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Role of Calcium Ions on Healthy and Diseased Pulmonary Fibroblasts from Idiopathic Pulmonary Fibrosis

This summer I researched the effect of calcium ions on both healthy and diseased pulmonary fibroblasts, a type of cell that resides in the connective tissue of the lung. The diseased fibroblasts were collected from patients with Idiopathic Pulmonary Fibrosis, an inflammatory lung disease that characteristically causes scarring of the lung tissue[1]. Pulmonary fibrosis is caused in part from the differentiation of fibroblasts into myofibroblasts in order to heal an injury. This process is a natural response to heal a wound, but fibrosis may develop when the differentiation from fibroblasts to myofibroblasts is uncontrolled. A way to control fibrosis would be to force cells into apoptosis, which is programmed cell death. Earlier research states that calcium chelators, such as ethylene glycol tetraacetic acid (EGTA) induce apoptosis in cancer cells[2]. Based on this evidence, we hypothesized that there may be an effect of calcium on our lung fibroblasts.

I grew both healthy (CCL-135) and diseased (CCL-191) lung cells in the Cell Culture Lab. I thawed a vial of cells from a liquid nitrogen cell storage tank, then transferred the cells to a flask. The cells are grown in growth media containing 10% Fetal Bovine Serum (FBS), which contains various growth factors that promote cell proliferation. The cells are incubated at 37 °C and 5% CO_2. The cells are given about 3 days to grow to at least 70% confluence before they are passaged 1:3. When the cells have been passaged four to six times, they are ready for experiments.

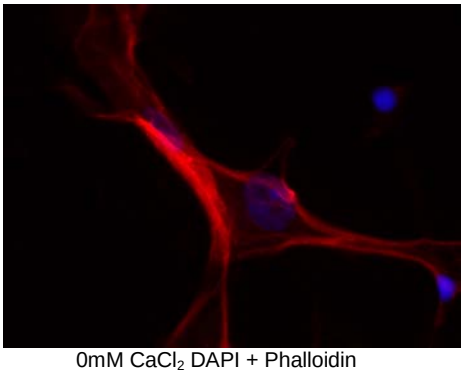
Both cell lines, CCL-135 and CCL-191, were treated with varying amounts of transforming growth factor-β1 (TGF-β1), EGTA, EDTA, $CaCl_2$, or left as a control with no

exposure to treatments. Most of the CCL-135, the healthy cell line, were lysed and run on SDS-PAGE gels. Most of the CCL-191, the diseased cell line, were immunostained and mounted on slides.

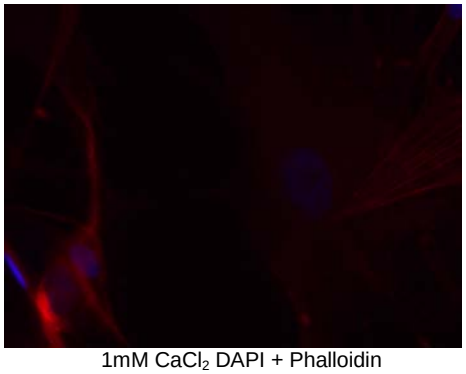
Western blotting gives information about the relative expression of a certain protein in a cell. We ran 15-well gels with samples of cell lysates that had been treated with varying amounts of TGF-β1, EGTA, EDTA, $CaCl_2$, or no treatment as a control. After running the gels and transferring the proteins to a membrane we blotted the membranes with antibodies to detect a specific protein. The primary antibodies we used were connective tissue growth factor (CTGF), TGF-β1, inducible nitric oxide synthase (iNOS) or α-smooth muscle actin (α-SMA). Using an α-SMA primary antibody will show a dark band on the transfer membrane if there is α-SMA in a quantifiable amount. Just because there is not a band there does not necessarily mean that there is not the specific protein, it means that there is not enough protein there to show as a band.

With immunostaining, we are able to see the nucleus, f-actin and α-smooth muscle actin of the cells. The fluorescence microscope shows each color separately: blue is the nucleus, green is the α-SMA, and red is the f-actin.

