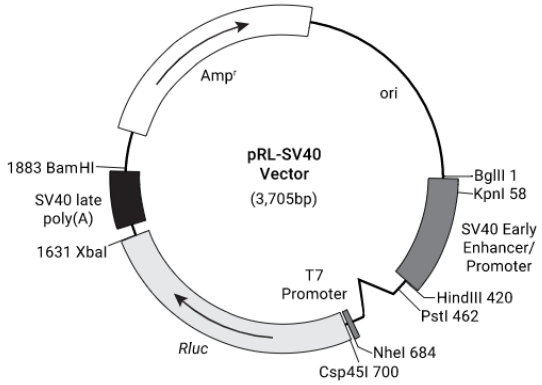


Figure 6: pRL Renilla Luciferase Control Reporter vector map

Materials and Methods:

Cell Culture:

HEK-293 cells were grown in cell culture. The media in which they grew consisted of 90% DMEM, 10% Fetal Bovine Serum (FBS) and 0.5% of Antibiotic Antimycotic solution of penicillin and streptomycin. Once the cells became confluent, they could be split and seeded into a six-well cell culture dish in preparation for transfection or into another dish for passaging and maintaining the cell line. The cells were split by washing the cells with 1x Phosphate-Buffered Saline (PBS). Trypsin was added to the cells and they were allowed to incubate for approximately two minutes. Then, 2 mL of media was added to the cell culture plate to neutralize the effects of the trypsin. The mixture of trypsin, media and cells were removed from the plate and placed in a conical tube. The mixture was centrifuged for approximately two minutes. The cells formed visible a pellet at the bottom of the conical tube. The supernatant was removed and

1 mL to 4mL of new media was added to the pellet depending on the size of the pellet. The pellet was then mixed well by gently pipetting the mixture until well mixed. The cells were counted using a hemocytometer. The cells were then added to each of the six wells along with 2 mL of media. The cells were incubated at approximately 37°C and 5% CO_2. To preserve the cells in a -80ºC freezer, the suspended cells were mixed with 10% DMSO as a cryoprotective agent.

Lipofectamine3000 Transfection:

In one microcentrifuge tube mix DNA, 5µL p3000 reagent and 150µL of media (DMEM and 10% FBS). In an additional microcentrifuge tube add 4.5µL of Lipofectamine3000 to 150µL of media. Mix. Then add the DNA mixture to the Lipofectamine3000 mixture and let incubate at room temperature for 15 minutes. After 15 minutes, dilute the mixture to 2mL. Remove the old media from the well of the 6-well plate and add the diluted mixture to the well. Multiply the amounts of reagents for the transfection of additional wells. Incubate for 24 hours at 37ºC and 5% CO_2. ("Lipofectamine 3000 Reagent", 2016).

PEI Transfection:

Transfection is completed in a six-well plate (surface area of 35mm per well). Add 200 µL of Opti-mem1 media to 2 µg DNA. Mix the solution well by pipetting the solution. Vortex the stock PEI (1µg/µL) and add 6µg to the Opti-mem1/DNA mixture and vortex again. Vortex the solution every 5 minutes for 20 minutes at room temperature.

Aspirate off the media that the cells were growing in and add 2mL of DMEM and 10% FBS media to each well. Be sure that the media does not contain the penicillin and streptomycin used in the normal growth media. Gently drop mixture of Opti-mem1, DNA and PEI into each well. Gently swirl plate to distribute the DNA mixture into the media. Incubate the cells in the standard cell culture incubation conditions for 24 hours. Optimum transfection efficiency can be seen between 24 hours and 36 hours.

Luciferase Assay:

After the treatment of the cells with inflammatory or anti-inflammatory agents, collect 200µL of the media at 0 hours, 6 hours, 12 hours, 24 hours, 36 hours and 48 hours. Freeze media at -80ºC after each collection. Once all samples are collected, begin the assay. Prepare the 10X substrate stock solution by dissolving the lyophilized secreted luciferase substrate in substrate buffer (250 µL). Store the 10X Substrate Stock Solution at –20° C. Then prepare the 1X Substrate/Reaction Buffer by diluting the 10X Substrate Stock Solution 1:10 in Reaction Buffer. To calculate the total volume of 1X Substrate/Reaction Buffer needed in your experiment, multiply the number of samples in your experiment by a factor of 5. Then transfer 50 µL of culture media from each sample into one well of a 96-well plate. Add 5 µL of the 1X Substrate/Reaction Buffer mixture to each sample well. Seal the plate and vortex the plate. Then spin down the 96-well plate in a centrifuge. Transfer the plate to a luminometer and record light signals immediately. Read the plate again 10 minutes after the initial addition to confirm results.

