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Summer 2011 Internship Report

For the summer of 2011, I was awarded funding by the Greene Science Research Internship Fund, giving me the opportunity to work under Dr. Bethel Sharma in the Chemistry Department at Sewanee. Our focus was the study of Idiopathic Pulmonary Fibrosis (IPF). IPF is a devastating illness belonging to a class of disorders known as Interstitial Lung Diseases, all of which lead to progressive scarring of the lungs. Why are we so concerned? There is currently no cure for this terrible disease. Patients typically only live three to five years after diagnosis. [2] Unfortunately, doctors do not know what causes IPF. Because nitric oxide (NO) has been shown to be one of the factors implicated in the advancement of fibrosis in the heart, we believe it may contribute to pulmonary fibrosis in the lungs. This summer we have focused on the impact of calcium, which affects the function of nitric oxide synthase (NOS), the enzyme that produces NO. Earlier literature suggests that calcium chelators such as EGTA induce apoptosis; therefore, our hypothesis is that the presence of calcium impacts apoptosis (programmed cell death) in myofibroblasts. [1]

Throughout the summer, we have cultured two cell lines: human lung fibroblasts from a healthy patient (CCL-135 LL 47 MaDo) and human lung fibroblasts from a patient suffering from idiopathic pulmonary fibrosis (CCL-191 LL97A AlMy). We kept the cells growing in an incubator until it was time to perform an experiment. We would feed them with growth media, which was replaced every three days to remove waste and add nutrients. After about 3 days of growing, we would passage (split) the cells to give them more space to continue growing. One flask was turned into three new flasks. The cells would then continue to grow.

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Once the cells were approximately 70-80% confluent, we transferred the cells to 12-well plates to grow onto small glass cover slips. Half of the cells remained in 10% growth media, while the other half were given 0% growth media. The difference between 0% growth media and 10% growth media is that 0% lacks Fetal Bovine Serum (FBS), which contains necessary growth factors for proliferation. After the cells were sorted into their growth medias, we decided which treatments we wanted to test. Since we focused on calcium, we added different concentrations of $CaCl_2$ and EGTA (a calcium chelator) to our wells.

The next day we would perform an experiment called Immunocytochemistry. We fixed the cells with 4% paraformaldehyde. Then, the cells were permeabilized with Triton x-100 (0.2% in PBS). The detergent, triton, was then aspirated off, and the cells were washed three times with PBS. The cells were then washed two times with 10% goat serum in PBS. An Oregon Green 488 phalloidin was pipetted onto the cells and after thirty minutes of incubation, the cells were washed three times with PBS once again. Then, the primary antibody (Abcam Anti-αSMA) was applied. This antibody was incubated for 1 hour, and then the cells were washed. The secondary antibody (Invitrogen Alexa Fluor 680 donkey anti-rabbit) was then applied. This antibody also incubated for an hour and then the cells were washed with more PBS. After the fixation of the cells, the cover slips were mounted onto glass slides using DAPI (nucleus stain) containing mounting media. The cells were then ready to be observed under the Zeiss Observer.A1 Axio series microscope. The microscope should have shown us pictures of the cell's nucleus

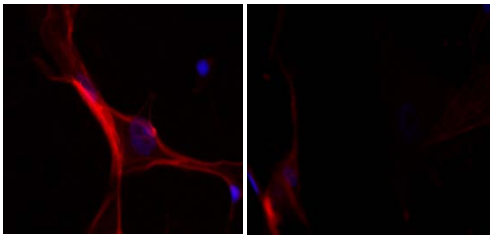
surrounded by alpha-smooth muscle actin (α-SMA) in the cytoplasm. The presence of α-SMA defines the cells as myofibroblasts, the overactive cells that cause IPF.

In order to show that our cells actually do have α-SMA, a process called electrophoresis was performed using lysates from cells treated with calcium and EGTA (calcium counteractive). An SDS-PAGE gel was run to separate the proteins by size. The proteins were transferred electrophoretically onto a membrane. Then we used a technique called western blotting to verify the presence of a particular protein. For western blotting, we blocked the membrane overnight in 1% milk to prevent non-specific interactions between the membrane and the antibody used to detect the protein. A primary antibody (α-SMA) was used first to bind to a protein of interest. Then the secondary (anti-rabbit alkaline phosphatase) was applied. It should bind to one of the primary antibodies. Last, an alkaline phosphatase substrate was added onto the membrane after being washed. A purple band indicates where the secondary antibody bound. We should be able to see a band that indicates that α-SMA is present, which confirms that what we see in our photos from the immunocytochemistry protocol is α-SMA as well.

Fibrosis is caused by the differentiation of fibroblasts into myofibroblasts. This process normally occurs in any wound repair. The myofibroblasts pull together to allow the skin to reform around an open wound. However, in this case of IPF, the myofibroblasts that are pulling produce the scarring of the lungs because once their job is done they are not reverting back into fibroblasts or experiencing apoptosis. This produces an overabundance of myofibroblasts, which produce excess collagen causing damage to the structure of the lung. Certain factors that lead to the proliferation of fibroblasts are TGF-β1, CTGF, and low calcium. EGTA is what we have

been interested with the most this summer, because it has binding sites that bind to calcium, not letting calcium enter the fibroblasts. According to our experiment, an increase in EGTA leads to proliferation or migration of the fibroblasts into protomyofibroblasts and then myofibroblasts at higher concentrations in CCL-191 cells. We treated the CCL-191 lung cells with calcium and took pictures of the immunostained cells. We saw that an increase in calcium concentration inhibits growth of myofibroblasts or α-SMA at high concentrations. Since high concentrations of calcium cause a decrease in α-SMA, then we can make conclusion that calcium may help revert myofibroblasts back into fibroblasts. The photos of the diseased cells (CCL-191) below show how without calcium, there is more α-SMA than the one treated with calcium.

No Ca^{2+} Added 1mM Ca^{2+}



Throughout the summer, I learned so much and had a lot of fun doing so. I feel like we accomplished a good amount. We still have more experiments to do in the future. More Western blots of the CCL-191 (diseased cells) need to be done. Also, we need to perform immunochemistry on the CCL-135 (healthy cells) to completely understand the differences between the two cell lines. We had some good photos of the CCL-191 cells showing that when there is an increase in calcium, there is a decrease in α-SMA or myofibroblasts, indicating that the myofibroblasts may be reverting back into fibroblasts or dying. Our hypothesis stated that

calcium impacts apoptosis in myofibroblasts. This experiment needs to be repeated to confirm that it is accurate, but we do believe that calcium levels do impact the myofibroblasts. Determining how and where in the cells this occurs will help to find potential targets for treatment of IPF in the future.

I have worked on this project for a year now, and it has really boosted my interest on becoming a doctor. I have become more acquainted with the materials in the lab and the experience has taught me so much. My major is biochemistry and my goal is to go to medical school. The experience I have had for the past year has given me an insight on what my future may be like.

References

1. Kluck, Ruth, Catherine McDougall, Brian Harmon, and June Halliday. "Calcium chelators induce apoptosis-evidence that raised intracellular ionised calcium is not essential for apoptosis." (1994): 247-254. Web. 3 November 1993.
2. "What is Pulmonary Fibrosis?." Pulmonary Fibrosis Foundation n. pag. Web. 28 July 2011. <http://www.pulmonaryfibrosis.org/education>.