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Andrew LaRow

John F. Harms

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CLARIFYING THE CONNECTION BETWEEN ONCGENIC K-RAS AND GASTRIN/CCK2R SIGNALING IN PANCREATIC TUMORIGENSIS

Andrew LaRow1, John F. Harms2
1Messiah College Department of Chemistry and Biochemistry
2Messiah College Department of Biological Sciences

Abstract
Pancreatic ductal adenocarcinoma (PDAC) is an extremely deadly cancer: the overall five-year survival rate is 8%. This is mainly due to the difficulty of diagnosing PDAC in its early stage: only 9% of patients are diagnosed with localized cancer. Cancer cells display rapid growth and cell division, due in part to oncogene activation and stimulation of mitogenic pathways. In PDAC, mutant K-ras, an oncogene and intracellular signaling protein, is present in 90% of all cases. Because of this high correlation and the role of mutant K-ras in PDAC, it has been repeatedly targeted for cancer therapy, but with little success. Interestingly, K-ras has been shown to increase gastrin expression in colon cancer. Gastrin is a peptide hormone also aberrantly expressed in PDAC cells. It plays a role in PDAC progression because it stimulates tumor cell proliferation in an autocrine manner, mediated through the gastrin receptor, CCK2R. It was hypothesized that K-ras increases gastrin expression in PDAC. Preliminary data showed the receptor, CCK2R, rather than gastrin, is upregulated by mutant K-ras. Further studies were undertaken using aggressive human pancreatic cancer cells as well as normal pancreatic ductal cells. No consistent pattern of altered CCK2R expression was observed, suggesting the preliminary data were not accurate. Additionally, COX-2, an enzyme downstream K-ras, showed no expression level changes after "tampering" with gastrin/CCK2R signaling. However, gene expression levels and protein levels do not necessarily correlate. Western Blotting is currently being undertaken to determine if a correlation between K-ras activation and CCK2R exists at the protein level.

Introduction

With a 5-year survival of 8%, pancreatic ductal adenocarcinoma (PDAC) is an extremely deadly cancer and is ranked fourth in cancer deaths (1). One reason for this is the difficulty of diagnosing PDAC before metastasis: only 9% of patients are diagnosed with non-metastatic cancer. As of now, the only true cure of PDAC is resection of the affected area and nearby tissues, but this is only achievable in less than 20% of patients. Even so, the cure rate after surgery is less than 25% (2).

PDAC originates in the pancreatic ductal epithelial cells. It starts as a lesion called pancreatic intraepithelial neoplasia (PanIN), which is considered to be pre-cancer (3). It then breaks through the epithelium to become invasive cancer. PDAC is the result of gene mutations. It has been shown that forty-three genes are affected in PDAC. Three key genes mutated include K-ras, CDKN2A, and TP53. In particular, the mutation and oncogenic activation of the K-ras protein has been found in 90% of PDAC cases (4).

K-ras is involved in communication between cell surface receptors and intracellular pathways. During normal cell signaling, the protein alternates between two configurations: “on” or “off”. If oncogenically activated, K-ras proteins will be “on” for a prolonged period of time leading to hyperactivation of downstream signal transduction. Interestingly, K-ras can become self-sustaining at a certain threshold meaning high levels of oncogenic K-ras allow for it to produce its own activators. K-ras is involved in pathways including apoptosis and cell proliferation (Figure 1).

Figure 1. A K-ras mutation can lead to uncontrolled cell proliferation, survival, and invasion (7).