Renilla Assay:

Lyse the experimental cells using a 5X lysis buffer diluted to 1X with autoclaved water. Add 500µL of the 1X lysis buffer to each of the wells in the six-well plate. Incubate on a rocker for 15 minutes and remove the lysate from each well into microcentrifuge tubes ensuring all wells are clear of cells. At this point, the lysate can either be stored at –80°C or can be used to begin the assay. For the assay, a Stop and Glo Assay Kit is used. Follow the instructions with the kit and mix the 50X Stop and Glo reagent with the buffer provided to dilute the solution to 1X. Then add 20µL of the lysate to 75µL of the 1X reagent to a 96-well assay plate. Seal the plate and vortex the plate. Then spin down the 96-well plate in a centrifuge. Transfer the plate to a luminometer and record light signals after a 30 second shake.

Protein Assay:

Concentration (µg/µL)	**BSA (µL) (2mg/mL)**	**Buffer (µL)**
Blank	0	10
2	1	9
4	2	8
6	3	7
8	4	6
12	6	4
16	8	2
20	10	0
Sample	2 of sample	8

Start a protein assay by thawing samples on ice and creating a standard curve. To make a standard curve, pipette known amounts of protein (BSA (2mg/mL)) and dilute with buffer (0.1% SDS in 1X PBS) in a 96-well plate. Perform this portion using the amounts seen in the table to the left of BSA and 0.1% SDS in 1X PBS. After pipetting the standard curve samples, add 2µL of the sample and 8µL of 0.1% SDS in 1X PBS into subsequent wells. Using the BCA protein assay reagents A and B (from Thermo Scientific) make a 50:1

dilution of reagent A:B so that you can add 200µL of the mixture to each well (standard and samples). After adding the reagents, incubate at 37ºC for 30 minutes and read the absorbance of the samples. Be sure that the R^2 value of the standard curve is above .99 for accurate estimations. Use the results and the standard curve to calculate the concentration of protein.

Results:

