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INHIBITING PROSTATE CANCER: THE EFFECTS OF THE SIGNALING RECEPTOR, RHAMM, ON PROSTRATE CARCINOMA GROWTH AND MOTILITY

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Prostate cancer is a disease that impacts millions of men around the world. The purpose of this research is to identify key targets of the cancerous cells in the prostate and to disrupt the signals that these cells use for invading the prostate gland and distant organs. This study examines the carbohydrate Hyaluronan (HA) and its receptor, RHAMM, as a target in order to inhibit prostate cancer. HA has been shown by many studies to help advance prostate cancer.

To study the effect of RHAMM on prostate cancer cell growth and migration, normal prostate tumor cells, mock prostate tumor cells, and CRISPR cells (Clustered Regularly Interspaced Short Palindromic Repeats) without RHAMM were used. The growth, migration, and protein expression of these cells was measured in 2-dimensional and 3-dimensional plates.

The results show that the RHAMM CRISPR cells displayed impaired motility and slower growth in the 3-dimensional and the migration assays. In 16 hours, the migration of the RHAMM CRISPR cells was more than 50% slower than the growth of normal and mock tumor cells. Therefore, it is apparent that removal of the receptor RHAMM is an important step to reduce prostate cancer metastasis.

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INTRODUCTION

Uncontrolled growth of abnormal cells in the body, or more commonly known as cancer, is one of the most dreadful diseases in the world. In 2015, there were approximately 1.6 million new cancer cases diagnosed worldwide, with more than half a million deaths in the United States. Prostate cancer is the most common cancer among men. In the United States, approximately one in seven men will be diagnosed with prostate cancer during their lifetime.

Given how many millions of men are impacted, it is imperative to find a suitable treatment that can reduce the mortality rate for prostate cancer patients and to improve their quality of life (American Cancer Society, 2016). Researchers across the world have been working tirelessly for many years, trying to find ways to prevent and cure this dreadful disease. Since research studies need to be focused at a molecular and cellular level in multiple dimensions, cancer research is particularly challenging.

A healthy prostate gland has a thin layer of interior cells called the secretory prostate epithelium. The job of these cells is to synthesize and secrete key molecular components of semen. However, these cells can also form prostate carcinomas (epithelial cancer cells) that ultimately can metastasize to kill patients.

The goal of this research is to identify key targets of the epithelial cancerous cells in the prostate and disrupt the signals that these cells use to their advantage for invading into the prostate gland and metastasizing to distant organs. The focus of the project is on a large carbohydrate termed hyaluronan (HA). The amount of HA is increased abnormally in prostate cancer tissues and functions to organize protumorigenic-signaling pathways in carcinomas. This means that HA has a high level of importance and uses special pathways for cell communication. (Heldin et al., 2009). So, the question is: how do prostate carcinoma cells exploit abnormally high levels of HA and a key HA receptor (RHAMM) to accelerate tumor growth and invasion in prostate cancer?

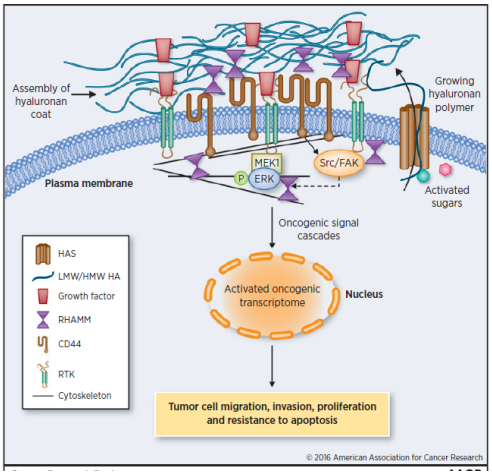

Figure 1. Hyaluronan and the plasma membrane. (McCarthy et al., 2016).