Consequently, a mutation leading to K-ras being “on” for a longer period of time causes anti-apoptosis and uncontrolled cell proliferation (5). Due to its importance in PDAC development, inhibiting oncogenic activation of K-ras has long been pursued as a potential therapy for PDAC. Unfortunately, targeting K-ras therapeutically has been a failure (6). Because K-ras is not a surface receptor, it needs to be targeted at the transcription level. This is done with small interference RNA (siRNA), which block expression of K-ras. This process has been shown to reduce K-ras expression and decrease cell proliferation. However, RNA is quickly degraded in the blood (7). Additionally, it would be extremely difficult to inject the siRNA into the PDAC cells in vivo. In light of this, focus has shifted to targeting downstream mediators of K-ras. One such pathway includes Cyclooxygenase-2 (COX-2), the prostaglandin biosynthesis enzyme.
COX-2 is known to play an important role in cancer progression (8). COX-2 is involved in biosynthesis of prostaglandins, which are lipid compounds with hormone-like purposes. Prostaglandins are involved in vasodilation, inflammation, and other important processes. One prostaglandin of interest is Prostaglandin E2 (PGE2) due to its activity in cancer progression. PGE2 is a mediator of inflammation and its biosynthesis is highly regulated. However, unregulated PGE2 can lead to uncontrolled cell proliferation, tumorigenesis, and apoptosis. Chronic inflammation, which can be caused by unregulated PGE2, has been connected to cancer development due to DNA damage and activation of survival pathways. PGE2 has also been shown to cause anti-apoptotic behavior by upregulating an anti-apoptotic protein, Bcl-2. Additionally, anti-apoptotic behavior can be caused by PGE2 activating an AKT-dependent survival pathway. PGE2 has also been shown to govern tumor growth. It activates the downstream product Src kinase, which has been shown to increase the cell cycle progression and also prevent p27, which inhibits cell cycle progression, from functioning. Due to the role of COX-2 in cancer progression, it has been the focus of cancer therapeutics. COX-2 inhibitors have been shown to improve the efficiency of radiotherapy and chemotherapy, however it has been unsuccessful in clinical trials with PDAC patients (9). Additionally, COX-2 inhibitor drugs, such as Celecoxib, have severe side effects such as increasing the risk of heart attack and stroke (8).

In addition to K-ras and COX-2, gastrin also plays a role in PDAC. Gastrin is a peptide hormone, which, among other digestive roles, plays a mitogenic role supporting the health and maintenance of the digestive mucosa. Gastrin signaling is mediated by a G protein-coupled receptor, CCK2R, located on the surface of responsive cells. Gastrin is not expressed by normal human pancreatic ductal epithelial (HPDE) cells, but is aberrantly expressed in pancreatic cancer cells (10). Upregulation of gastrin, or its receptor, has been repeatedly implicated in tumorigenesis, driving cell proliferation by an autocrine mechanism (11-13). For example, introducing progastrin, which is a peptide that stimulates gastrin expression, created tumorigenic and metastatic behavior in embryonic epithelial cells (12). As with COX-2, gastrin expression can be inhibited with siRNA but this is extremely difficult to do in vivo. However, there is progress in finding a way to deliver siRNA into PDAC cells. Importantly, it has been demonstrated that K-ras activation and higher gastrin expression are connected (13). Colon cancer cells with K-ras mutations were shown to have high gastrin mRNA levels. When the cells were treated with K-ras mRNA inhibitors, there was a decrease in gastrin expression. Additionally, introducing oncogenically-activated K-ras increased gastrin expression. Even though this was shown in a colon cancer model, it makes it possible that K-ras oncogenic activation in PDAC may be mediated through changes in the gastrin signaling pathway.

This possible relationship has been tested in our lab utilizing a human pancreatic ductal epithelial (HPDE) cell line, H6c7 (14). This line, immortalized with E6E7, is non-tumorigenic and “near-normal.” Furthermore, activated K-ras was introduced into these H6c7 cells, generating the H6c7-Kr (cells containing the mutant k-ras) line which showed 50% tumorigenicity. With the knowledge that K-ras mutation can lead to uncontrolled cell signaling, this outcome makes sense. Our lab, demonstrated gastrin was highly upregulated in these cells even before K-ras activation (15). However, a 2.5-fold upregulation of the gastrin receptor, CCK2R, which is exhibited in the H6c7-Kr cells, suggests K-ras activation may increase gastrin/CCK2R signaling by affecting receptor levels.

For further analysis, CCK2R antagonists were used to block the receptors on both cell strains to determine if cell growth is affected by this gastrin/CCK2R expression. When cells were treated with YM022 antagonist, cell proliferation was decreased in both strains. At the same time, several genes were measured for changes in expression with treatment. Gastrin production in both strains was significantly decreased with antagonist treatment, showing that gastrin/CCK2R signaling is involved in an autocrine signaling loop driving itself (15). This decrease also occurred in K-ras normal H6c7 cells, showing that blocking the gastrin/CCK2R decreases cell proliferation regardless of K-ras status. Importantly, COX-2 was also shown to be downregulated (Figure 2). This is data further suggests that K-ras mediates some tumorigenic effects through gastrin/CCK2R signaling.