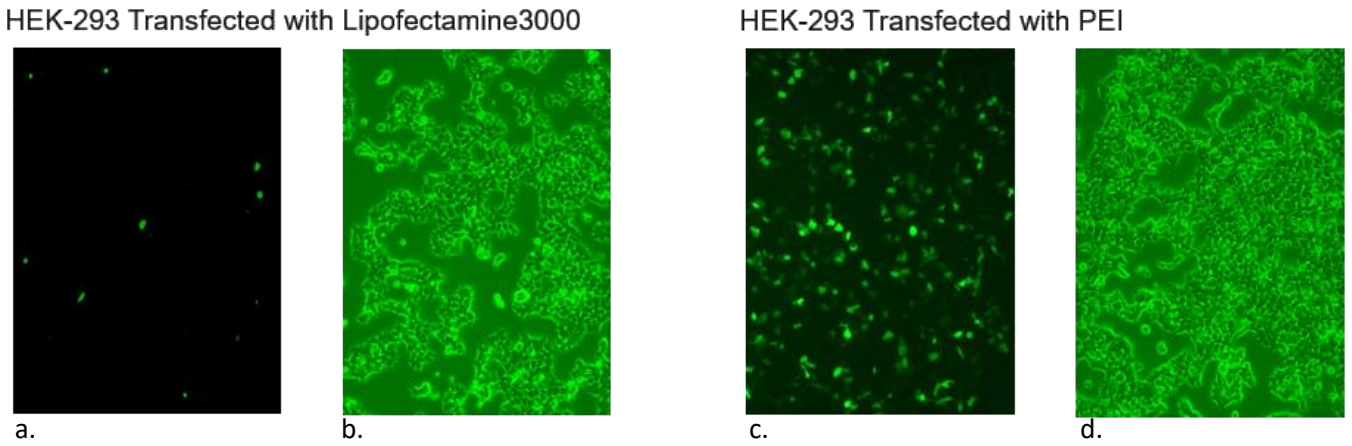
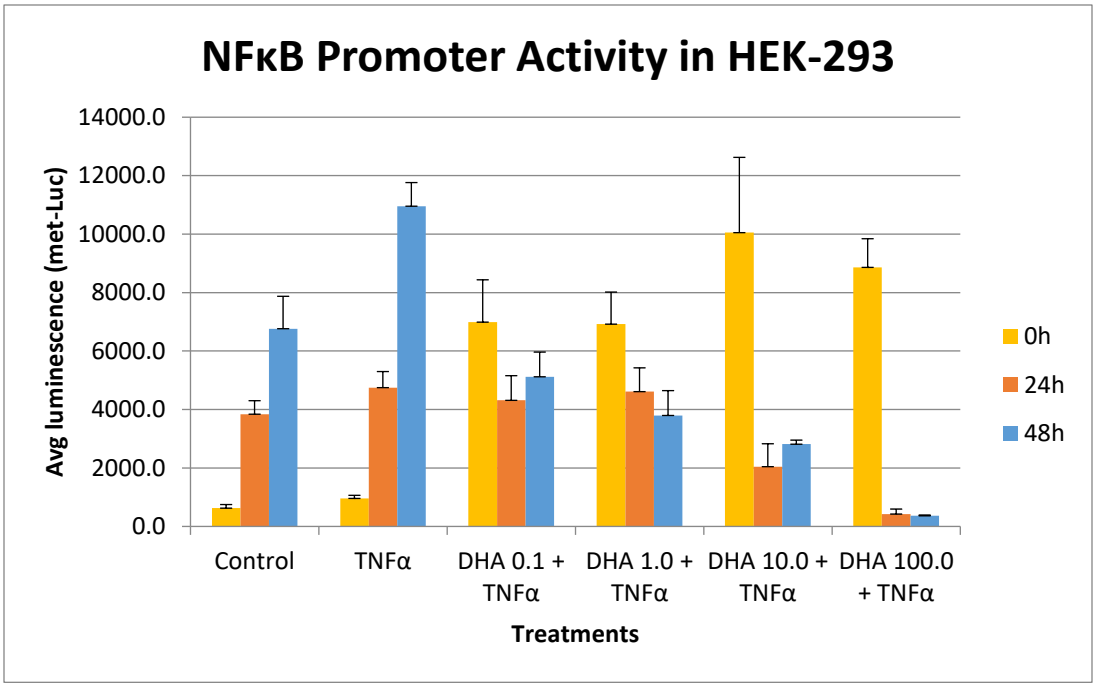
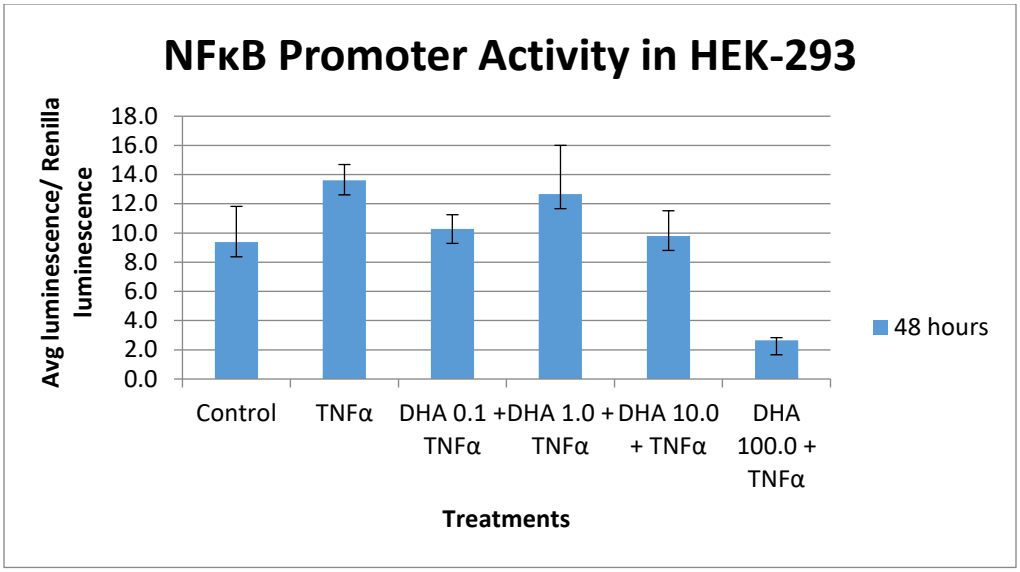


