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Metastasis Suppression in Carcinoma and Melanoma Cells

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**METASTASIS SUPPRESSION IN CARCINOMA AND
MELANOMA CELLS**

by

Sherry Marie Baker

**Thesis submitted in partial fulfillment
of the requirements for the degree**

of

**HONORS IN UNIVERSITY STUDIES
WITH DEPARTMENTAL HONORS**

in

**Public Health Education
in the Department of Biology**

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ABSTRACT

The BRMS1, Breast cancer Metastasis Suppressor 1 gene suppresses cancer metastasis of the MDA-MB-435 human breast carcinoma cell line and the *KiSS-1* gene suppresses cancer metastasis of the C8161 human melanoma cell line. While both metastasis suppressor genes suppress secondary tumor formation without preventing orthotopic tumorigenicity, their mechanisms of action are still largely unknown. Cell culture and SDS-PAGE zymography were performed to analyze matrix metalloproteinase (MMP) secretion. Zymography gels were then analyzed by densitometry utilizing the Quantity One software package. Results of the data analysis showed the expression of the BRMS1 and *KiSS-1* genes correlate with decreases in secretion of specific MMPs from the cells. In MDA-MB-435, expression of the metastasis suppressor gene BRMS1 reduces secretion of MMP-9 and MMP-2. In C8161, the expression of the metastasis suppressor gene *KiSS-1* reduces secretion of MMP-9 and MMP-2. The MMPs are believed to play a role in the invasive potential of cells. By utilizing wound healing assays, the invasive potential of the MDA-MB-435 cells were compared to the non-metastatic MDA-MB-435/BRMS1 cells. Results showed that expression of BRMS1 in MDA-MB-435 human breast carcinoma cells decreased the initial invasiveness of the cells. These data show that BRMS1 and *KiSS-1* impact MMP secretion, suggesting this is part of their mechanism of suppression of invasive potential and metastasis.

DEDICATION

This thesis is dedicated to my family. To my mom, who has helped me get through hard times and has taught me how to thrive. Your ability to make self-sacrifices to help others around you improve is an inspiration. To my dad, who has always helped me to laugh in the face of disappointments. Your brilliance will constantly motivate me. To both of my parents, who have always been supportive figures, cheering from the sidelines and encouraging me to make decisions for myself. To my big brother, who has always been such a smarty pants. You made me work hard to avoid your shadow. Your example of being a good person and a good brother will forever influence how I treat others. Thank you for everything you have done and the sacrifices you have made. I love and appreciate you!

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LIST OF ABBREVIATIONS

APMA: Aminophenyl mercuric acetate

BRMS1: BReast cancer Metastasis Suppressor 1

DME/F-12: Dulbecco's Modified Eagle's medium and Ham's F-12 medium (1:1 mixture)

ECM: Extracellular matrix

FBS: Fetal bovine serum

FISH: Fluorescence *in situ* hybridization

EDTA: Ethylenediaminetetraacetic acid

HBSS: Hanks buffered saline solution

KP: Kisspeptins

MMPs: Matrix metalloproteinases

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

SDS: Sodium dodecyl sulfate

TIMPs: Tissue inhibitors of metalloproteinases

INTRODUCTION

Cancer Metastasis

Over 10 million people in the United States have cancer (1). As a major public health burden, cancer accounts for one in four deaths in the United States. Breast cancer is the most common form of cancer in women. In 2004, almost one-third of the new cases of diagnosed cancer in women were breast cancer and it was the second leading cause of cancer death behind lung cancer (2). While incidence of breast cancer has been increasing, advances in prevention, detection, and treatment have contributed to declines in mortality during the past few years. However, metastatic breast cancer is still incurable (3).

Metastasis is the primary cause of mortality in the more than five hundred thousand patients who pass away each year due to cancer (1, 2, 4). Cancer metastasis involves many highly regulated steps. Cells multiply in the absence of control mechanisms to produce a tumor. Tumor cells break off from a primary tumor, invade the surrounding tissue, and migrate through the basal membrane and into the circulatory system (intravasation) or lymphatic system. The cells travel and arrest in the blood or lymph vessel before migrating out of the circulatory system (extravasation) or lymphatic system. For some metastatic cells, extravasation is not required as tumor cells arrested in the blood vessel can proliferate (5). Secondary tumor cells must be able to grow and thrive in this new secondary environment and secrete signal molecules to induce formation of blood vessels (angiogenesis) to provide nutrients to the newly growing tumor (6). The many steps of metastasis make it a highly inefficient process.

