MATRIX DEGRADATION AND INVASION

IN BREAST CANCER

BY

ELLA STAROBINSKA

A Thesis Submitted to The Honors College

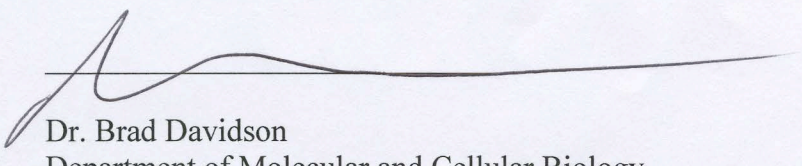
In Partial Fulfillment of the Bachelors degree
With Honors in

Physiology

THE UNIVERSITY OF ARIZONA

MAY 2012

Approved by:



Dr. Brad Davidson
Department of Molecular and Cellular Biology

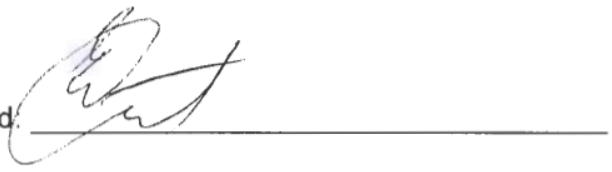
The University of Arizona Electronic Theses and Dissertations
Reproduction and Distribution Rights Form

Name (Last, First, Middle) Starobinska, Ella, I	
Degree title (eg BA, BS, BSE, BSB, BFA): BS	
Honors area (eg Molecular and Cellular Biology, English, Studio Art): Physiology	
Date thesis submitted to Honors College: 5/2/12	
Title of Honors thesis: Matrix Degradation and Invasion In Breast Cancer	
:The University of Arizona Library Release	I hereby grant to the University of Arizona Library the nonexclusive worldwide right to reproduce and distribute my dissertation or thesis and abstract (herein, the "licensed materials"), in whole or in part, in any and all media of distribution and in any format in existence now or developed in the future. I represent and warrant to the University of Arizona that the licensed materials are my original work, that I am the sole owner of all rights in and to the licensed materials, and that none of the licensed materials infringe or violate the rights of others. I further represent that I have obtained all necessary rights to permit the University of Arizona Library to reproduce and distribute any nonpublic third party software necessary to access, display, run or print my dissertation or thesis. I acknowledge that University of Arizona Library may elect not to distribute my dissertation or thesis in digital format if, in its reasonable judgment, it believes all such rights have not been secured. Signed: _____ Date: 5/2/12

Last updated: Nov 15, 2009

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for a degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Signed: 

Abstract:

In order to metastasize, cancer cells need to invade and degrade matrix. Previous research showed that Epidermal Growth Factor Receptor (EGFR) is an oncogene, a member of ErBb family, that is over-expressed in aggressive cancers. EGFR mediates cell survival, proliferation, and motility through different signaling pathways. Located on the basolateral membrane of the cell, EGFR can be either translocated to the nucleus, degraded by the lysosome or recycled. However, in cancerous cells, EGFR activity is altered by MUC1, which associates itself with EGFR. Research suggested that this pathway acts in a Met-dependent manner. We conducted matrix degradation and invasion assays to see whether MUC1/EGFR activity has affect on these processes. Matrix degradation assay showed that Muc1 and EGFR inhibit matrix degradation and PMIP promotes it. However, Muc1/EGFR regulated matrix degradation is not Met-dependant. Meanwhile, the transwell invasion assay provided variable and statistically insignificant results.

Introduction:

Epidermal Growth Factor Receptor (EGFR) is a tyrosine kinase receptor which is frequently deregulated in cancer, being overexpressed in invasive carcinomas (Pochampalli et al. 2007). Normally, EGFR is localized to the basolateral cell membrane, while in cancer its localization is altered. In healthy epithelial cells, EGFR dimerizes upon ligand binding, is endocytosed and degraded by lysosomes. In cancer cells this pathway is altered such that EGFR is constantly trafficked to the nucleus, where it initiates transcription of cyclin D1 (Lin et al. 2001).

