Demonstrating the Link Between MUC15 Expression and Ovarian Cancer Metastasis Through Downregulation of PI3K/AKT Signaling

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ABSTRACT

Ovarian cancer is a highly aggressive and deadly form of cancer. Diagnosis at early stages is difficult, leading to limited treatment options and low survival rates. Therefore, studies to understand both progression and treatment are necessary to improve outcomes for individuals affected by this disease. Previous work has shown that there is a relationship between surface shear stress and the initiation of epithelial to mesenchymal transitions which increases the migratory and invasive potential of the primary tumor with subsequent metastasis. Additionally, expression of the surface marker Mucin-15 (MUC15) has been shown to downregulate the migratory potential of the primary tumor. This project attempts to explain the relationship between MUC15 expression in ovarian cancer cells, and the downregulation of the proliferative PI3K/AKT signaling pathway. Amounts of phosphorylated and unphosphorylated AKT were measured via western blotting of samples taken from cell lines engineered to express MUC15 at varying levels. While results did not show the expected expression patterns, future work should be done to continue to analyze the molecular mechanisms of shear initiated transformation and metastasis.

INTRODUCTION

Ovarian cancer is the most deadly form of cancer of the female reproductive tract [1]. It has one of the lowest survival rates of any form of cancer and treatment options are limited. This is primarily due to diagnoses at late stages after the disease has progressed to be more invasive than is sufficiently treatable. For this reason, it is imperative to understand more about the disease at all stages, as well as to introduce novel treatment options to help improve patient outcomes.

Many factors are involved in the metastasis of cancers. However, the basis of cancers being localized to a specific organ or region in the body to become invasive to other more distant parts of the body lies in the epithelial to mesenchymal transition (EMT). This occurs when normal, healthy epithelial cells lining the walls of an organ or cavity are transformed to demonstrate a mesenchymal phenotype [2]. This enables the cells to become invasive, migrating to distant organ systems using the host vasculature and establishing new tumor sites to perpetuate the continuation of cancer.

Recently, the role of mechanical forces to promote EMT in ovarian epithelial cells has been examined. In particular, the ascitic microenvironment resulting from improper fluid buildup in the abdominal cavity is of interest due to the shear forces, both interstitial and vascular mediated, that tumors experience. Studies have shown that introducing shear stresses to mimic fluid flow in the ovarian ascitic microenvironment increases the migration potential of these cells [3]. Ascitic channels that form from leaky vasculature have long been proposed as a pathway by which ovarian cancer cells migrate from the primary tumor. Efforts to understand how these physical cues from mechanical shear forces can be converted into chemical cues to promote increased

transcription of pro-oncotic factors in ovarian epithelium are of great interest in microenvironment research.

Mucin-15 (MUC15) is a glycoprotein and is a member of the domain of cellular surface markers of the mucin protein family. The presence of this surface marker has been shown to decrease metastasis in renal cancer cells by inhibiting the activities of the PI3K/AKT signaling pathway (Figure 1) [4]. This pathway is known to promote invasion and metastasis in other cancer types as well, such as in breast cancers and hepatocellular carcinomas [5] [6]. The inhibition is experienced due to the fact that there is decreased phosphorylation of AKT which prevents transcription of oncogenes and genes linked to the EMT.