Figure 7: HEK-293 Transfection Results: a. HEK-293 cells transfected with pBABE GFP using the Lipofectamine 3000 protocol seen under a fluorescent microscope. A minimal number of cells were successfully transfected using this method. b. The bright field view of a. for comparison. c. HEK-293 cells transfected with pBABE GFP using the PEI protocol seen under a fluorescent microscope. A significantly greater number of cells were successfully transfected using this method. d. The bright field view of c. for comparison.



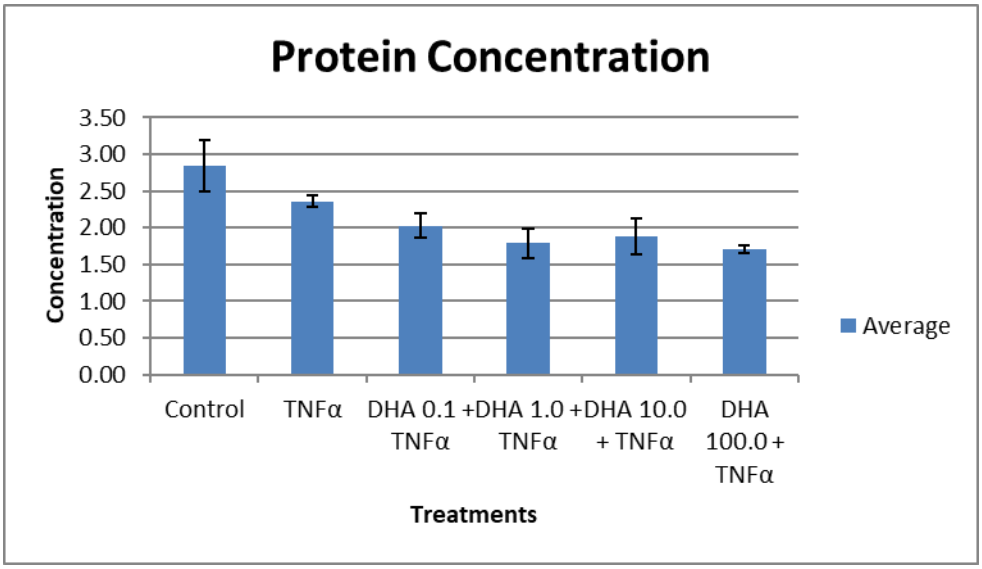
AVERAGE	Control	TNFα	DHA 0.1 +TNFα	DHA 1.0 +TNFα	DHA 10.0 +TNFα	DHA 100.0 +TNFα
0h	626.25	959.00	6991.00	6921.00	10051.75	8862.33
24h	3840.50	4749.50	4314.50	4612.00	2045.50	425.50
48h	6765.25	10956.67	5119.67	3797.25	2814.33	364.00
SD						
0h	212.47	182.31	2504.56	2194.48	5148.22	1697.78
24h	921.89	1098.34	1685.84	1623.84	1564.99	339.55
48h	2215.70	1397.20	1466.84	1697.78	241.22	55.18
SEM						
0h	122.67	105.26	1446.01	1097.24	2574.11	980.21
24h	460.95	549.17	842.92	811.92	782.50	169.78
48h	1107.85	806.67	846.88	848.89	139.27	27.59

Figure 8: The average luminescence was plotted against the different treatments at 0 hours, 24 hours, and 48 hours. It can be seen that as the dose of DHA increases, it significantly decreases the effect of the TNFα. The averages, standard deviation and standard error can be seen in the tables. It was expected that there should be equal luminescence at 0 hours in each of the groups. This experiment will need to be conducted again to confirm the results of this experiment.



