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Professor Sharma

Summer Internship

30 July 2012

Summer 2012 Internship Report

At the end of the 2011-2012 school year, the Science Research Internship Fund granted an award to me for summer research. This internship gave me the opportunity to study under Dr. Bethel Sharma for a period of ten weeks. During this time, we researched the effects of calcium ions in both diseased and healthy pulmonary fibroblasts. The diseased cells were obtained from a patient with a condition called Idiopathic Pulmonary Fibrosis (IPF). IPF is a deadly disease that belongs to a class of disorders known as Interstitial Lung Diseases, all of which lead to progressive scarring of the lungs. As of now, there is no known cure, and only palliative treatments are available. Fibrosis occurs when fibroblasts differentiate into myofibroblasts in order to heal a wound or repair damaged tissue, and due to some unknown circumstance, the myofibrolasts fail to die or revert back to fibroblasts once the wound is healed. Then, these myofibroblasts begin to accumulate in large amounts creating scar tissue, hindering whatever organ the fibrosis is occurring in. In our case, this scar tissue accumulates in the lungs, severely hindering the affected's lungs. It is believed that the nitric oxide synthase (NOS) plays a role in the advancement of fibrosis. Likewise, we believe it may play a role in the pathogenesis of IPF. To understand this further, we decided to examine the effects of calcium in IPF cells, as it affects the function of some forms of nitric oxide synthase. Furthermore, some literature has suggested that calcium chelators like ethylene

glycol tetraacetic acid (EGTA) can induce apoptosis (cell death) in cancer cells. From this evidence, we have hypothesized that different levels of calcium could have substantial affects on the growth and development of fibroblasts.

I began the summer by growing both CCL-135 (healthy) and CCL-191 (diseased) pulmonary fibroblasts. These cells were obtained from a liquid nitrogen container, and thawed at 37 º C in a warm water bath. The quicker the cells are thawed, the less cell death occurs. Once the cells are thawed, they are transferred to a small flask where they will be grown in 10% FBS in DMEM/F12 cell media. Fetal Bovine Serum (FBS) contains many of the necessary nutrients for the cells to proliferate. Without the FBS, or what we call 0% media, the cells will survive, but not proliferate. This allows us to control the conditions in which we test the cells. All cell culturing is performed inside a sterile hood that prevents contamination from reaching the cells. The cells are incubated at 37 º C with 5% CO_2 and a high humidity. The cells are given new media every 3 days, and are passaged into new flasks once they reach 60-70% confluence. After five or six passages, the cells are ready to be treated and used for experiments.

For the most part, our treatments were with $CaCl_2$ and EGTA. $CaCl_2$ would increase the amount of calcium in the media and inside the cells, whereas EGTA would decrease the amount of calcium inside the cells by forcing it to stay in the media. Using different concentrations of the treatments, we would allow the cells to incubate with the treatments for 24 hours, and then we would perform our experiments.

Many of our treated CCL-135's were used for western blotting. First the cells are lysed (burst open) and then run on a SDS-PAGE gel. This gel captures the proteins and s separates them based on relative size. Next, the proteins are transferred to a PVDF

membrane. This membrane is exposed to antibodies that bind to the specific protein we are trying to analyze. Once they are bound, the membrane can be treated to show the specific protein. This gives us information on how much of the specific protein is expressed relative to the same cells under different conditions. Our interest this summer was in CTGF, α-SMA, and TGF-ß. Tubulin was used as our housekeeping gene, to make sure that the protein expression was due to our treatments, and not a nonspecific circumstance. With varying degrees of success, we found a few patterns in protein expression. The blot for CTGF suggested that the protein is more highly expressed in .5 mM and 1 mM $CaCl_2$ treated cells in 0% media. But, more research into this finding must be done to confirm our inquiry.

Immunocytochemistry was performed on CCL-135 in an attempt to provide the second half of the experiment that was begun last summer. The cells were treated with varying levels of EGTA and $CaCl_2$ and then stained with antibodies that bound to α-SMA (green), f-actin (red), and the nuclei (blue), as shown in figure 1 below.

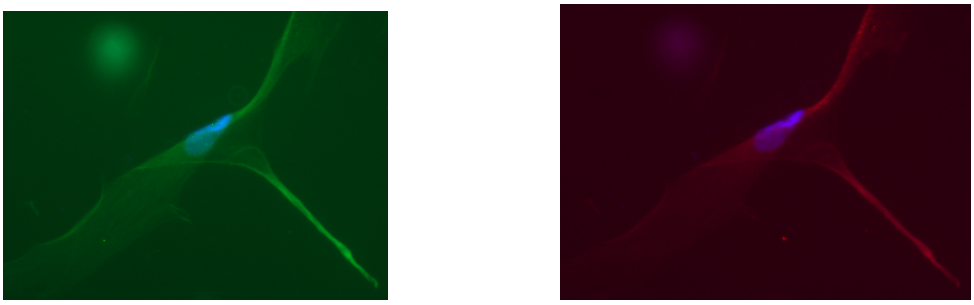

Figure 1: CCL-135, passage 6, grown in 0% media, no treatment.

Figure 2: CCL-135, passage 6, grown in 0% media and treated with overnight with 2 mM EGTA.

This was done to see if the fibroblasts had differentiated into myfibroblasts. A defining marker of a myofibroblast is high α-SMA expression. One can peer into a fluorescence microscope and determine if the cells have differentiated based on the amount of α-SMA expressed. It appeared as if the more $CaCl_2$ given to the fibroblasts, the more α-SMA was expressed. This means that higher levels of calcium may cause the cells to differentiate into myofibroblasts. Likewise, the more EGTA introduced into the cells, the less α-SMA was expressed. This means that low levels of calcium may have prevented differentiation.

Scratch Wound Assays were performed to determine the motility of the cells under varying concentrations of EGTA and $CaCl_2$. Our successful scratch wound assays were performed on CCL-135. This assay required a scratch to be made in the confluent layer of cells, and pictures were taken at different time periods to record the amount of cell movement into the wound. A trend was found that the more calcium added, the more motile the cells were. Likewise, the more calcium that was pulled out of solution, the less motile the cells were.

A cell cytotoxicity assay was performed to determine if certain concentrations of treatment were killing the cells, which may end up skewing our results. The cells were

treated with varying levels of $CaCl_2$ and EGTA and then treated with antibodies that could be detected by the fluorescence microscope. Cells that were dead would appear with a red stained nuclei, and cells that were alive would appear with green stained cytoplasm. We found that higher concentrations of all treatments tended to result in more cell death, with each one having a threshold that resulted in all of the cells dying. This occurred at around 2 mM EGTA, and 5 mM of $CaCl_2$. With that, we know where to stop our treatments before our data becomes skewed by cell death.

Over the summer, Scott Ward and I had the pleasure of researching ways to combat this terrible illness. Although we spent all summer researching this topic, there is still much to be done to confirm out hypotheses. I hope that the information that was gathered can be used to further Dr. Sharma's research, and eventually create a breakthrough in the battle against IPF. In the future, I would like to see experiments run to confirm the trends that we have seen, and further our research in a way that may one day find a way to revert myofibroblasts back into fibroblasts, or even cause the myofibroblasts to commit apoptosis.

This research has given me insight into the early stages of drug development, which is basically discovering what treatments affect the cells in what ways. These early stages are essential to deciding what treatments should be pursued further. This information is very broad, but very critical in developing targeted therapies. Furthermore, this research has confirmed my interest in drug development. As of now, I plan to pursue drug development as a potential career option.