Figure 2. The change in COX-2 after CCK2R inhibition suggests gastrin/CCK2R signaling mediates the effects of K-ras on COX-2 expression. COX-2 expression before (purple) CCK2R antagonist was higher than COX-2 expression after (green) CCK2R antagonist in H6c7-Kr cells (15).

This data implicates gastrin/CCK2R is a key mediator for K-ras driving COX-2 and cancer progression. This could mean that gastrin/CCK2R signaling is a crucial upstream change leading to COX-2 upregulation and prostaglandin-driven tumorigenesis (Figure 3).
K-ras and COX-2 inhibition have not been successful in treating PDAC (6,9). Therefore, research must be undertaken to find new therapeutic targets for PDAC therapy. Validation of a relationship between K-ras, gastrin/CCK2R, and COX-2 will be important for this search. In his research, Nevin utilized the H6c7 lines—modeling “early” pancreatic tumorigenesis. However, we seek to validate the K-ras → gastrin/CCK2R → COX-2 relationship in fully-progressed, pancreatic adenocarcinoma. This would be done with PANC-1 cells. PANC-1 harbors activated K-ras and high CCK2R, but low gastrin and low COX-2. By modifying the signaling pathway in the cell lines we hope to induce changes in COX-2 expression. We hypothesize that K-ras uses gastrin/CCK2R signaling as a key mediator in driving COX-2, which in turn drives cancer progression. If successful, our research would demonstrate that influencing the gastrin/CCK2R autocrine pathway may be an effective target for PDAC therapy.

My research will test this hypothesis with two parallel approaches. One approach is to take the preliminary data to fully advanced human cancer cells, such as PANC-1. PANC-1 harbors activated K-ras and high CCK2R, but low gastrin and low COX-2 (see Table 1).

Both of these expression profiles agree with our hypothesis. If K-ras does mediate some of its effects through gastrin/CCK2R signaling, then giving the cells the “juice” (gastrin) to increase signaling will allow K-ras to mediate its effects. I will try exogenous treatment of the cells with gastrin hormone. Additionally, cells will be transfected with gastrin to test the first approach in a different way. I will then analyze the effects this has on COX-2 expression, which can be used as a “readout” for K-ras activity. The second approach is to test all the H6c7 cells available in the lab for CCK2R expression. If the hypothesis is correct, there should be higher CCK2R expression levels in the K-ras activated cells as opposed to the non-K-ras activated cells.

Table 1. PANC-1 expression profile fits the hypothesis of this study. Expression levels of relevant genes and K-ras status PANC-1 cells.

<table>
<thead>
<tr>
<th>Expression Profile</th>
<th>PANC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK2R levels</td>
<td>High</td>
</tr>
<tr>
<td>K-ras status</td>
<td>G12D mutation</td>
</tr>
<tr>
<td>Gastrin levels</td>
<td>Low</td>
</tr>
<tr>
<td>COX-2 levels</td>
<td>Low</td>
</tr>
</tbody>
</table>

Materials and Methods

Quantitative PCR

In order to measure target gene expression in the cells, I utilized real-time qPCR. I used real-time qPCR as opposed to end-point qPCR technique for two reasons. First, real-time qPCR is extremely sensitive and can quantify RNA levels from a single cell. Second, it collects data from the exponential phase of the reaction, which end-point PCR cannot do. This allows for accurate, reproducible data (18). The qPCR instrument I used is StepOnePlus (Applied Biosystems). Additionally, the TaqMan Probe Set (Applied Biosystems) and SYBR Green (Applied Biosystems) were used in order to quantify RNA levels during qPCR via measuring fluorescence (19).

Cell Culture

In order to continue the cell lines, it was necessary to continually split cells. PANC-1 cells were split every 3-4 days. was aspirated and cells were washed with PBS. Two mL Trypsin was added and the plates were placed in incubator for five minutes in order to detach the cells. After five minutes, 8 mL of DMEM media was added to create a 10mL suspension. Cells were split 1:5 (2mL of suspension in 10mL of new media) or 1:10 (1mL of suspension in 10mL of new media) into new plates. H6C7 cells, human pancreatic ductal epithelial
Western Blot

Samples were resolved for 1 hour. DTT Sample Buffer prior to introduction into the wells. The acrylamide:bisacrylamide. Cell lysates were mixed with 5x DTT Sample Buffer prior to gel electrophoresis via semi-dry transfer. Membranes were transferred to nitrocellulose membranes.