The studies done by tumor researchers should help characterize the importance of HA in prostate carcinoma histology. HA functions in normal tissues to maintain the tissue structure function, tissue homeostasis. Changes in the synthesis and degradation of HA are also very important for promoting tissue repair and wound healing. However, studies have shown that abnormal changes in HA metabolism within tumors are used by tumor cells to grow, invade distant organs, and metastasize (McCarthy et al., 2016). Metastasis is the process of cells expanding into their microenvironment and traveling to distant tissues and organs. Metastatic cells travel by invading normal tissue and the bloodstream. Prostate carcinoma cells synthesize and deposit abnormally high levels of tissue associated HA, which function to organize and activate important protumorigenic signaling pathways in tumor cells; these signaling pathways promote tumor growth, motility and invasion. Studies have shown that changes in HA metabolism within tumors is associated with malignant progression and these changes in the peritumoral prostate stroma and parenchyma are related to tumor initiation, progression, and poor clinical outcomes (McCarthy et al., 2016). The goal of our work was to inhibit key signaling proteins within tumor cells that are activated by HA, thereby stunting the ability of HA to promote tumor formation and metastasis.

In a cancerous prostate microenvironment, HA acts as signaling pathway for cells to multiply, which causes abnormal growth and tumor cell invasion leading to metastasis and resistance to therapies (Heldin et al., 2009). HA binds to specific tumor cell receptors, such as RHAMM, that function to initiate the signaling (oncogenic) pathways important for the tumor cells. RHAMM binds to HA and interacts with the machinery within tumor cells to promote their abnormal growth, motility and invasion. Studies have shown that carcinoma cells from advanced metastatic tumors have abnormally high levels of RHAMM (McCarthy et al., 2016), suggesting that RHAMM may be critically important for maintaining malignant prostate tumors. The specific focus of this research is to determine if RHAMM expression is important for stimulating malignant prostate carcinoma cell growth and invasion.

MATERIALS

Three primary cell lines were used in this study. The cells used include PC3M-LN4 parent, mock, and CRISPR cells. The parent cells are the unmodified PC3M-LN4 prostate carcinoma cells. The mock cell lines differ from the parents because they do not express RHAMM, but it is still included. The CRISPR cells differ from the parent and mock cells because the CRISPR cells have RHAMM knocked out. These cells have been genetically engineered to knock out RHAMM using CRISPR technology. The CRISPR cells are the primary indicators of the growth and migration of PC3M-LN4 cells without RHAMM.

Cell lines: The PC3M-LN4 prostate tumor cell line was provided by a University of Minnesota laboratory partner. The RHAMM CRISPR cell line was

generated by transfection with paired guide RNA's (5' GTATTGTATTTGATTAGAAT 3', and 5' GAATTTGAGAATTCTAAGCT 3' in plasmid pCR4-TOPO-U6-HPRT-gRNA), that bind in exons 3 and 6, respectively. The guide RNAs are co-transfected with the plasmid expressing the CAS9 enzyme (pT3.5 Caggs-FLAG-hCas9) as well as two plasmids for Puromycin and GFP selection, pcDNA-PB7 and pPB SB-CG-LUC-GFP (Puro)(+CRE). Transfection was performed using the UltraCruz transfection reagent (Santa Cruz Biotechnology) following the manufacturer's suggested protocol. Mock cells were transfected with control plasmids. Clonal cell lines were screened for loss of RHAMM by Western Blot and genomic DNA PCR analysis. All statistical analysis were performed using the Graphpad Prism 6 software.

The antibodies used for Western Blots include: peroxidase-conjugated AffiniPure donkey anti-rabbit IgG, peroxidase-conjugated affinipure donkey anti-mouse IgG, HAS2 (S-15): Sc-34067, RHAMM Anti-CD168 antibody [EPR 4055] ab108339, CD44 (156-3C11) mouse mAb, and c-Myc (D84C12) Rabbit mAb. The reagent used in the 2-D Growth Assay is the CellTiter 96® AQueous One Solution Cell Proliferation Assay purchased from Promega (Madison, WI).