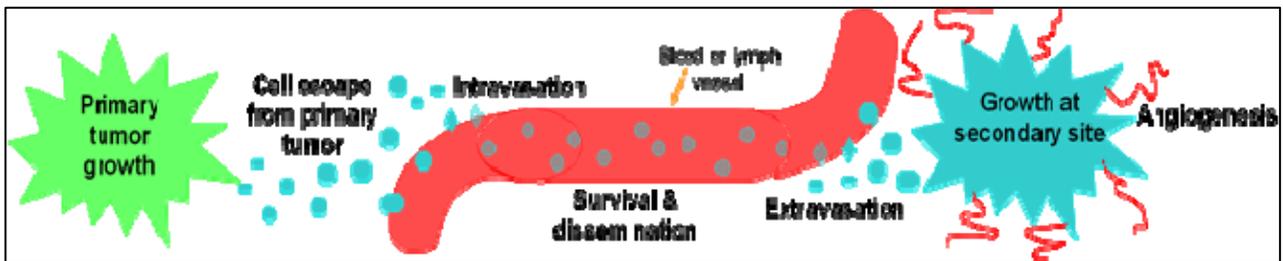


Figure 1. Cancer metastasis involves many highly regulated steps.

Cell Lines

Two of the cell lines chosen for these studies were established in the 1970s. MDA-MB-231 and MDA-MB-435 are breast adenocarcinoma cells that were obtained from Caucasian females in 1973 and 1976, respectively. Both cell lines were obtained from a metastatic tumor and appeared to be epithelial in origin. These cells form gradually growing tumors that result in metastases when they are injected into the mammary fat pads of immunocompromised mice. The MDA-MB-231 cell line was from a 51 year-old patient, while the MDA-MB-435 cell line was from a 31 year-old patient (7). In recent years, argument about the classification of MDA-MB-435 as a breast carcinoma cell line has surfaced (8). However, it has been shown that MDA-MB-435 cells express milk proteins and metastasize from mammary fat pad but not subcutaneous tissue. This is consistent with breast carcinoma cell lines (9).

Metastasis Suppressor Genes

Over the years, metastasis suppressors have been significant in metastasis prevention research. Metastasis suppressors, a fairly new class of genes with predominately unknown mechanisms of action, prevent secondary tumor formation without affecting primary tumor growth (4, 10). Metastasis suppressors are different from oncogenes, which promote cellular transformation, and tumor-suppressor genes, which suppress tumor growth (11). This family of more than 25 known genes includes the BRCA cancer Metastasis Suppressor 1 (BRMS1) and *KiSS-1* (12).

The BRMS1 gene is mapped to chromosome 11q13. It was identified by comparing occurrence of metastases in metastatic MDA-MB-435 and metastasis suppressed chromosome 11-MDA-MB-435 microcell hybrids using a differential display technique (13). BRMS1 has been shown to associate with the SIN3-histone deacetylase complexes possibly altering the transcription of some genes (14). Additionally, BRMS1 has been shown to regulate phosphoinositide signaling (15), expression of epidermal growth factor receptor (9), osteopontin (16), NFκB (17), and connexins (18), all of which are known to play a significant role in cancer development. These results implicate mechanisms by which BRMS1 suppresses metastasis.

KiSS-1 is a metastasis suppressor gene known to suppress metastasis of the C8161 human melanoma cell line without suppressing orthotopic tumorigenicity. While the mechanism of

metastatic suppression by this gene is partially understood, our knowledge is still incomplete. The *KiSS-1* gene produces the KISS1 metastasis suppressor protein which is secreted and converted to kisspeptins (19). Four biologically active kisspeptins (KP) have been identified: KP-54 (metastin), KP-14, KP-13, and KP-10 (20, 21). It has been shown that the presence of kisspeptins is correlated with the suppression of metastasis (19).