Epithelial cacinomas also have high expression of the mucin MUC1 transmembrane protein (Bitler et al. 2010). It is normally expressed on the apical side of epithelial cells. In breast carcinomas it loses its normal localization and is highly overexpressed. (Bitler et al.2009). It's overexpression is associated with various leukemias, myelomas, and lymphomas (Bitler et al. 2009). Muc1 has been proven to be a potent oncogene. As such, its overexpression in mouse mammary glands induced breast cancer (Schroeder et al 2004).

MUC1 and EGFR interact in breast cancer cell lines (Bitler et al. 2009). Muc1 enhances nuclear translocation and recycling of EGFR, while inhibiting its degradation (Pochampalli et al. 2007). The interaction of these two proteins results in increased transformation and metastasis (Bitler et al. 2009). The MUC1-EGFR protein interaction is blocked by the MUC1 inhibitory peptide, PMIP, which binds the cytoplasmic domain of MUC1 (Bitler et al. 2009). PMIP binding to MUC1 Destabilizes the MUC1-EGFR interaction and leads to reduction of EGFR levels. Consequently, PMIP inhibited breast cancer progression in mice as well as breast cancer cell migration (Bitler et al. 2009).

Microarray analysis identified the target genes downregulated in cells treated with PMIP. One of these genes is the Met receptor, which is an oncogene involved in cell motility (Bitler, unpublished). This led to the hypothesis that EGFR and MUC1 function in a Met-dependent manner. Various studies have shown that the Met receptor tyrosine kinase induces murine mammary tumors, which are similar

to human breast cancer (Ponzo et al. 2010). Furthermore, human basal tumors have elevated levels of Met (Ponzo et al. 2010).

Two biological processes characteristic of cancer cells are degradation of extracellular matrix and tissue invasion. In our studies, we examined these processes with respect to EGF, MUC1, and Met activity. Using the gelatin matrix degradation assay, published by Artym et al. 2009, we looked at the ability of a specific breast cancer cell line (MDA-MB-231) to degrade gelatin matrix. Gelatin is denatured collagen and therefore serves as a biologically relevant extracellular matrix substitute. We found that an increase in Muc1 and EGFR activity significantly decreased punctual degradation, while blocking their activity with PMIP reinstated matrix degradation.Yet the impact of EGFR/MUC1 activity on matrix degradation is not Met-dependent. A transwell invasion study was used in order to examine invasion of these cancer cells. However, the data gathered over three trials had very high variability and were found to be statistically insignificant. This result may be due to the experimental error innate variability of the cancer cell line we employed.

Materials and Methods:

Cell Culture. Our studies were conducted using metastatic breast cancer cell line MDA-MB-231 from the American Tissue Culture Collection. These cells were cultured in RPMI 1640 media in the 37^{o}C humidifier incubator with G418 antibiotic added to them with final concentration of 1mg/mL.

Transfection with siRNA. Transfection was performed as described by Bitler et al 2010. MDA-MB-231 were transfected with pCDNA3 vector to create the "-Muc" cell line and pCDNA3-MUC to create +Muc cell line.

Matrix Degradation Assay. Matrix Degradation studies were conducted in accordance to Artym et al, 2009. +Muc cell line was treated with 10 ng/mL EGF for the duration of this experiment to create "+Muc+EGF" sample. "+Muc+EGF-Met" was created by treating the +Muc cell line with 10 ng/mL EGF and 5uM SU11274, while "+Muc+EGF+PMIP" was created by treating +Muc cell line with 10 ng/mL EGF and 50uM PMIP for the duration of this experiment. The samples were imaged with Zeiss LSM 510 META NLO confocal microscope. 10 images per sample were taken for each of the three trials. We scored each image according to the following scale: 5.5 points- strong punctual and uniform degradation,5- strong punctual, 4.5- weak punctual and uniform degradation, 4- weak punctual degradation, 3- strong uniform degradation, 2- weak uniform degradation, and 1- no degradation. We counted the number of cells belonging to each category. These values were added and assigned to two categories: non-punctual degradation of scores 1-3 and punctual degradation of 4-5.5 points. We calculated the percent of cells exhibiting punctual degradation and non-punctual degradation by dividing the number of cells in each category by the total number of cells and multiplying by 100. Standard deviation values and p-values (student's t-test) were calculated using Microsoft Excel.