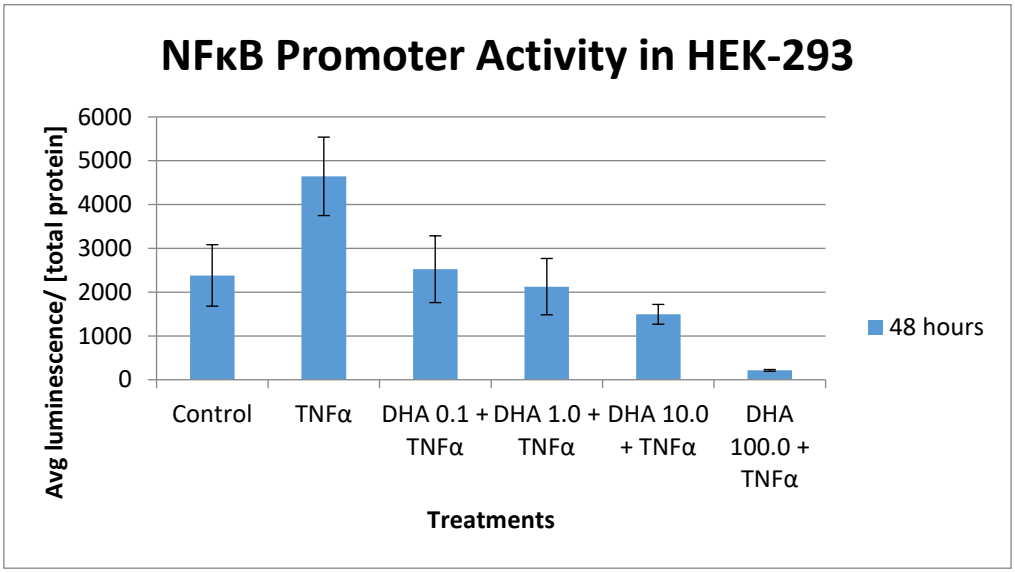
48 Hours	Control	TNFα	DHA 0.1 +TNFα	DHA 1.0 +TNFα	DHA 10.0 +TNFα	DHA 100.0 +TNFα
Normalized Average	9.37	13.61	10.29	12.67	9.81	2.66
SD	4.91	1.87	1.67	5.78	3.44	0.30
SEM	2.45	1.08	0.97	3.34	1.72	0.18

Figure 9: The 48 hour pNFκB- MetLuc Reporter Assay data normalized to the Renilla activity. Renilla luminescence was assayed and then used to normalize the data. It was determined that the Renilla was an inadequate normalizer due to the great variation in the data.



48 Hours	Control	TNFα	DHA 0.1 +TNFα	DHA 1.0 +TNFα	DHA 10.0 +TNFα	DHA 100.0 +TNFα
Average (µg/µL)	2.84	2.36	2.03	1.79	1.88	1.71
SD	0.70	0.16	0.32	0.39	0.48	0.09
n	4.00	4.00	4.00	4.00	4.00	4.00
sqrt(n)	2.00	2.00	2.00	2.00	2.00	2.00
SEM	0.35	0.08	0.16	0.19	0.24	0.05

Figure 10: Total protein content of cells after 48 hours of treatment. A protein assay was performed to determine the concentration of total protein. A standard curve was used to determine the concentrations. The average of the 4 samples per treatment group was taken and the standard deviation and SEM was also determined. This data appears to be a strong normalizer since the protein content across the treatment groups is similar. There was a slight decrease of protein in the higher concentrations of DHA where some cell death was observed.



48 Hours	Control	TNFα	DHA 0.1 +TNFα	DHA 1.0 +TNFα	DHA 10.0 +TNFα	DHA 100.0 +TNFα
48h Average	6765.25	10956.67	5119.67	3797.25	2814.33	364.00
[protein] (µg/µL)	2.84	2.36	2.03	1.79	1.88	1.71
normalized value	2381.18	4643.98	2522.73	2125.44	1494.35	213.33
SD	701.17	895.76	762.17	643.39	225.61	20.77
SEM	8.52	8.56	10.65	10.44	4.25	1.09

Figure 11: The 48 hour pNFκB - MetLuc Reporter Assay data normalized to the total protein concentration of each of the treatment groups. This method of normalization is a stronger normalizer since the data across the groups is similar.