MMPs

The first matrix metalloproteinase (MMP) was characterized by Gross and Lapiere in 1962. They found that during metamorphosis, tadpole tails secreted an enzyme that could degrade fibrous collagen, the first collagenase (22). Since that initial study, further research has led to the classification of a family of structurally related proteinases that includes at least 23 different MMPs in humans and at least 24 different MMPs in mice.

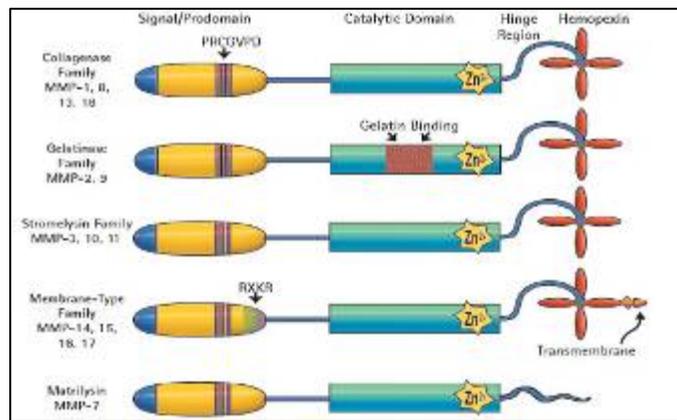


Figure 2. Structural domains of MMPs (http://www.emdbiosciences.com/html/cbc/matrix_metalloproteinase_MMP.htm).

Gross and Lapiere stated that “precisely timed and localized removal of structural tissue

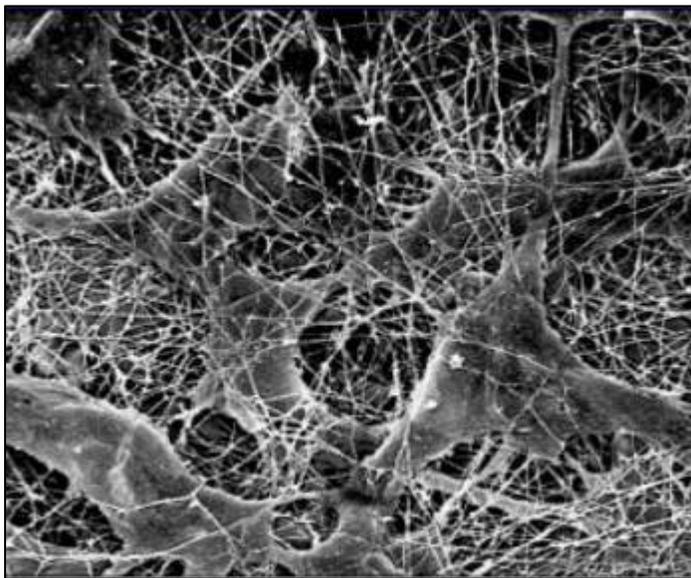


Figure 3. A Scanning Electron Microscope image of the extracellular matrix (Figure 19-35, Molecular Biology of the Cell, 4th Edition).

elements in delicate balance with synthesis is essential to normal growth and development (22).” Previous research has revealed that some metastatic cancer patients have shown secretion of abnormally high levels of MMPs from their cells (23).

Additionally, in animal models, it has been shown that relatively benign cancer cells acquire malignant properties when MMP expression is increased and that

malignant cells become less aggressive when MMP expression is decreased (24). MMPs are proteolytic molecules that are believed to be secreted by all mammalian cells (25). MMPs are secreted from cells in a latent proform and require proteolytic processing for conversion to an