Resolving gels (8%) were made with 29:1 and 37.5:1 acrylamide:bisacrylamide. Cell lysates were mixed with 5x DTT Sample Buffer prior to introduction into the wells. The samples were resolved for 1 hour.

Results

PANC-1 Gastrin Treatment and Transfection Data

In order to see if K-ras mediates its effects through gastrin/CCK2R signaling, PANC-1 cells were treated with exogenous gastrin hormone. PANC-1 cells express low gastrin levels and low COX-2 levels, so according to my hypothesis, I expected to see an increase in COX-2 expression after exogenous gastrin treatment. Two treatment groups (CCK and gastrin) and a control were used in this experiment. Hormones were added every 12 hours at concentrations consistent with previous studies. Cells were treated for 72 hours and RNA was isolated to use for PCR. RNA from BxPC3 cells (known COX-2 expressers) were included in the PCR in parallel with the PANC-1 RNA. The results showed no COX-2 expression in the PanC1 cells after gastrin treatment. I reran the experiment to see if the data is consistent. Unfortunately, during the RNA isolation procedure, I neglected to dilute RPE buffer with ethanol and the RNA was lost. I reran the experiment for a third time and successfully isolated RNA. This time, however, I tested for COX-2 expression of PANC-1 cells that had been transfected with gastrin in parallel with the RNA isolated from my hormone treated PANC-1 cells. Altogether, the data showed no change in COX-2 expression in both the exogenously treated PANC-1 cells and the PANC-1 cells transfected with gastrin (see Figure 4).

Figure 4. No significant change in COX-2 expression in PANC-1 cells was observed after exogenous treatment with gastrin (A) or high endogenous expression of gastrin (B). PCR was run with exogenously treated PANC-1 cells in parallel with transfected PANC-1 cells that expressed high gastrin levels. BxPC3, a known COX-2 expresser, was run in parallel.

The transfected cells were expressing gastrin well over 3,000 times than normal, so if there was any connection between gastrin/CCK2R signaling, I would expect to see it in the PCR results. Based on the results, Dr. Harms and I concluded the

Exogenous Treatment of PANC-1 cells with Gastrin

After 24 hours, the media in the PANC-1 cell culture plates were aspirated and replaced with serum-free media. PANC-1 cells were grown for 48-72 hours in media containing gastrin peptide (G-17), which was added every 13 hours. To simultaneously test for a dose-dependent response, PANC-1 cells were cultured in different media containing 1 μM, 100 nM, and 1 nM gastrin. Additionally, PANC-1 cells were cultured for 6 days in gastrin free media to serve as a control.

Transfection of PANC-1 cells

PANC-1 cells were transfected with a gastrin by fellow lab mate Rebekah Jones.

RNA Isolation, cDNA synthesis, and qPCR Analysis

RNA was purified from cells using the RNeasy Kit and Qiashredder Homogenizer Kit (Qiagen). After purification, RNA was synthesized to cDNA via reverse transcription in preparation for qPCR. H6c7 RNA samples from previous studies will also be converted to cDNA for qPCR. I used TaqMan Probe Sets (Applied Biosystems) and SYBR green (Applied Biosystems) for quantitation. PPIA, an endogenous "housekeeping" gene, will serve as a control for qPCR.

Alamar Blue Growth Assay

Pilot data has suggested CCK2R has implications in cancer cell growth. For example, when the receptor was blocked by antagonists (Proglumide and YM022) there was a decrease in growth. However, this data was obscure and a rerun was necessary. For the rerun, I used the same drug concentrations: two concentrations of Proglumide (10 and 100mg/L) and two concentrations of YM022 (200nM and 2μM). Both H6C7-Kr and H6C7-Ak cell lines were subject to the drugs over a period of four days. Every 24 hours, 10μL of Alamar Blue was added to each well. Four hours later, the cell lysates were mixed with 5x DTT Sample Buffer prior to gel electrophoresis via semi-dry transfer. Membranes were blocked using 10% milk in 1X TBST. Two primary antibodies were used: 28H8 and 7B8.8. Secondary antibodies were added after the appropriate time. The membranes were rinsed twice for 1 minute with 1x TBST and then washed twice for 5 minutes with 1x TBST. X-ray film was exposed for varying time periods (30 seconds, 1 minute, and until completion).