Other materials used include: 1% & 20% sodium dodecyl sulfate , 2-mercaptoethanol, 10x electrode buffer, 10x TBST (Tris Buffered Saline with Tween), acrylamide, ammonium persulfate, double distilled water, dextrose, difco trypsin, ethylene diamine triacetic acid (EDTA), fetal bovine serum, potassium chloride, minimal essential medium, nonessential amino acids, non-fat dry milk powder, octyl phenoxypolyethoxylethanol (NP-40), phosphate buffered saline, 1x penicillin-streptomycin, phenol red, protease inhibitor, sodium bicarbonate, sodium chloride, sodium deoxycholate, sodium lauryl sulfate, sodium orthovanadate, sodium pyruvate, tetramethylethylenediamine (TEMED), trypan blue, and tris buffer pH 6.8 and 8.8.

METHODS

The methods we applied allowed us to measure the growth, migration, and protein expression of the parent, mock, and CRISPR cell lines. For example:

- Migration assay was performed to study the rate of cell motility over a period of time.
- Two-dimensional growth assay was conducted to analyze how fast cells grew when adhered to a tissue culture plate for a certain period of time
- Three-dimensional growth assay was used to simulate close to real-life growth patterns.

The collected data can be used to form conclusions about the differences between the RHAMM CRISPR cells and the parent and mock cells with respect their growth, migration, and protein expression. This will help to understand how tumor growth can be inhibited.

Media: To make media, a filter was screwed tightly onto the glass bottle. The bottle cap was kept face down on the lid of the filter, so that the materials remained sterile. 500 mL of minimal essential medium was poured carefully into the filter, followed by 50 mL fetal bovine serum.

Next, 5 mL of the pen strep was pipetted carefully into the filter. Afterward, 5 mL of nonessential amino acids and 5 mL of sodium pyruvate was pipetted into the filter. The vacuum was then connected to the protruding part of the filter on the cap. The vacuum was turned on to start the filtering process.

Trypsin/EDTA (2L): Trypsin was used to cleave cells off the walls of the flask. Prior to mixing all of the ingredients to create Trypsin, the following ingredients were measured precisely:16 mg NaCl, .8 mg KCl, 2 mg dextrose, 1.16 mg $NaHCO_3$, 1 mg difco trypsin, .4 mg ethylene diamine triacetic acid, and .9 mg phenol red . After the ingredients were measured, they were poured into a glass vial on a head shaker with a stirring rod in the solution. The top of the vial was wrapped with aluminum foil to ensure the bottle was sterile. After the solution was done mixing, the solution was transferred into a 250 mL glass bottle in the chemical hood.

Cell culture (1:10): First, the media-trypsin solution was discarded. The cells were washed with 3 mL trypsin on the side with adhered cells, and then discarded. 3 mL of trypsin was added to release cells. Then, the flask was hit a couple times to start the releasing. 9 mL of media was added to a new flask. After, 7 mL of media was pipetted into the flask with trypsin and was pipetted up and down on the side with cells to obtain all cells. Then, 1 mL of solution was pipetted into the flask with media depending on cell confluence. The liquid in the flask

was gently stirred to mix solution well, and then placed in an incubator.

Cell lysis: The cells were washed with 5 mL of cold Phosphate Buffered Saline (PBS). Then, 5 mL of cold PBS was added and sat for 3 minutes at 4°C in a refrigerator. All PBS was removed with a vacuum. 300-400µL of lysis buffer was added to flask and was made sure to tilt the flask around so that the buffer completely covered the side with cells. The lysis buffer sat on the cells for 1 to 2 minutes. Then, the cell solution was scraped into a corner of the flask and collected into a microfuge tube. The samples were sonicated for 30 seconds at 25% amplitude. Then, they were incubated at 40°C for 10 minutes. The samples were spun in a centrifuge for 10 minutes at maximum speed in 4°C cold room. The supernatant was transferred to a fresh tube, and then kept on ice.

2-D growth assay: The purpose of the two-dimensional growth assay was to see how fast cells grew when adhered and anchored to a tissue culture plate for 1 week. To prepare this assay, the cells were harvested by pipetting trypsin (3 mL) and were washed and pipetted along the sides of the flask with 7 mL of media. After harvesting the cells, 10 mL of cell/media/trypsin solution was pipetted into a filter top tube. Next, to count the cells in the tube, the tube was inverted several times, and then 10 µL of this solution was pipetted into a microfuge tube, then 10 µl of Trypan Blue was pipetted in the same tube with a new pipette tip to prevent contamination.