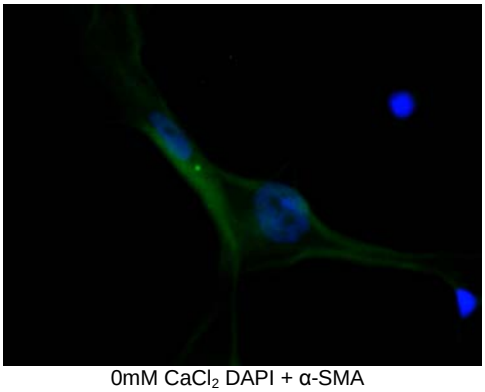
CCL-191 P.6 0% Growth Media



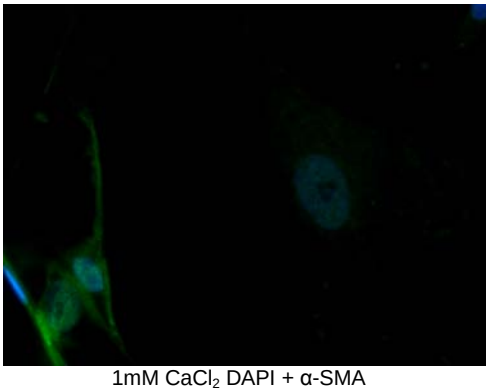
0mM $CaCl_2$ DAPI + Phalloidin



1mM $CaCl_2$ DAPI + Phalloidin



0mM $CaCl_2$ DAPI + α-SMA



1mM $CaCl_2$ DAPI + α-SMA

We use immunostaining to help determine if the cell is a fibroblast or if it has differentiated into a myofibroblast. The way to tell by using a fluorescence microscope to determine the amount of α-SMA seen. Since myofibroblasts have higher expression of α-SMA, cells that show brighter and/or more green signifies the cells are myofibrblasts, not fibroblasts. The pictures can tell us under which conditions the cells differentiate from fibroblasts to myofibroblasts. So far we see that high concentrations of calcium 1mM, 2mM, and 10mM, show a lower amount of α-SMA compared to the control which could mean that high levels of calcium may revert myofibroblasts back into fibroblasts. We will need to repeat this aspect of the experiment to ensure that our results are consistent.

From our various experiments we were able to suggest that for the cell line CCL-135, low concentrations of calcium 0 μM and 100 μM, increases the expression of TGF-β1. This is relevant to our research because TGF-β1 impacts conversion of fibroblasts to myofibroblasts[3] so if the expression of TGF-β1 increases then there will be differentiation from fibroblasts to myofibroblasts. From the immunocytochemistry procedure, we see that in the cell line CCL-191, high concentrations of calcium, 1mM, 2mM, and 10mM, there is less α-SMA visible compared to the control with no added calcium concentration. With this, we can suggest that high concentrations of calcium may revert myofibroblasts back into fibroblasts. This piece of information is really interesting

because it shows that the diseased cells can potentially be forced to revert back to fibroblasts. When this differentiation is out of control fibrosis occurs and can cause scarring in the lungs; therefore, finding a pathway involved in the reversion of myofibroblasts back to fibroblasts is a step closer to developing a treatment for Idiopathic Pulmonary Fibrosis.

Over my 8 weeks researching in the Biochemistry Lab, my research partner and I gained a lot of information and data which we hope may help to combat this devastating illness. Although a lot was done there is still much more to do over the year. I look forward to western blotting the CCL-191 cell line to see if there is a quantitative difference in α-SMA expression based on the different treatments, and immunostaining the CCL-135 cell line to see if there is a visible difference in α-SMA expression. With the data collected over this summer, we will start to look into which one of the many pathways in which calcium is involved, is affecting the α-SMA expression and TGF-β1 expression.

When the summer began I was not completely sure what type of pharmaceutical research I would like to form into a career. I was not sure at which stage of drug development interested me. The research I completed this summer was the earlier stage of drug development for Idiopathic Pulmonary Fibrosis. I cannot say without a doubt that early stage of drug development is what I have a passion for but, that does not mean it is not an option for my future career. I learned valuable information about pharmaceutical research and with more time I will learn even more about research and may come to love researching in the early stage of drug development.

[1] Diseases and Conditions Index. National Institutes of Health. May 2009. National Heart Lung and Blood Institute. July 28, 2011 http://www.nhlbi.nih.gov/health/dci/Diseases/ipf/ipf_whatis.html

[2] Kluck, Ruth M.; McDougall, Catherine A.; Harmon, Brian V.; Halliday, June W. "Calcium chelators induce apoptosis – evidence that raised intracellular ionized calcium is not essential for apoptosis." Elsevier Science (1994): 247-254.

[3] Tomasek, James J.; Gabbiani, Giulio; Hinze, Boris; Chaponnier, Christine; Brown, Robert A. "Myofibroblasts and mechanoregulation of connective tissue remoldelling." Nature Reviews. Vol. 3. (2002): 349-363.