Invasion Assay. The matrigel was thawed overnight and was then diluted to 1 mg/mL in serum free RPMI 1640 media. 100 uL of this solution was placed into the upper chamber of a 24-well transwell plate. The matrigel was left to incubate overnight at 37^{o}C. Both -Muc1 and +Muc1 cell lines were treated with 50uM PMIP, 5uM SU11274, and 10 ng/mL EGF respectively. Cells were trypsinized and resuspended in serum free RPMI media at the density of 1 x 10^{5}. The lower chamber was filled with 600 uL of 10%FBS media and 50 ng/mL EGF. 100 uL of cell suspension was then added to the upper chamber. The plate was incubated for 24 hours at 37^{o}C.The transwells with cells were fixed in 50% acetone-methanol for 5 minutes The matrigel was then removed using cotton swabs. The filters on the transwells were cut out from the baskets using a scalpel, were treated with Prolong Gold Anti-Fade Reagent with Dapi, and finally mounted on microscope slides. Images of the cell nuclei on each filter

were taken at 5x magnification on the Leica HBO 0100 microscope and were then analyzed with ImageJ. They were converted into 8-bit type files, which were then inverted. The optimal threshold was found, allowing us to count the nuclei using the function "Analyze Particles." Statistical analysis was performed by comparing -Muc1+EGF, -Muc1+PMIP, and -Muc1+Su1147 to -Muc1. +Muc values were compared to -Muc, while +Muc1+PMIP, +Muc1+Su1147, +Muc1+EGF were compared to +Muc. Each experiment was done in triplicate.

Results:

We looked at the ability of MDA-MB-231 cells to degrade gelatin matrix by the matrix degradation assay. The cells, labeled green with FITC phalloidin, were placed on the fluorescently red labeled matrix. We were able to assess degradation by observing dark spots under the cells. Interestingly enough, these spots correlated with the bright green spots observed on the cell periphery. Bright phalloidin staining in these areas indicates accumulated monomeric actin, which might be attributed to the formation of cell invadopodia in those regions. The MDA-MB-231 cells have very low levels of Muc1 and we refer to them as "-Muc" cells. Figure 1 shows that these cells exhibit punctual degradation. When the cells are transfected with a plasmid that highly increases intracellular levels of Muc1, there is a visible loss of this punctual degradation pattern. When EGF is added in order to activate the EGFR receptors in the cells transfected with Muc1, the cells show no punctual degradation as well. However, when both Muc1 and EGFR levels are high but Met receptor is blocked with the SU1147 inhibitor, some cells recover their ability for punctual degradation. A similar impact is observed when PMIP is added to those cells with high levels of Muc1 and EGFR.