Trypan Blue was included in the assay to identify dead cells which were not counted in the total. 10 microliters of the mixed solution was pipetted in a cell counter slide. Using the cell count (cells/mL), the cells were suspended in equal numbers across 6 wells for each day of the assay. Then 100 µl of media were added in each well, then placed all the plates for the various days of assay in the incubator. For each day of the assay, after day 0, the old media was removed using a glass pipette attached to a vacuum flask located inside the chemical hood. Each well was replaced with 100 mL of fresh media, and added with 20 µL of dying reagent. The samples were placed in a 37oC incubator for 2 hours. After the incubation period, the samples were taken to the Tecan plate reader to quantify the number of cells. This was done for every day of the 2-D Growth Assay.

3-D growth assay lower layer: 1 g of agarose was added to a glass bottle, and .6g of agarose was added to another glass bottle. Next, 50 mL of filter-sterilized water was added to the both glass bottles. Each glass bottle was microwaved and stirred until agarose was completely dissolved in water. 20 mL of 2x media and 20 mL of 1g agarose was added into a 40 mL conical tube. The tube was inverted to mix the solution. 2 mL of solution was pipetted into six wells. This was repeated for three plates. There were three 6-well plates and each well was filled with 2 mL of solution. This created a gel that would serve as the lower layer in this assay.

3-D Growth Assay Upper Layer: To add top layer, pipette 4 mL of 2x media, a variable amount of cell-media solution, and 4 mL of .6g agarose was pipetted into one conical tube. To calculate the amount of cell-media solution, the cells have to be counted (cells/mL). The tube was inverted to mix up the solution. Then, 2 mL of the solution was added to each well on top of the lower layer. The wells had 2 mL of lower layer, and 2 mL of upper layer. To collect cells, the cells were washed with 3 mL of trypsin, cells were then released with 3 mL of trypsin. 7 mL of 1x media was added once the cells had released, and then centrifuged for 5 minutes. Then, each tube was decanted and 2 mL of media was added to each tube. The cell pellet was disrupted by vigorous pipetting to make a single cell suspension. The cell number was quantified and the concentration adjusted for dispensing into the upper agarose solution. determined. Then, the three plates were refrigerated for 15 minutes. After, 2 mL of 1x media was added to each well. Finally, each plate was placed in the incubator and the number of colonies was quantified after 2 weeks.

Migration assay: The purpose of the migration assay was to see the rate of cell motility over a 48-hour period. To prepare the assay, the cells were released and harvested with 3 ml of Trypsin, and then added with 7 mL of media to the trypsin to neutralize the solution. The solution was transferred out of the flask, into a 15 mL tube, spun down in a centrifuge, the media decanted and repeated once. After decanting, 2 mL of media was added and then transferred into a FACS filter top tube for counting. While the cell counter was running, the gel inserts were placed in the bottom of each well, firmly placed so that there was no way the cells would escape the confinement of the inserts. After the inserts were placed in the wells, the appropriate amount of cells

were equally pipetted in the inserts. The inserts were removed to create a space into which the cells would migrate and the plates were placed in the incubator.

Cells were photographed at Time 0, 12, 24, 32, and 48 hour time points, pictures of the cells were taken. Using Photoshop software, the cell free space over the time points was measured.

Western Blot: The protocol used for the western blot was based on of the University of Minnesota Western Blot protocol. Modifications pertain to only chemical concentration for separation and stacking gel, the amount of marker in lysed cells, the machine used to measure samples, milk concentration, washing and incubation timing, and a secondary antibody was added in the protocol for this research. The full western blot protocol used in this research can be found in Appendix A.