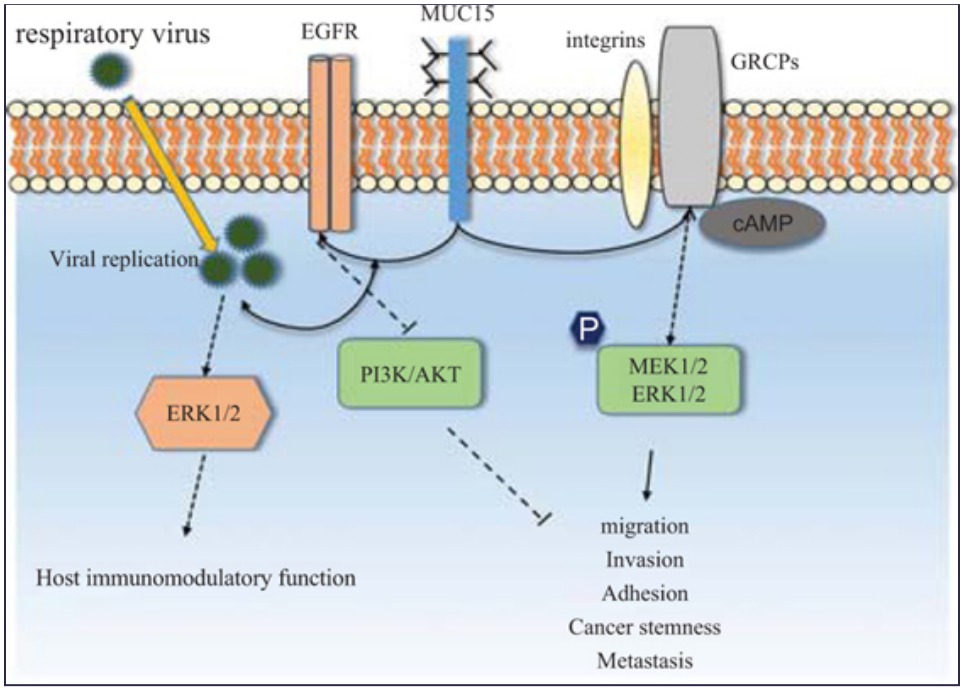

Figure 1: Demonstration of the possible link of the MUC15 surface marker to the PI3K/AKT signaling pathway. Signaling is prevented in the presence of MUC15 because there is decreased phosphorylation of AKT which is a step required to initiate genes responsible for invasion and metastasis [7].

Recently, our lab has been interested in expanding these trends that have been seen between shear stress, EMT and the PI3K/AKT signaling cascade in other cancers to investigate whether these trends hold for ovarian cancer as well. Previous work has shown that in RNAseq experiments performed for ovarian cancers, there is a decreased expression of MUC15 in sheared cancer cells relative to cancer cells that have not been exposed to shear stresses. Additionally, increased activation of the PI3K/AKT pathway has indicated that possible shear sensing by MUC15 might be a factor in the proliferation and metastasis of ovarian cancer cells exposed to

ascitic shear stress. Finally, the end product of PI3K/AKT signaling is NFκB. Preliminary data from our lab shows that in sheared cells, this molecule shows high localization in the nucleus which is consistent with PI3K/AKT signaling.

In order to answer this question we have created five genetically transduced cell lines with different expression levels of MUC15 used to compare relative expression to the invasion potentials of these cell lines (Figure 2).

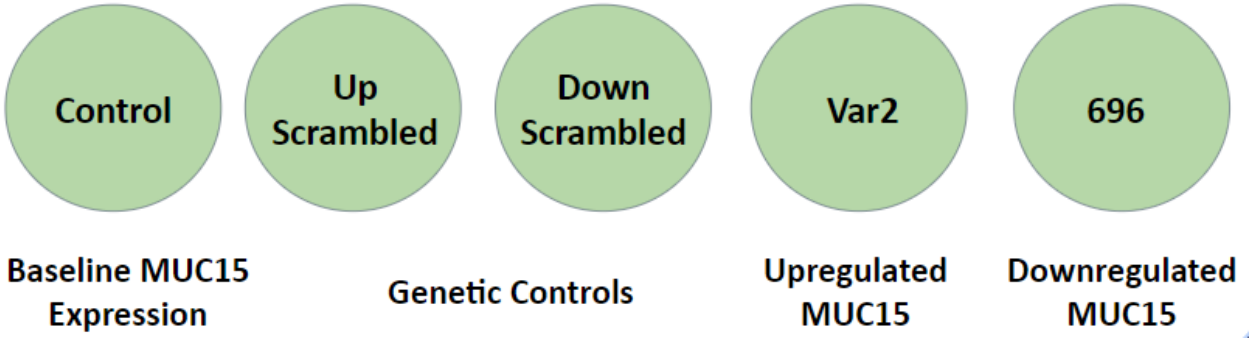

Figure 2: Explanation of each of the different genetically engineered cell lines used in this project.

In this project my role was to examine the expression levels of AKT and pAKT in each of these cell lines to demonstrate an inverse relationship between MUC15 expression and the expression of pAKT. Expression was measured using western blots. Controls were composed of standard ovarian cancer cells and up-scrambled and down-scrambled genetic controls to provide base levels for MUC15 expression. Var2 had increased MUC15 expression and expected decreased pAKT expression levels. The opposite expression pattern was expected for the 696 cell line.