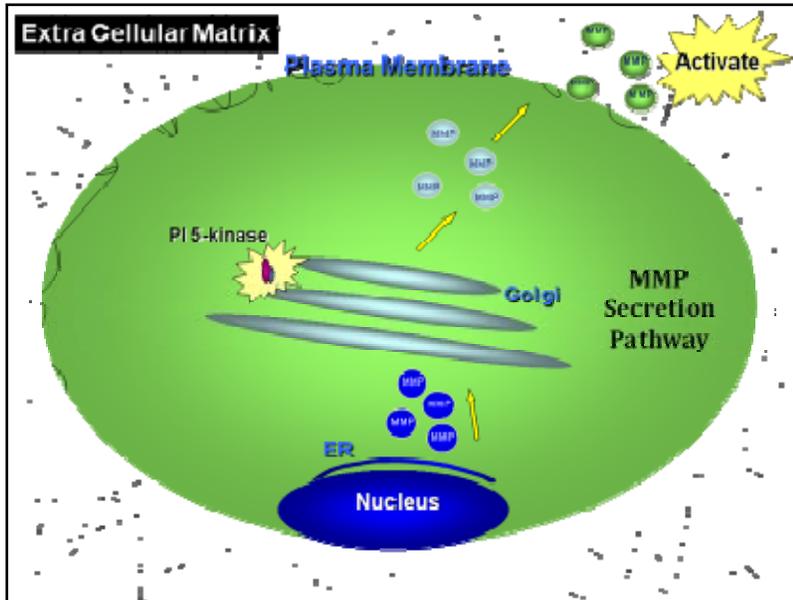


Figure 4. A possible model of the secretion pathway of MMPs to the extracellular matrix for activation.

enzymatically active form. They contain a zinc ion at their active site (24, 26). MMPs function at neutral pH and require calcium for stability (27). MMPs function as enzymes which dynamically regulate the extracellular matrix (ECM) by degrading its fibrous components (28). MMPs are important in tissue remodeling, angiogenesis, ovulation and wound healing, but they have also been implicated in

many disease processes such as rheumatoid arthritis, tumor cell metastasis, atherosclerosis, and cardiac disease (24, 25, 26, 28, 29). MMPs are secreted in varying concentrations, and the amount of secretion dictates how heavily the ECM is remodeled. Specific tissue inhibitors of metalloproteinases (TIMPs) have been found to inhibit the actions of MMPs, but also have been shown to function in the activation processes of specific MMPs (24, 27). Several clinical trials with first generation MMP inhibitors have shown limited effects on suppressing cancer metastasis (24, 30).

Hypotheses

- Metastasis suppressors reduce matrix metalloproteinase secretion in metastatic breast carcinoma and metastatic melanoma cells.
- Metastasis suppressors reduce the initial invasive potential in metastatic breast carcinoma cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

MDA-MB-435, MDA-MB-231, and C8161 human breast carcinoma and melanoma cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DME/F-12) with 5% fetal bovine serum (FBS). Cells were passaged at 80-90% confluency using 2.5% Trypsin in Hanks buffered saline solution (HBSS) without calcium or magnesium and with 1% EDTA (Hyclone, Logan, UT). No antibiotics were added to the medium. Cells were cultured in 25-cm² Corning tissue culture dishes at 37°C with 5% CO₂ in a humidified atmosphere.

MDA-MB-435 and MDA-MB-231 cells were transfected with full length BRMS1 cDNA under the control of the cytomegalovirus promoter. Cell lines were confirmed to be free of *Mycoplasma* contamination using PCR (TaKaRa, Japan). The C8161 cells underwent microcell-mediated transfer of a 40-megabase region of chromosome 6 (chromosomal bands 6q16.3-q23). Donor cells containing the neomycin-resistance gene-tagged chromosomal bands 6q16.3-q23 were used for preparation of the microcell. After transfection, the C8161 *KiSS-1* cells were selected in G418-containing media. Stable microcell hybrids were tested for the region utilizing fluorescence *in situ* hybridization (FISH), karyotyping, and PCR amplification. The presence of the gene was tested for by subcutaneous injection of the cells into the flanks of immunodeficient mice (11, 31).

Cell Starvation and Zymography Sample Collection

Confluent cells in 25-cm² Corning tissue culture dishes were washed with serum free DME/F-12 and incubated with serum free DME/F-12 for 6 hours. Serum free DME/F-12 was removed and saved to analyze secreted MMPs. New serum free medium was added and cells were scraped from bottom of dish utilizing a rubber cell scraper. The lysate was vortexed on high to further lyse the cells and determine retained MMPs within the cells. Microcon Centrifugal Filter Devices, YM-10 (Millipore Corporation, Bedford, MA) were used to concentrate collected samples. Sodium dodecyl sulfate (SDS) sample buffer was added to samples at a 1:1 concentration.