RESULTS

Figure 2 shows the doubling rate of the parent, mock, and RHAMM CRISPR cells in a 96-well plate for the 2-D growth assay. The parent cells grow at a slower rate than the mock and RHAMM CRISPR cells when all of the cells are provided with an adhesion induced reorganized cytoskeleton to support growth.. The data points were combined from four experiments on days 0, 1, 2, 3, 4, and 6.

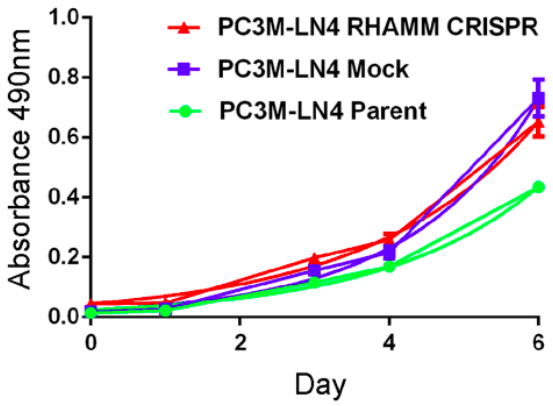

Figure 2. 2-D Growth Assays (figure by authors)
Due to the variance in start values for each of the cell lines across the four independent experiments, the data were analyzed by nonlinear best fit analysis to calculate the relative doubling time of each cell line using the Graphpad Prism 6 software. The estimated doubling time for each cell line of 1.443 days for parent cells, 1.204 days for mock cells, and 1.557 days for CRISPR cells.

Figure 3 shows the average number of colonies in triplicate wells across the three cell lines studied for the 3-D growth assay. The graph shows that while the parent and mock cells were both averaging almost 70 colonies per well, the gene-edited RHAMM CRISPR cells averaged a minimal 30 colonies per well. Unlike the 2-D growth assay, the cells in the 3-D growth assay have no external cytoskeletal support because they are suspended in gel.

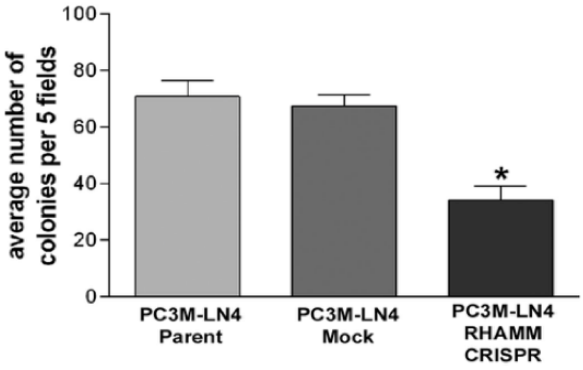

Figure 3. 3-D growth assay (figure by authors)
Bars represent the average number of colonies counted from five random fields/well from triplicate wells, +/- s.e.m, from four independent experiments. ANOVA $p<0.001$, *$p<0.0001$ vs. parent and mock cells by Bonferroni's adjustment for multiple comparisons.

Figure 4 displays the results of the migration assay, comparing the parent, mock and CRISPR cells after 16 hours of migration. Using "time zero" as the start point of the assay, the percentage of confluence was calculated after 16 hours. Figure 4 shows that the parent cells were completely confluent after 16 hours, with the mock cells being almost as confluent as the parent cells. The CRISPR cells are shown to be reduced to less than half the amount of the parent cells.

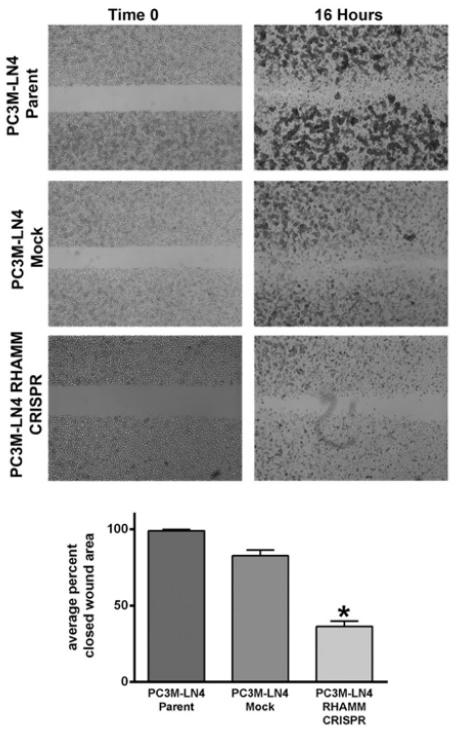

Figure 4. migration assay (figure by authors)