After careful tuning of western Blot parameters, protein expression was able to me measured for the two proteins of interest (AKT, pAKT) as well as for the housekeeping gene -actin. The inconclusiveness of these results points to the need for further tuning of western blot parameters and possible re-transduction of the cell lines to respond to possible contamination issues that cells faced during the project. Overall, the lessons learned through this project will be useful for continuing this research to determine the mechanisms for invasion and metastasis of ovarian cancers.

METHODS

In order to prepare cell lysates for analysis with western blotting, cell stocks were grown on 10 cm plates for one week splitting as necessary. When cells had grown appropriately, they were

lysed using a cocktail of RIPA Buffer (ThermoFisher, PI89900) with added HALT inhibitors , and Phenylmethylsulfonyl fluoride phosphatase inhibitor to prevent dephosphorylation of protein samples. Cell protein concentrations were measured using BCA protein assay. Normalized sample lysates were prepared using a 4x Laemmli Buffer (BioRad, 161-0747) with final concentration of added 2-mercaptoethanol at a concentration of 2.5%. Cell lysate samples were stored in a -20 ℃ freezer until use in western blotting experiments.

Lysates were loaded along with protein standard ladders into 10% MiniProtean TGX Precast protein gels to complete protein separations. Voltages were set to 140 volts and samples were allowed to separate along the length of the gel. Following protein separations transfer to membranes was completed using membrane and filter paper sandwiches (Immun-Blot LF PVDF Membrane BioRAD) and a Tris/Glycine 10x Buffer with added methanol at a concentration of 20%.

Blotting followed using primary antibodies at the concentrations recommended by the manufacturer and listed in Table 1 below. Blotting of primary antibodies was allowed to incubate overnight and was followed by three wash steps using 10x Tris Buffered Saline (TBS) with a concentration of 1% added Tween 20 (TBST). HRP-conjugated secondary antibody incubations followed immediately after the wash steps using the antibodies and concentrations recommended by the manufacturer and listed in Table 1 below. Incubations occurred for 60 minutes at 4 ℃ and were washed with three additional wash steps before imaging.

Table 1: List of primary and secondary antibodies, manufacturers and concentrations used for western blotting

Primary Antibody	**Manufacturer**	**Concentration**
Beta Actin Monoclonal Antibody	Invitrogen	1:1000
Phospho-Akt (Ser473) Antibody	Cell Signaling	1:1000
Akt Antibody	Cell Signaling	1:1000
Secondary Antibody	**Manufacturer**	**Concentration**
Goat anti mouse IgG (H/L) HRP	BioRAD	1:2000
Goat anti Rabbit IgG (H/L) HRP	BioRAD	1:2000

Incubations were completed for each primary antibody separately in order to achieve the best resolution from blotting and staining steps. The order of incubations were consistent: pAKT followed by -actin, and concluding with AKT. This was to prevent dephosphorylation from occurring in the protein samples before images were acquired. Between incubations, stripping of the membrane was completed using 0.2X NaOH solution composed of diluted 1X NaOH and deionized water.

Imaging was completed using the LiCOR Odyssey CLx Infrared Imaging system and corresponding software packages.

RESULTS

Western blotting as a protein analysis technique requires a lengthy process of fine-tuning in order to obtain reproducible results. Many rounds of analysis were performed in order to improve the blotting parameters and to gather data with protein signatures for each of the proteins of interest. Figure 3 highlights some of the issues that were faced during the process of tuning parameters.

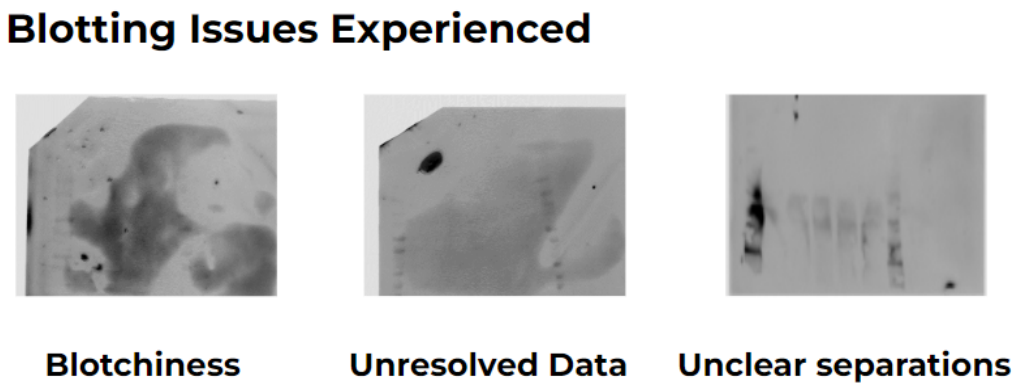

Figure 3: Blotting issues experienced during the early rounds of blotting which were refined in subsequent rounds of experimentation in order to produce results.