SDS-PAGE Zymography

SDS-PAGE zymography was used to visualize and detect specific MMPs. Zymogram gels were prepared with 10% separating gel [stock solution: 30% acrylamide mix, 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate, Gelatin Type A, TEMED] and 5% stacking gel [stock solution: 30% acrylamide mix, 1.0 M Tris (pH 6.8), 10% SDS, 10% ammonium persulfate, TEMED]. Samples were activated by incubating MMP samples in 10 mM aminophenyl mercuric acetate (APMA) at a 10:1 concentration for 2 hours at 37°C. Samples were loaded and run at 120 mV in 1x SDS Running Buffer. Gels were removed from glass plates, rinsed with water, then soaked in 2.5% Triton-100x for 45 minutes on a shaker. Gels were then transferred to a digesting solution [50 mM Tris-HCL (pH 8), 10 mM CaCl₂, 1μM ZnCl₂] and incubated at 37°C for 24 hours with light shaking. Following incubation, gels were stained with Coomassie Brilliant Blue overnight and destained with destaining solution [40% methanol, 10% acetic acid] until bands of lysis were clear.

Statistical Analysis

SDS-PAGE zymography gels were analyzed utilizing densitometry (Quantity One software). MMP secretion densities could be quantitatively measured by recording the transparency of each band with beams of light. The data were analyzed in Microsoft Excel using box plots and a pooled variance t-test. T-tests evaluated the statistical significance between the metastasis suppressor and control cells and were performed assuming equal variances.

Wound Healing Assay

MDA-MB-435 human breast carcinoma cells were cultured in DME/F-12 with 5% FBS. Cells were passaged at 80-90% confluency using 2 mM EDTA in 0.1 μM phosphate buffered saline solution (PBS) without calcium or magnesium. Cells were cultured in flat bottom 6-well tissue culture plates at 37°C with 5% CO₂ in a humidified atmosphere.

MDA-MB-435 and MDA-MB-231 cells were transfected with full length BRMS1 cDNA as stated above and cultured parallel to the MDA-MB-435 cells and MDA-MB-231 cells. The cells were cultured to 90-100% confluency in 6-well plates and a linear streak was created by scraping across the cell culture with a 0.1-10 μL sterile pipette tip. The wells were washed with DME/F-

12 with 5% FBS and new 5% FBS supplemented DME/F-12 was replaced on the cells. The wound was imaged with a microscope at five locations along the wound at 0 hours and 18 hours. In between imaging, the cells were incubated at 37°C with 5% CO₂ in a humidified atmosphere. Following imaging, the width of the wounds were measured and statistically analyzed with replicates.

RESULTS AND DISCUSSION

Data from in-gel zymography have shown that in MDA-MB-435, the expression of the metastasis suppressor gene BRMS1 reduces secretion of MMP-9 and MMP-2 (Fig. 5 and Fig. 6). However, in MDA-MB-231, the expression of the metastasis suppressor gene BRMS1 does not

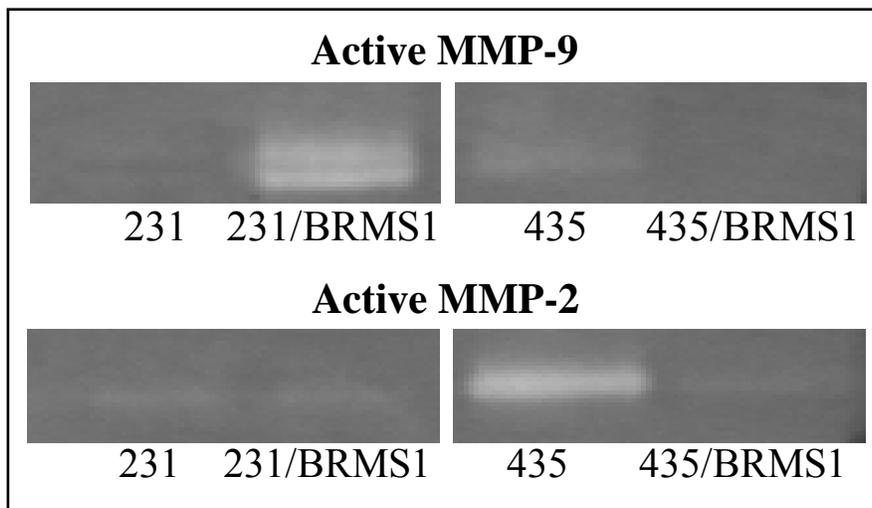


Figure 5. In-gel zymography showing MMP activities in human carcinoma cell lines MDA-MB-231, MDA-MB-231/BRMS1, MDA-MB-435, & MDA-MB-435/BRMS1.

show the same reduction in secretion of MMPs (Fig. 5 and Fig. 6).