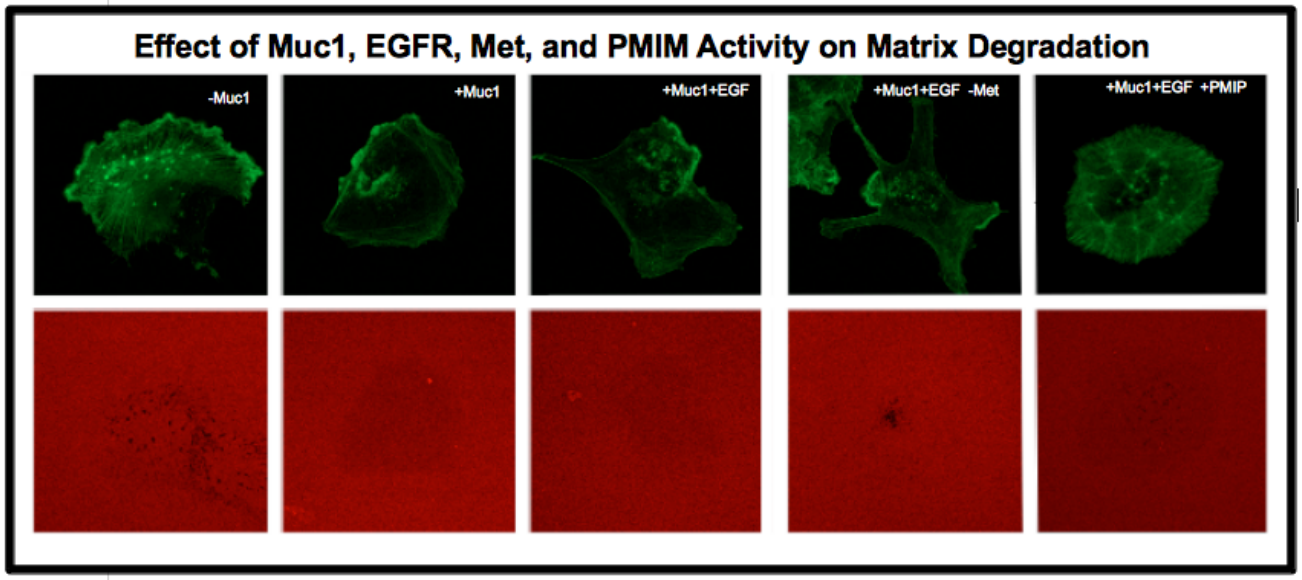


Figure 1. Effect of Muc1, Met, and PMIP Activity on Matrix Degradation. MDA-MB-231 cells show punctual degradation. When Muc1 is overexpressed, this punctual degradation disappears. The same effect is observed with increased levels of MUC1 and EGFR. Some cells seem to regain punctual degradation phenotype when Met is inhibited but levels of EGFR and Muc1 remain high. A similar phenotype is seen when PMIP is used to block MUC1 and EGFR interaction.

Quantification of the matrix degradation data in Figure 2 showed that the punctual degradation occurred in 61.67% of the wildtype MDA-MB-231 cells. An increase in Muc1 levels led to a significant reduction in the incidence of punctual degradation, (10 %, p=0.025). Only 3% of cells over-expressing both Muc1 and EGFR had punctual degradation. The p-value was also found to be significant, p=0.025. When the Met receptor was blocked in the cells with high MUC1 and EGFR levels, punctual degradation occurred in 69.16% of the cells. However, these data had a high standard deviation value and an insignificant p-value, p=0.11. When PMIP blocked the interaction of MUC1 and EGFR, punctual degradation levels increased significantly to 80%, p=0.03.