In order to obtain resolved data various adjustments were made to the protocol. Improvements included adjusting the concentration of the washing buffer, increasing the incubation time of the primary antibody incubations from one hour to overnight, and by increasing the amount of protein used in the electrophoresis separation steps.

The amount of protein that obtained the first set of resolved data was 35 mg. The results from an overnight incubation with all of the proteins of interest probed is included in Figure 4.

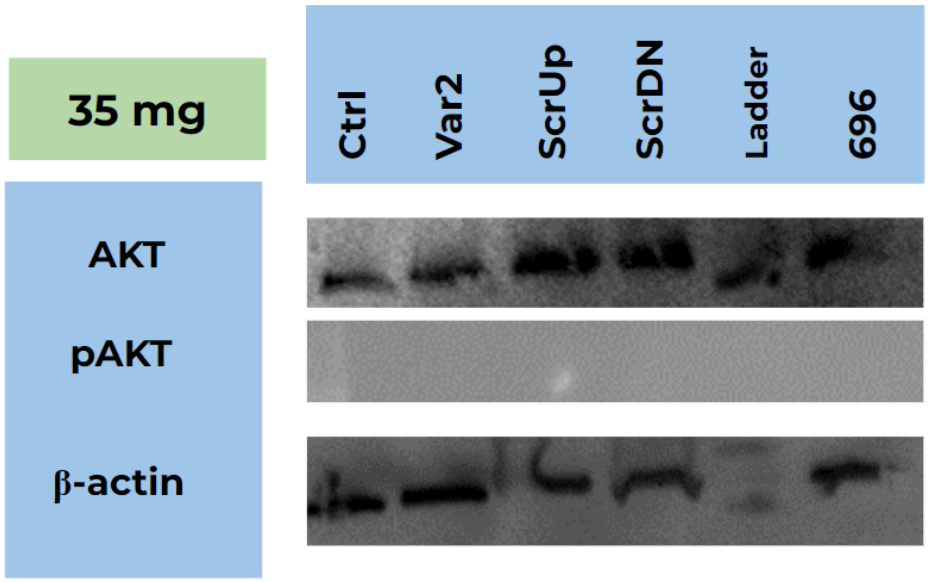

Figure 4: Western blot results of each of the proteins of interest for the five cell lines explored. The amount of protein used in this experiment was 35 mg.

Since pAKT expression was not detected with the use of 35 mg of protein, subsequent incubations used larger amounts of protein.

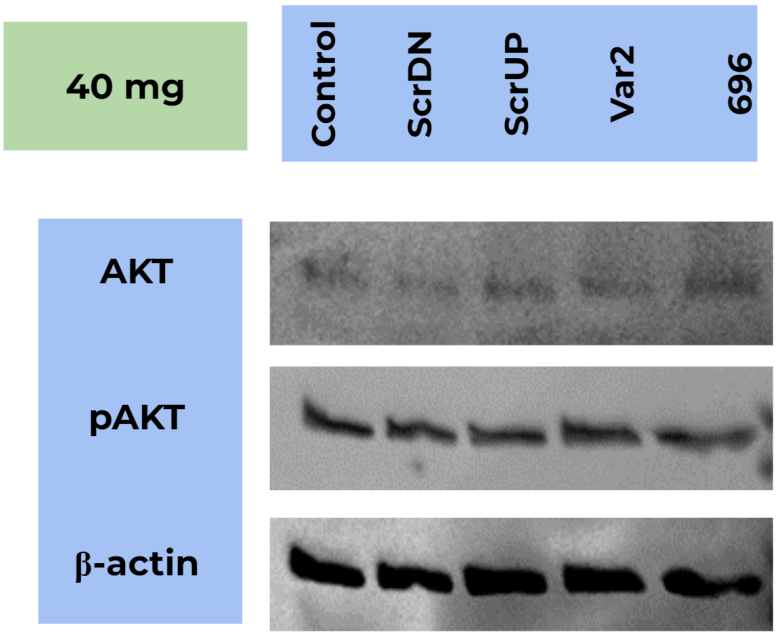

Figure 5: Western blot results of each of the proteins of interest for the five cell lines explored. The amount of protein used in this experiment was 40 mg.