Polyacrylamide Gel Electrophoresis

Resolving gels (8%) were made with 29:1 and 37.5:1 acrylamide:bisacrylamide. Cell lysates were mixed with 5x DTT Sample Buffer prior to introduction into the wells. The samples were resolved for 1 hour.

Western Blot

Gels were transferred to nitrocellulose membranes after gel electrophoresis via semi-dry transfer. Membranes were blocked using 10% milk in 1X TBST. Two primary antibodies were used: 28H8 and 7B8.8. Secondary antibodies were added after the appropriate time. The membranes were rinsed twice for 1 minute with 1x TBST and then washed twice for 5 minutes with 1x TBST. X-ray film was exposed for varying time periods (30 seconds, 1 minute, and until completion).

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PANC-1 cell model showed no evidence suggesting K-ras mediates some of its tumorigenic effects through gastrin/CCK2R signaling. This finding is important because it contradicts previous experimental data collected by a former lab member.

**COX-2 Expression in H6c7 Cells After Inhibitor Treatment**

A former lab member had treated H6C7 cells with CCK1R inhibitor (Devazipide) and CCK2R inhibitor (YM022) in parallel. This was done in order to specify if CCK1R or CCK2R played a role in COX-2 expression in a H6C7 cell model (as opposed to a PANC-1 cell model). I ran a cDNA reaction and PCR analysis to determine any changes in COX-2 expression. The PCR data showed two things: 1) gastrin participates in an autocrine feedback loop in which gastrin drives gastrin and 2) COX-2 expression was unchanged in both H6C7-Kr and Ak cell lines suggesting K-ras does not mediate its effect on COX-2 through gastrin/CCK2R signaling. It is also important to note that the expression levels of COX-2 in the Ak cells was shown to be higher than the cancer cells in this study. This suggest the expression levels are not consistent across the board. Ultimately, this data suggests K-ras does not mediate some of its tumorigenic effects through gastrin/CCK2R signaling in an H6C7 cell model (see Figure 5).

![Figure 5](image)

**Figure 5.** Repeat analysis cannot confirm decrease in COX-2 expression levels via blocking gastrin/CCK signaling. Pilot data had previously shown the prog lumide—a general antagonist of both gastrin receptors, CCK1R and CCK2R—decreased COX-2 mRNA expression in H6c7-Kras* cells. Here, H6c7 cells were placed on two receptor-specific inhibitors, YM022 (CCK2R) and Devazepide (CCK1R). In contrast to pilot data, COX-2 expression was higher in normal H6c7 cells than in cells with activated K-ras, and no inhibition was observed with antagonist treatment.

**Alamar Blue Growth Assay**

Pilot data has suggested CCK2R has implications in cancer cell growth. For example, when the receptor was blocked by antagonists (Progumide and YM022) there was a decrease in growth. However, this data was obscure and a rerun was necessary. For the rerun, I used the same drug concentrations: two concentrations of Proglumide (10 and 100mg/L) and two concentrations of YM022 (200nM and 2uM). Both H6C7-Kr and H6C7-Ak cell lines were subject to the drugs over a period of four days. Every 24 hours, 10uL of Alamar Blue was added to each well. Four hours later, the plate was visualized using plate reader software. Unfortunately, my cell count calculations were off by a factor of ten and too few cells were added to each well (250 cells/well instead of 2500). This error effectively disrupted normal cell growth. Despite this, H6C7-Ak cells showed normal growth until Day 3. On the other hand, the H6C7-Kr cells immediately went into the death phase. Due to these factors, I deemed a rerun necessary. The data from the rerun suggested 200mg/L Proglumide effectively inhibits cell growth (see Figure 6).

![Figure 7](image)

**Figure 7.** Proglumide 200mg/L inhibited cell growth in both H6C7 cell lines. Alamar Blue growth assay of H6C7-Kr (A) and H6C7-Ak (B) in the presence of CCK2R inhibitors to see contribution of CCK2R to cell growth.