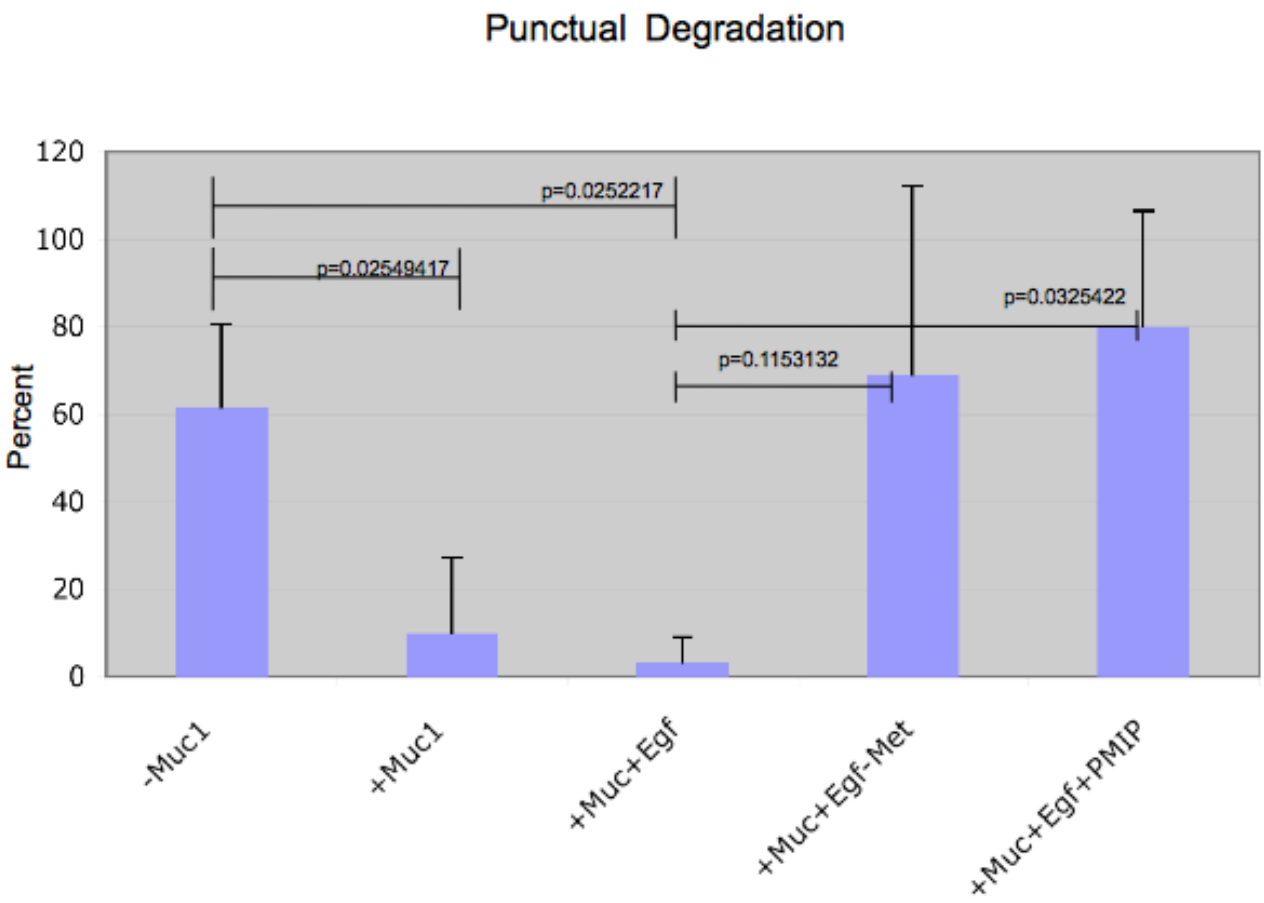


Figure 2. shows the average percent of punctual degradation present in each of the experimental conditions tested in triplicate. For +Muc1 and +Muc1+EGF, p values were calculated with respect to -Muc1; while for +Muc+EGF-Met and +Muc+EGF+PMIP they were found compared to +Muc+EGF.

We also assessed the ability of MDA-MB-231 cells to invade using the transwell invasion assay (Figure 3). $1x10^5$ cells with various levels of protein activity were placed on the transwells and allowed to invade through the filters in the direction of EGF present in the lower chamber. We found that the average value in the three trials conducted showed that 980 wildtype cells invaded. The standard deviation value of this set was 352, implying large variability within the trials. When Muc1 was overexpressed, 1426 cells invaded, on average, showing a slight increase. However, the standard deviation value of this set was 2088, showing even higher variability. The p-value compared to the wildtype was found to p=0. 74, showing that these data are statistically insignificant. When PMIP was added to to the MUC1- positive cells, on average, 206 cells invaded. The standard deviation value for

this set was 188, while the p-value compared to the cells with overexpressed Muc1, was p=0.41. When the Met receptor was blocked with the SU1147 inhibitor, 320 cells invaded. The standard deviation value was 105, while the p-value compared to Muc1 overexpressing cells, was p=0.45. We also tested whether desensitized cells still invade towards the EGF-containing serum by pretreating the cells with EGF. 572 EGF-treated cells invaded. The standard deviation was 447 cells, while the p-value compared to Muc1 overexpressing cells was also insignificant, p=0.55. The controls for this experiment also showed high variability in cell numbers. As such, when PMIP was used to treat wildtype cells, on average 654 cells invaded. The standard deviation was high, 352, and p=0.32. When the Met inhibitor was used for the wildtype cells, the number of invading cells increased to 1809. The standard deviation of 1846 showed high variability, while the p=0.52 compared to wildtype made it statistically insignificant. The treatment of wildtype cells with EGF seemingly increased the number of invading cells to 2234. However, the standard deviation was 2597 showing high variability in the three data sets and thus the increase in invasion was statistically insignificant, p=0.49