Finally, to explore further increases in the amount of protein and the quality of the western blot resolution additional incubations for 45 mg of protein were examined (Figure 6). These proved to be less successful than the experiments using 40 mg of protein, with pAKT and AKT expression not being well resolved, despite good display of the housekeeping gene -actin.

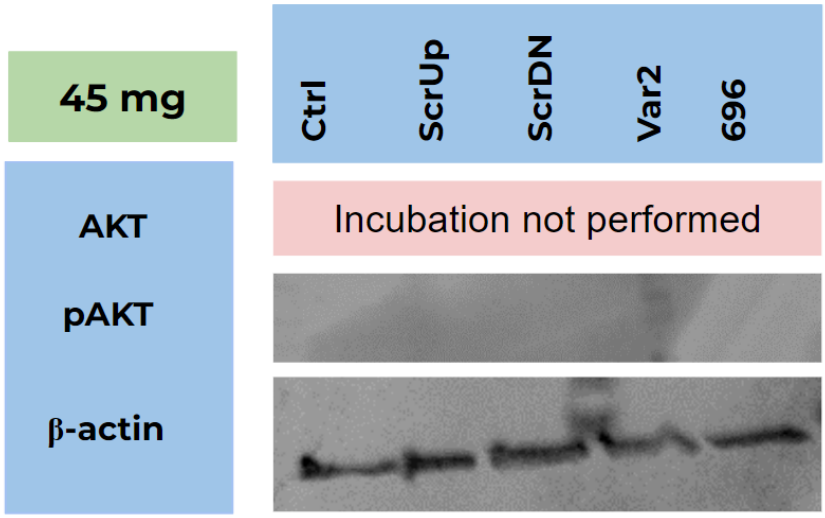

Figure 6: Western blot results of each of the proteins of interest for the five cell lines explored. The amount of protein used in this experiment was 45 mg. Incuations were not performed for AKT due to the limited success experienced in pAKT analysis.

DISCUSSION

Based on the results shown above it is clear that efforts to obtain information about protein expression via western blotting, require significant amounts of tuning in order to produce meaningful and reproducible results. At the start of this project, the western blot membranes that were produced showed no meaningful data due to large degrees of blotchiness in the membrane, failure of the protein of interest to be detected and incomplete separations in the protein bands. Each of these issues was addressed systematically by adjusting parameters of the protocol for each problem that was faced.

Blotchiness in the membrane is attributed to an overly diluted washing buffer. Increasing the concentration of the washing buffer used decreased the amount of blotchiness that was seen on the membrane. Additionally, preventing the membrane from drying out between the final wash step following secondary antibody incubations is also a factor that contributes to blotchiness.

Since the LiCOR Odyssey is located far from the ECM lab, leaving the membrane soaking in TBST during transport became an essential part of the procedure.

Failure to detect the protein of interest is due to a variety of factors such as the incubation time of the primary and secondary antibodies, length of time of the wet transfer to membrane following the electrophoresis step, the amount of protein used in the electrophoresis separations, and image analysis settings to measure the fluorescence of the HRP conjugated secondary antibodies. The incubation time was a factor that was adjusted between rounds of western blotting. Primary antibody incubations were tested for 60 minutes, 90 minutes and overnight. Overall, the overnight incubations showed the most promise in resolving the protein expression bands and these were pursued for subsequent experiments.

Finally, separation of the protein bands was primarily an issue that was experienced when attempts to show the expression of different proteins on the same membrane at the same time was attempted. Since staining was unclear, and it was not necessary to show multiple proteins at once, a sequential incubation strategy was employed for subsequent rounds of blotting.

Once the membranes were able to be resolved by adjusting the factors above, the amount of protein lysate used for the electrophoresis step was varied. From the data above it is clear that the best expression patterns can be deduced from 40 mg protein lysate samples. This showed the clearest expressions for all of the proteins of interest. When protein levels were increased to 45 mg this resulted in a decreased expression of the protein, and no expression of the pAKT protein at all. The reasons for this are likely due to other factors than the amount of protein used, and it is recommended that in future experiments a larger amount of protein is used.