Additionally, in C8161, the expression of metastasis suppressor gene *KiSS-1* reduces secretion of MMP-9 and MMP-2 (Fig. 7 and Fig.

8). These results suggest that BRMS1 may reduce the invasive potential of

MDA-MB-435 cells by reducing the expression of MMP-9 and MMP-2, but may function differently in MDA-MB-231 cells. *KiSS-1* may reduce the invasive potential of C8161 by reducing the expression of MMP-9 and MMP-2. In some metastatic tumor cells, MMP-9 and MMP-2 have been found to be secreted at abnormally high levels. In MDA-MB-435 and C8161 cells, reducing MMP secretion may contribute to metastasis suppression.

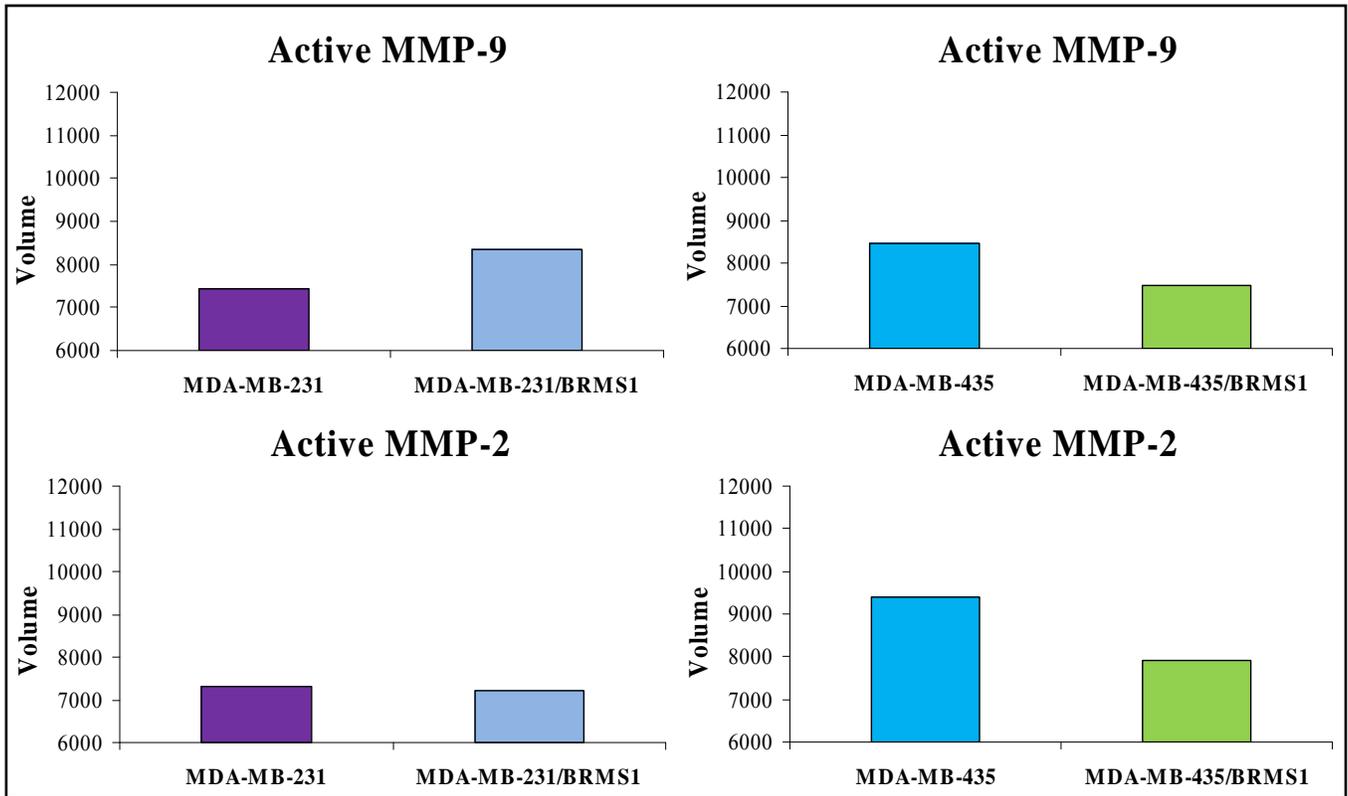


Figure 6. Graphs showing the densitometry analysis of activated MMP-9 and MMP-2 from the in-gel zymography of human carcinoma cell lines MDA-MB-231, MDA-MB-231/BRMS1 MDA-MB-435, & MDA-MB-435/BRMS1. Volume refers to the volume integration method that was used to compare the relative intensity of different bands on the scanned gel. Each pixel of the scanned gel was assigned a numerical value corresponding to the optical density of the