I was also expecting to see both cell lines show marked growth inhibition in 2uM YM022, which was only observed in the H6C7-Kr cell line. In order to get more conclusive data, a rerun is necessary.
Testing CCK2R Expression in H6c7 Cells

In order to see if Nevin’s preliminary data was statistically significant, CCK2R expression analysis was run on each H6c7 cell available in the lab. The RNA was already isolated and in storage so cDNA synthesis was the only thing necessary in preparation for qPCR. The lab has nine different H6c7 RNA samples that have only been used as controls in past experiments. Taqman and SYBR were used in some of the PCR’s in order to see if the data was consistent. In the results there is evidence of an upregulation of CCK2R in K-ras activated cells; however, this is not seen across the board. Additionally, the curves were consistently crossing threshold at around 36 cycles, which means expression was very low. Also, these low expression levels caused the inconsistently in the expression analysis data (see Figures 8).

Figure 8. No consistent upregulation of CCK2R expression levels was observed in H6c7-Kras* cells. Pilot data suggested activated K-ras increases CCK2R expression levels in H6c7-Kras* cells. In contrast, expression analysis of multiple RNA isolates from both H6c7 and H6c7-Kras* revealed barely detectable levels of receptor expression (C_T = 36-40; PPIA, C_T =15-17), high variability, and no consistent pattern of upregulation with K-ras activation

Discussion

The purpose of this study was to clarify the connection between oncogenic K-ras and gastrin/CCK2R signaling. Preliminary studies gave evidence that activated K-ras causes an upregulation in CCK2R. This allows K-ras to mediate some of its tumorigenic effects through the gastrin/CCK2R signaling pathway. Supporting data showed that there was a 2.5-fold increase in CCK2R in H6c7-Kr cells as compared to H6c7-Ak cells. Additionally, Nevin showed that blocking gastrin/CCK2R signaling decreased cell proliferation, giving evidence to the biological significance of gastrin/CCK2R signaling in the H6c7 cells. Nevin also had evidence that placed gastrin/CCK2R signaling upstream COX-2 despite K-ras status.

Taking the preliminary data into account, the original hypothesis of this study was to test whether or not gastrin/CCK2R signaling is upstream COX-2; however, after looking into Nevin’s data, I decided that the experiments needed to be rerun for reproducibility. Frist, I used a PANC-1 model. Exogenous treatment of and transfection with gastrin did not lead to an increase in COX-2, which we used as a “readout” for K-ras activity. We then looked at H6c7-Kr cells that had been treated with CCK2R inhibitors. There was no decrease in COX-2 expression and there were higher levels of COX-2 in the H6c7-Ak cells. Both of these findings are contrary to the preliminary data.

The second way Nevin’s hypothesis was tested was by looking at CCK2R expression in all H6c7 cells available in the lab. There were nine samples in total that had only been used as controls available in the lab. After running multiple PCR reactions, I determined that there was no consistent pattern that showed higher CCK2R expression in K-ras activated H6c7 cells.

The results of this study suggested a connection between gastrin/CCK2R signaling may not be as relevant as previously shown. However, it is important to note that mRNA levels do not necessarily correlate with protein levels. Therefore, it was necessary to determine CCK2R protein levels via Western Blot. Previous studies in the lab attempted to optimize the Western Blot procedure. There were issues with getting the proteins to migrate into the resolving gel. A previous member had success in migration; however, too many parameters at one time to determine which were the most effective.

The goal is to perform Western Blot analysis on H6c7-Kr and H6c7 cells. However, there is too many complications with these cells lines. Because of this, they are not ideal for optimizing the Western Blot parameters. PANC02 cells, a murine cancer cell line, were chosen. This is because the antibodies used to detect CCK2R protein should not bind to any other proteins on the cell. PANC02 cells are therefore ideal for negative controls. PANC02 cells transiently transfected with CCK2R and PANC02 cells stably expressing CCK2R were to serve as positive controls.

Previous Western Blot studies suggested changing the polyacrylamide:bisacrylamide solution from 29:1 to 37.5:1 allowed the protein to migrate in the gel better. Two Western Blot experiments were run to test the preliminary data. Unfortunately, the first run, although separation was achieved, suffered from spilling over of cell lysate into the other wells. As a result, the negative control tested positive for protein. The second run was a failure due to the gel failing to transfer to the membrane. Taken together, this data suggests further studies are needed to determine whether or not 37.5:1 is the reason for the protein migrating into the resolving gel.

AUTHOR INFORMATION
Author Contributions

Andrew LaRow carried out the procedure and data analysis. Dr. Harms provided advice and guidance.

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