In terms of the expression of the protein levels among cell lines, it was found that there was no change in expression based after MUC15 expression was upregulated or downregulated. While this might suggest that MUC15 has little effect on the promotion of the PI3K/AKT pathway, there could be other factors that are affecting the expression levels as well.

One thing to consider is the limited expression of pAKT throughout the trials. This could be due to dephosphorylation of the protein samples during storage, electrophoresis, transfer, or blotting. While steps were taken to decreased the rate of this potential dephosphorylation such as incorporating a phosphatase inhibitor, and completing the experiments in colder temperatures, further steps could be taken to decrease the likelihood of dephosphorylation such as completing the electrophoresis and transfer steps at 4 ℃ as well.

Another factor that could have been impacting the expression of the different proteins is due to mycoplasma contamination that was experienced in the cell lines of the lab during the semester. Mycoplasma is a difficult contaminant to control given its inability to be treated by most

antibiotics, and its ability to permeate and proliferate inside of cells themselves. Mycoplasma primarily impacts the metabolic function of the cells, though it could also have an effect on the signaling pathways as well which might impact expression levels. However, from the data one of the protein expressions that was relatively consistent across cell lines and trials was the -actin protein which is a measure of the metabolic function of the cells indicating that this was likely not impacted.

Finally, one other reason that cellular AKT and pAKT expressions could have been unexpected relative to the MUC15 expression patterns is because MUC15 is not the sensor for shear stress in the cells themselves. This would mean that MUC15 expression is independent of the promotion or inhibition of the PI3K/AKT pathway. One way to test for this would be to target other surface markers from the list provided in the upregulated and downregulated RNA constructs identified in RNAseq experiments, create similar transduced lines with increased or decreased expression of these surface markers and test the expression patterns of these proteins relative to activation of the PI3K/AKT pathway. While the work done on this project does not rule out the possibility of MUC15 acting as the receptor for shear stress, it does suggest that examining other potential signaling molecules might yield additional insight into the promotion of this metastatic pathway.

CONCLUSIONS

Ovarian cancer has disastrous impacts on many people. With limited diagnostic capabilities, few treatment options, and low chances of survival, this disease is deadly for many that contract it. Therefore, increasing an understanding of the progression and treatment of this cancer is of high importance for the medical community.

Previous work has shown that shear stress caused by fluid flow in ascites that form intraperitoneally has a correlation with increased migratory potential through promotion of the EMT. This increases the invasion of the cells in far organ systems and metastasis from the primary tumor site. The role of MUC15 as a possible receptor for shear stress is motivated by a downregulation of MUC15 expression in sheared ovarian cancer cells, as well as display of this phenomenon in other cancer types. Expression of MUC15 has been shown to decrease metastasis due to a decreased phosphorylation of AKT in the PI3K/AKT signaling pathway.

In order to examine if the same expression pattern correlation is consistent in ovarian cancer cells, five transduced cell lines with different MUC15 expression levels were created. Western blotting was done on each of these cell lines to determine whether a decreased expression of pAKT was demonstrated for increased MUC15 expressions. Tuning of the parameters of these western blots showed that overnight incubations with higher levels of protein lysates was effective at improving the resolution and reproducibility of protein expression results. From the datasets explored in this work, 40 mg of protein lysate showed the best expression results in western blotting.

In terms of expression patterns no discernable difference was shown for expression of pAKT across cell lines. There are several reasons why this might have occurred such as contamination to the cell stocks impacting expression, a need to further tune western blotting parameters, or a possible use of a different sensing mechanism for shear stress.

For future work, it is important to obtain transduced cell lines that have not been exposed to mycoplasma contamination. Completing analysis on these lysates would remove the possibility of influence of this contamination on the western blot results. Further tuning of western blot parameters, specifically in the image analysis parameters would also be beneficial. Finally, remaining the RNAseq data and the upregulated and downregulated transcripts between sheared cells and control cells might provide other possible candidates for the shear stress sensing cellular structure. Localizing these structures and creating transduced cell lines that allow for increased or decreased expression might provide a more concrete answer to the question of PI3K/AKT signaling.

Understanding the role that external cellular forces play in promoting the metastasis of cancers is essential for improving treatment options for patients that experience ovarian cancer. In particular, the conversion of mechanical and physical signals to chemical and genetic cues that initiate phenotypic changes might help physicians to better predict the rate at which ovarian cancer is progressing, and therefore prescribe next steps appropriately.

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