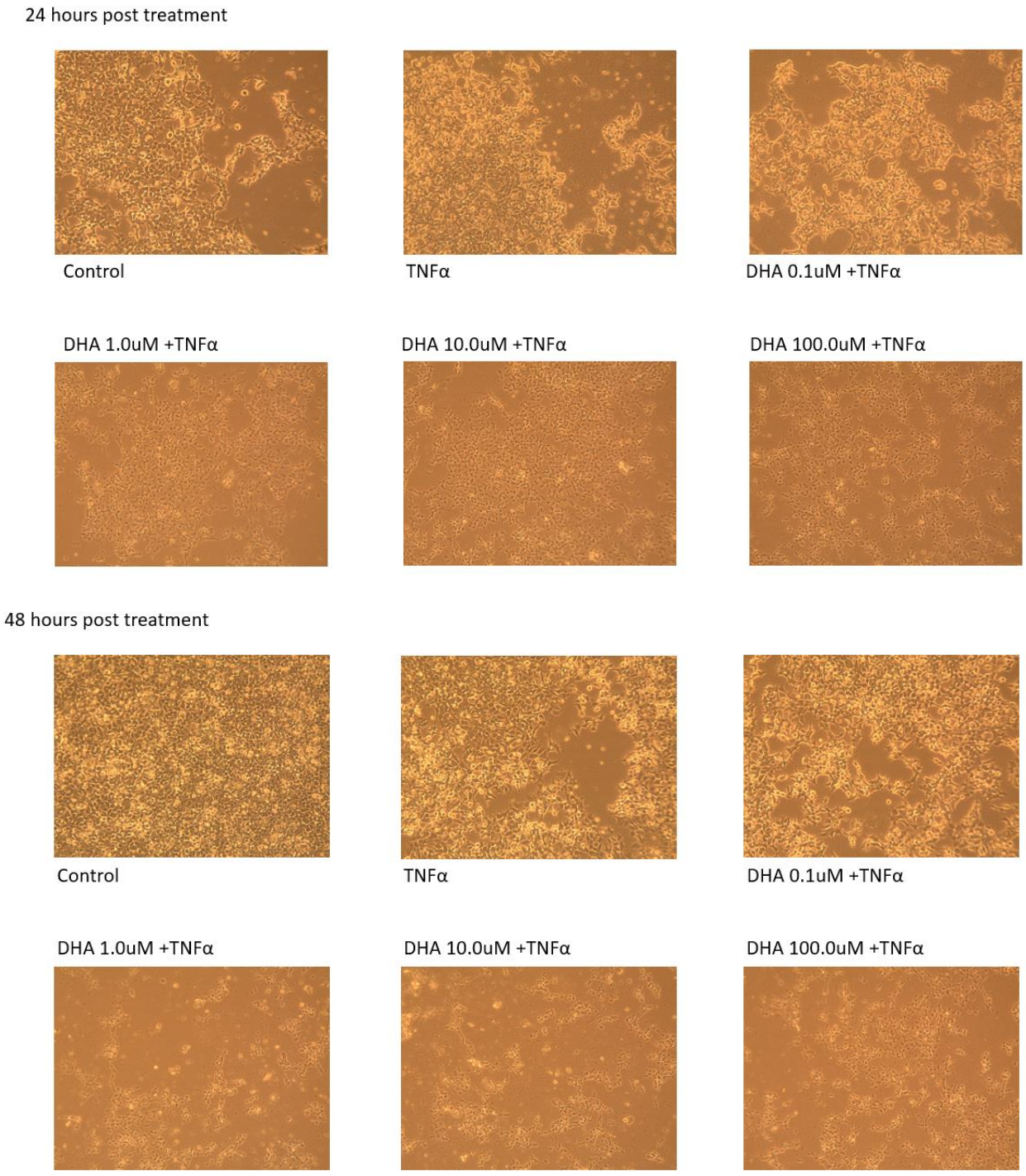


Figure 12: Images of the HEK-293 cells 24 hours and 48 hours after treatment of TNFα and DHA. The DHA treated cells show a decrease in cellular viability as the dose of DHA increased.