Legend: Images of the wound area were collected using a 4x objective at 0 and 16 hours and the cell free area quantitated by tracing the open wound area using Adobe Photoshop™. Bars represent the percentage loss in cell free area at 16 hours from triplicate inserts, +/- s.e.m. *p<0.001 vs. parent and mock cells by Student's two-tailed t-test.

Figure 5 is a western blot developed with the RHAMM, HAS2 (Hyaluronan Synthase 2), CD44 (another receptor for Hyaluronan) and c-Myc (a transcription factor) primary antibodies. The primary antibodies were then detected using a biotinylated secondary antibody. As expected, the CRISPR cells contain no RHAMM because the the RHAMM gene has been edited using CRISPR/Cas9 technology. Turning to the HAS2, CD44, and c-Myc results we see that the bandwidths and relative intensities are all the same. The Tubulin blots also had the same bandwidths and relative intensities; Tubulin antibodies are used to verify equal loading levels among all wells in the gel.

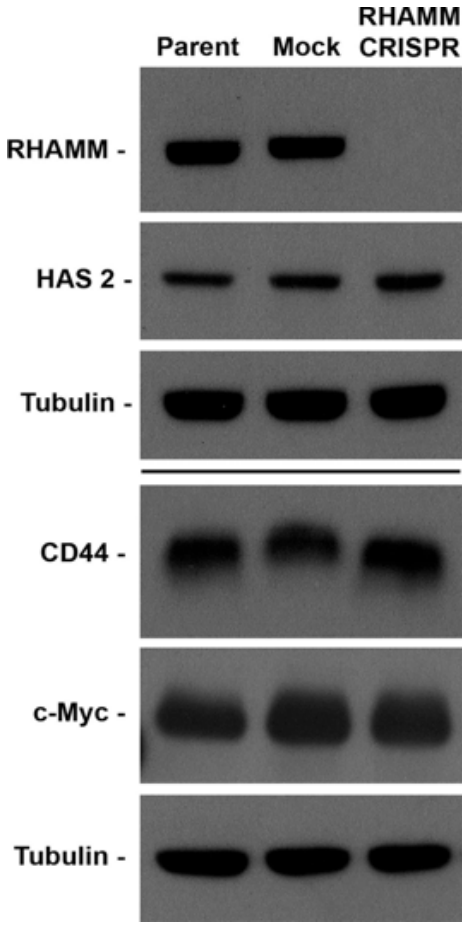

Figure 5. western blot readings (figure by authors)

DISCUSSION AND CONCLUSIONS

2-dimensional growth, 3-dimensional growth, migration, and western blot assays were successfully performed on three different PC3M-LN4 cell lines: normal prostate tumor cells, mock cells, and RHAMM CRISPR cells. These assays were meant to test the growth, migration, and protein expression of these three cell lines.

The Western Blots were successful in showing that RHAMM had been knocked out of the CRISPR cells. Knowing this, the 2-dimensional and 3-dimensional growth assays were performed to find out if taking RHAMM out would inhibit cell growth in these CRISPR cells, as hypothesized. The 2-D growth assay results showed that for two dimensions, where cells only have to grow latitudinally, growth is not inhibited by removing the RHAMM gene, as shown in

Figure 2. In contrast, the CRISPR cell struggled to grow in a 3-D environment, showing extremely stunted growth, and in some cases, inability to form colonies, as shown in Figure 4. This can be attributed to the fact that in the 2-D assay, the plate gave the cells a cytoskeletal structural integrity that helped the cells grow. While in a 3-D Assay, these CRISPR cells had no support to grow, and could not sustain themselves at the same rate that parents and mock cells did. While the 2-D growth assay results may seem to contradict the 3-D Growth Assay results, the 3-D Growth Assay is a better indicator because the cells are suspended. Growth in 3-D is also used as a correlate for the ability to form tumors, suggesting that RHAMM expression is an important contributing factor to tumor formation and growth in vivo. This is closer to a real-life scenario of tumor metastasis. Therefore, the 3-D Growth Assay truly shows how the RHAMM CRISPR cells are expected to act in a human body.