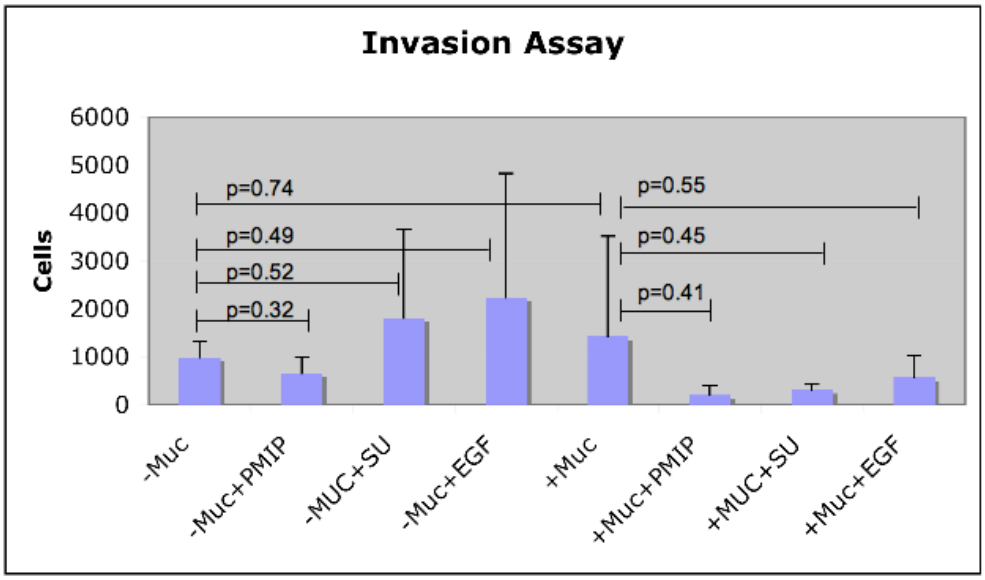


Figure 3. The transwell invasion assay examined invasion in MDA-MB-231 cells, showing high variabiliy and insignificant results.

Discussion:

According to the National Cancer Institute, 1,638,910 people will be diagnosed with cancer in 2012, while 577,190 of them die of cancer. This year, 226,870 women will be diagnosed with and 39,510 women will die of breast cancer. Meanwhile, 1 out of 8 women will develop breast cancer in their lifetime (National Cancer Institute). Metastasis is one of the main critical contributors to poor prognosis in cancer patients. Cancer cells undergo metastasis by degrading the extracellular matrix and invading the tissues. In order to better understand the molecular basis of cancer, we have conducted matrix degradation and invasion assays to examine the role of Muc1, EGFR, and Met proteins in these processes.

Our in vitro matrix degradation experiments have shown that Muc1 and EGFR inhibit the ability of cells to perform matrix degradation. The blocking of Muc1 and EGFR interaction with PMIP also blocked Muc1/EGFR mediated inhibition of degradation. However, the hypothesis that this pathway works through the Met protein was not supported by the data due to the insignificant p-values obtained by comparing the three sets of results. If Muc1 and EGFR normally promote cells to migrate and cancer, then they should increase matrix degradation, and thus amplify the cancerous nature of MDA-MB-231 cells. In turn, PMIP, a cancer drug, should inhibit matrix degradation, therefore inhibiting cancer. In fact, the results we obtained were completely opposite to our predictions. Previous studies on MUC1/EGFR showed that these proteins directly increase cell migration, while PMIP inhibits MUC1/EGFR mediated cell migration (Schroeder, unpublished). This suggests that cell migration and matrix degradation are inversely coupled processes. As such, the more cells migrate, the less time they have to degrade matrix due to their constant movement. However, once migration is inhibited with PMIP, the cells will be more static and thus will have more time to degrade the matrix around them. Further studies need to be done in order to assess whether there is a link between matrix degradation and migration. Another, more precise way to test the effect of MUC1/EGFR activity on

matrix degradation would be a 3D matrix cell assay. In vivo, tissues have a very complex 3D structure, where cells are surrounded by various extracellular matrix components. In such an environment, cells are exposed to various mechanical and biochemical signals, which influence their behavior (Sala et al. 2011). Because cell invasion and matrix degradation happen in a 3D environment, cells have to move and degrade matrix in a 3D fashion. The 2D matrix degradation studies performed here mirror cell motility due to focal adhesion formation on one surface, but do not accurately model migration in a 3D environment, which relies on the interaction of cell protrusions with the matrix (Mak et al. 2011) Therefore, the studies we conducted need to be repeated in a 3D matrix of similar composition. Additionally, we would have to perform live imaging of these matrix degradation experiments with 2D and 3D matrices, which would allow us to link matrix degradation with migration and invasion.