Discussion:

Transfection:

The transfection optimization of the HEK-293 cells was completed through a series of experiments utilizing different protocols. The images in Figure 7 are the optimized results of each of the two main protocols (Lipofectamine3000 and PEI). Variables tested within each protocol for the optimization of the protocol include varying the ratio of the reagent to DNA, the confluency of the cells, the length of time after transfection before visualizing the cells, and the amount of media diluting the reagents. Each of the experiments where repeated to ensure accuracy of the results. It was found that the PEI transfection protocol using a 6:2 ratio of PEI: DNA, 2mL of media total per well and a cell count of $5x10^4$ cells per well when seeded 24 hours before transfection proved to be the optimum conditions for the transfection of HEK-293 cells. It was also observed that 24 hours after the initial transfection, the highest transfection efficiency was observed.

pNFκB- MetLuc Reporter Activity:

This experiment utilized the optimized transfection protocol using HEK-293 cells and PEI described above. The cells were seeded for transfection on day one at $5x10^4$ cells per well. After 24 hours, the cells were transfected with the pNFκB- MetLuc plasmid and the Renilla Luc plasmid. 24 hours after transfection, the cells were then treated with nothing (control only replacing the media), TNFα (10μL of 100ng/μL), 0.1μM DHA + TNFα, 1Mm DHA + TNFα, 10μM DHA+ TNFα, and 100Mm DHA + TNFα respectively. The media was then collected from

the wells at 0 hours, 24 hours and 48 hours. At 48 hours, after the media was collected, the cells were lysed. The assays were then completed utilizing the collected samples.

Figure 8 shows the average luminescence plotted against the different experimental treatments at 0 hours, 24 hours, and 48 hours. It can be seen at 24 hours and at 48 hours that as the dose of DHA increases, it significantly decreases the effect of the TNFα on the pathway. The results can be more clearly seen when looking at the 48-hour data. The averages, standard deviation and standard error can be seen in the tables below the graph. It was expected that there should be equal luminescence at 0 hours in each of the groups. That, however, was not the results that were obtained. This was puzzling because at that point the cells did not have time to secrete any luciferase into the media. This experiment will need to be conducted again to confirm the results of this experiment and to answer any additional questions.

The data shown in Figure 8 was not normalized and therefore many assumptions are being made with this data. Assumptions being made include the number of cells in each well is consistent and the transfection efficiency in each well is exactly the same. To normalize the data, the cells were also transfected with Renilla luciferase. This type of luciferase is not secreted from the cells and can only be assayed after the cells were lysed at 48 hours. Therefore, only the 48-hour data can be normalized. Using the normalized 48-hour data, assumptions can be made about the other timepoints. Figure 9 shows the 48-hour data normalized to Renilla. Once the Renilla assay was completed, it was determined that the Renilla was not an adequate normalizer. The reason this was chosen in the first place was that Renilla is continually expressed. However, in this experiment, it was clear that it was not equally expressed in all of the samples. There was significant variation across all samples of the experiment, even the samples within the same treatment group.

After the results of the Renilla assay came back varied, another normalizing technique was used. An assay of the total protein concentration was performed. Using the same cell lysate from the 48 hour samples used in the Renilla assay, a basic protein assay was conducted. A standard curve was created and the protein content of each sample was determined based on the standard curve. Figure 10 shows the average total protein content of the samples from each experimental group. The protein content was relatively equal across the groups, meaning that it is a good normalizer. There was a slight decrease in protein content as the amount of DHA increased. This was expected based on the images of the cells at 48 hours, seen in Figure 12. Finally, the 48 hour pNFκB MetLuc assay data could be normalized using the total protein content. This normalized data, seen in Figure 11, indicates that the DHA significantly decreases the activity of the NFκB pathway, after amplification of the pathway by TNFα, in a dose dependent manner. This experiment will need to be repeated two more times in order to confirm the promising results seen in this study.

Conclusion:

With the promising information obtained in this study, the experiment should be repeated in order to confirm the obtained data. Through the NFκB pathway, it is thought that omega-3 fatty acids, such as the DHA in flax seed, could help in reducing the inflammatory response associated with ovarian cancer. We propose that flax seed acts via DHA-inhibition of NFκB activity which is likely important to the inhibition of PGE2 production. Inhibition of PGE2 is critical to flax seed amelioration of ovarian cancer. Further studies could include an assay of the PGE2 levels in the same cellular model used in this experiment. The effects of DHA could then

be studied in vivo in the chicken model used in our laboratory. After further studies and clinical trials, the consumption of flax seed or the omega 3 fatty acids associated with flax seed could be used as a critical preventative method for ovarian cancer in women.

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