The migration assay was conducted to measure the differences of distance spread by the three PC3M-LN4 cell lines over a 16 hour period. The experiment showed that the motility of CRISPR cells was severely stunted because the CRISPR cells were unable to close the gap over 16 hours; the Parent and Mock cell lines were able reach 100% confluence. This means the CRISPR cells have successfully been inhibited, as they cannot move and migrate in a simulated environment. This result suggests that RHAMM expression is important for the ability of tumor cells to migrate and invade into other tissues/organs. This result can be applied by people to create clinical therapies inhibiting prostate cancer metastasis; this is something that can help 1 in 7 men in the United States (American Cancer Society, 2016).

In patients with cancer, hyaluronan concentrations are usually higher in malignant tumors than in corresponding benign or normal tissues, and in some tumor types the level of hyaluronan is predictive of malignancy and/or adverse clinical outcomes (Toole, 2004). Hyaluronan is especially known to drive tumor metastasis in prostate carcinomas. Hyaluronan is able to transmit signals to activate cellular adhesion and metastasis largely because of its ability to stimulate specific receptors RHAMM and CD44. In this study, the hypothesis was that PC3MLN4 Prostate Carcinoma cells that lack RHAMM would have slower growth and migration than the parental RHAMM expressing RHAMM cells. After completing and analyzing several assays, it has become apparent when RHAMM is deleted, protumorigenic functions of hyaluronan can be blocked, indicating that RHAMM is a potential clinical target for treatment of patients with metastatic prostate cancer.

Possible Sources of Error

Some possible sources of error could include incorrect pipetting or measurements. Contamination is also a possibility when working with cell lines. In the 3-D Growth Assay, the colonies were manually counted out, so there is a possibility of counting error. To minimize the potential of technical errors, the assays were completed multiple times to ensure reproducible and reliable data.

Future Work

Future continuations of this research would include testing other effects of removing RHAMM. RHAMM may have a large impact on other components such as the invasion, resistance to therapies of a cell that promotes metastasis and/or causes recurrence and relapse.

Other future work would include specifically targeting the removal and/or function of RHAMM in prostate tumor cells. Ideally, we would also want to look into slowing down the progress of malignant cancerous tumors even more by targeting other signaling pathways that also may contribute to rapid progression of cancerous tumors. One of these may be C-Myc, a protein we found in a high concentration in correlation with HA.

ACKNOWLEDGEMENTS

Dr. J. McCarthy, the program co-leader of tumor microenvironmental studies at the Masonic Cancer Center and a professor at the University of Minnesota, served as our mentor. He allowed us to work in his laboratory, and assisted us with our research paper. Mr. M. Price, a lab scientist working under Dr. McCarthy, trained us to work in the laboratory and supervised our work. Mr. J. Yang, another scientist in Dr. McCarthy's lab, helped us perform assays. Lastly, P. Hansen provided guidance for our research paper and helped organize our project.

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18. Western Blot Protocol (University of Minnesota): College of Biological Sciences. Western Blot. http://cbs.umn.edu/gale-lab/protocols/westernBlot, 7/14/16.

APPENDIX

Western Blot (Apparatus): The required materials include 3.3 mL 30% acrylamide, 2.5 mL Tris Buffer PH 8.8, 2.5 mL Tris Buffer PH 6.8, 1% Sodium dodecyl sulfate, Double Distilled Water, Tetramethylethylenediamine, Ammonium persulfate, 1x Electrode Buffer, 1x Tris-buffered saline Tween, Nonfat Dry milk powder, Antimony, Phosphate Buffer Solution and lysed cell samples, and materials for running gel. The first step is to set up the electrophoresis apparatus. Clean two glass slides with ethanol and wipe until dry. Repeat for two dividers that will separate the glass slides. Insert the dividers in-between the glass slides, and insert slides in a mechanism that holds the slides in place. Tighten the hold on the slides by twisting the screws on the mechanism. Insert the mechanism into a stand that makes the slides and mechanism flush.