To examine cell invasion with respect to the MUC1/EGFR pathway, we used a transwell invasion assay. However, the data collected in the three trials were completely different for each trial in addition to being statistically insignificant. This variability within trials suggests that the assay might have been flawed. This may be due to experimental error, such as variability in the starting cell number within each trial. Also, the cancer cell line we used in our experiments, (MDA-MB-231), is known to be highly metastatic (Mak et al. 2011). Heterogeneity within a single cell line affects mechanical invasiveness among a common population of cells (Mak et al. 2011). This heterogeneity maybe attributed to the genetic variability within cancer cells. Cancer cells constantly undergo uncontrolled cell division and therefore experience heritable changes. Moreover, cancer cells are genetically unstable and fail to maintain the integrity of their DNA. Such cells accumulate mutations, which impact cell signaling, in turn changing the very nature of the cancer cells and thus giving rise to heterogeneity within a single cell line. For the three trials that we have conducted, we used different generations of MDA-MB-231 cells, which could have had different cell identities and resulted in dramatic differences in cell behavior.

Further experiments need to be conducted to help us draw clear conclusions about how

Muc1/EGFR interaction impacts matrix degradation and cell invasion. By studying which proteins are involved in and regulate these processes, we hope to create a better understanding of the molecular basis of cancer as well as potential treatments.

References:

Alberts, Bruce. Molecular Biology of the Cell. New York: Garland Science, 2008. Print.

Artym, V. V., K. M. Yamada, and S. C. Mueller. "ECM Degradation Assays for Analyzing Local Cell Invasion." Methods in Molecular Biology 522.3 (2009): 211-19. Web.

Bitler, B. G., A. Goverdhan, and J. A. Schroeder. "MUC1 Regulates Nuclear Localization and Function of the Epidermal Growth Factor Receptor." Journal of Cell Science 123.10 (2010): 1716-723. Print.

Bitler, B. G., I. Menzl, C. L. Huerta, B. Sands, W. Knowlton, A. Chang, and J. A. Schroeder. "Intracellular MUC1 Peptides Inhibit Cancer Progression." Clinical Cancer Research 15.1 (2009): 100-09. Print.

Cancer Statistics. National Cancer Institute. Web. 19 Apr. 2012. <http://seer.cancer.gov/statfacts/html/breast.html>.

Heino, Jyrki. "Biology of Tumor Cell Invasion: Interplay of Cell Adhesion and Matrix Degradation." International Journal of Cancer 65.6 (1996): 717-22. Print.

Lin, S. Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K. Y., Bourguignon, L. and Hung, M. C. (2001). Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat. Cell Biol. 3, 802-808.

Mak M, Reinhart-King CA, Erickson D (2011) Microfabricated Physical Spatial Gradients for Investigating Cell Migration and Invasion Dynamics. PLoS ONE 6(6): e20825. doi:10.1371/journal.pone.0020825

Pochampalli, M. R., B. G. Bitler, and J. A. Schroeder. "Transforming Growth Factor Dependent Cancer Progression Is Modulated by Muc1." Cancer Research 67.14 (2007): 6591-598. Print.

Pochampalli, M. R., R M El Bejjani, and J. A. Schroeder. "MUC1 Is a Novel Regulator of ErbB1 Receptor Trafficking." Oncogene 26.12 (2006): 1693-701. Print.

Ponzo, Marisa G., and Morag Park. "The Met Receptor Tyrosine Kinase and Basal Breast Cancer." Cell Cycle 9.6 (2010): 1043-050. Print.

Sala, Ana, Patrick Hanseler, Adrian Ranga, Matthias P. Lutolf, Janos Voros, Martin Ehrbar, and Franz Weber. "Engineering 3D Cell Instructive Microenvironments by Rational Assembly of Artificial Extracellular Matrices and Cell Patterning." Integrative Biology 3 (2011): 1102-111. Web.

Sharona Even-Ram and Vira Artym (eds.), Methods in Molecular Biology, Extracellular Matrix Protocols, vol. 522 © Humana Press, a part of Springer Science + Business Media, LLC 2009 DOI: 10.1007/978-1-59745-413-1_15

Schroeder, Joyce A., Azzah Al Masri, Melissa C. Adriance, Jennifer C. Tessier, Kari L. Kotlarczyk, Melissa C. Thompson, and Sandra J. Gendler. "MUC1 Overexpression Results in Mammary Gland Tumorigenesis and Prolonged Alveolar Differentiation." Oncogene 23.34 (2004): 5739-747. Print.