Western Blot (Separating Gel 10%): First, pipette 3.3 mL of acrylamide, 3.5 mL of Tris Buffer PH 8.8, 4 mL of Double Distilled Water, 100 µL of SDS, 50 µL of APS, and 10 µL of TEMED into a 15 mL tube. Invert the tube several times to properly mix solution. Then, pipette the solution in-between the two glass slides in the stand until the solution takes up 60-70% of space. Then, add 600 µL of Double Distilled Water on top of the separating gel. Wait 15-20 minutes for

gel to harden. Check for solid gel by looking at tube with rest of solution.

Western Blot (Stacking Gel 10%): First, pipette 4 mL of acrylamide, 2.5 mL of Tris buffer PH 6.8, 3.35 mL of Double Distilled Water, 100 µL of SDS, 50 µL of APS, and 10 µL of TEMED into a 15 mL tube. Invert tube several times to mix solution. Pipette the solution on top of the separating gel once the separating gel has solidified. Pipette it carefully and evenly across the top of the separating gel to get a nice and even layer. Let sit for 15-20 minutes until stacking gel solidifies. Check for solid gel by looking at tube with rest of solution.

Western Blot Loading and Electrophoresis: Once the stacking gel has solidified, insert a comb into the stacking gel and wait 30 minutes. Once complete, take the slides out of the stand and mechanism and secure slides in a gel electrophoresis stand. Once this is done, take the comb out of the gel and mark the wells. Fill the slides with 1x electrode buffer until the slides are completely full; overflowing is fine. To prepare appropriate amounts of sample; dilute sample, PBS, and SB to a desired dilution. Vortex this solution in a microfuge tube to mix the solution. Them, carefully pipette each sample into its own individual well in the gel. Once done loading, add the cover on top of the electrophoresis mechanism and start electrophoresis. Let gel run for three hours. After three hours, remove the membrane from the slides and then put on top of piece of gel paper. Put the gel paper on top of sponge. Pour TBST on top of membrane, and then add the top sponge over the membrane. Soak the membrane in electrode buffer overnight.

Western Blot Blocking, Washing, and Antibodies: Take the membrane out of the electrode buffer. Take the gel off the gel paper. The membrane should have transferred to the gel paper. Make up a 5% Milk/TBST solution. This will be used as the blocking buffer. Put paper in an open-top container and let rock at a slow speed for 1 hour with blocking buffer. After an hour has been completed, pour out the blocking buffer, and add TBST. Let the membrane rock for 5 minutes and pour out TBST. Add new TBST and let rock for 5 minutes again. Repeat this process until the membrane has rocked in TBST for 1 hour total. The antibody used will vary depending on the experiment. After an hour is complete, pour out the TBST in the container, and then prepare a primary antibody dilution. The dilution should consist of 10 mL of Milk/TBST solution, and 2 µl of a desired antibody. Invert the tube to mix the antibody in the solution. Pour the primary antibody on the membrane and let sit overnight in a 4°C cold room. The next day, pour out the antibody and start the TBST washes for an hour again. Next, repeat the same process for the primary antibody, but use the second antibody instead. Let the antibody sit for 1 hour. Pour out the antibody and then repeat another hour of washes. Once complete, pour out TBST and let membrane sit on scanning board. Prepare 1 mL from two chemiluminescent reagents in a microfuge tube. Then, vortex the microfuge tube to mix the reagents. Pour the solution on the membrane and coat the membrane evenly. Seal the membrane in plastic and put in scanning clipboard. Take the clipboard to a dark room and obtain a scanning using the proper machine. The readings should come out on a blue sheet with the Western Blot markings on them. These readings can be read in amounts, and then can be produced into graphs and tables.