gel at that point. The numerical value of each pixel within a rectangle was added together to determine the volume (the amount of sample contained within the rectangle).

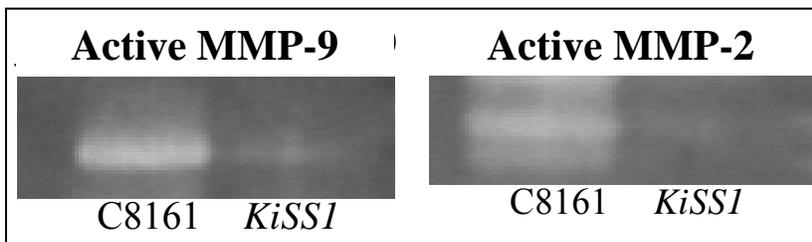


Figure 7. In-gel zymography showing MMP activities in human melanoma cell lines C8161 & C8161-KISS1.

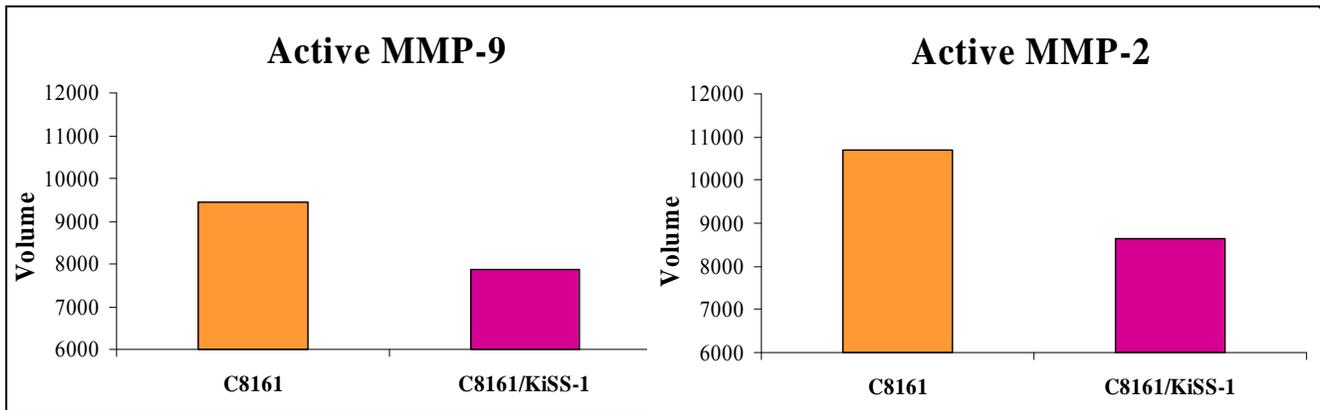


Figure 8. Graphs showing the densitometry analysis of activated MMP-9 and MMP-2 from the in-gel zymography of human melanoma cell lines C8161 & C8161-KISS1. Volume refers to the volume integration method that was used to compare the relative intensity of different bands on the scanned gel. Each pixel of the scanned gel was assigned a numerical value corresponding to the optical density of the gel at that point. The numerical value of each pixel within a rectangle was added together to determine the volume (the amount of sample contained within the rectangle).

To determine potential differences in motility between the MDA-MB-435 and the MDA-MB-435/BRMS1 cells, a wound-healing assay was performed. The wound was created with a pipette

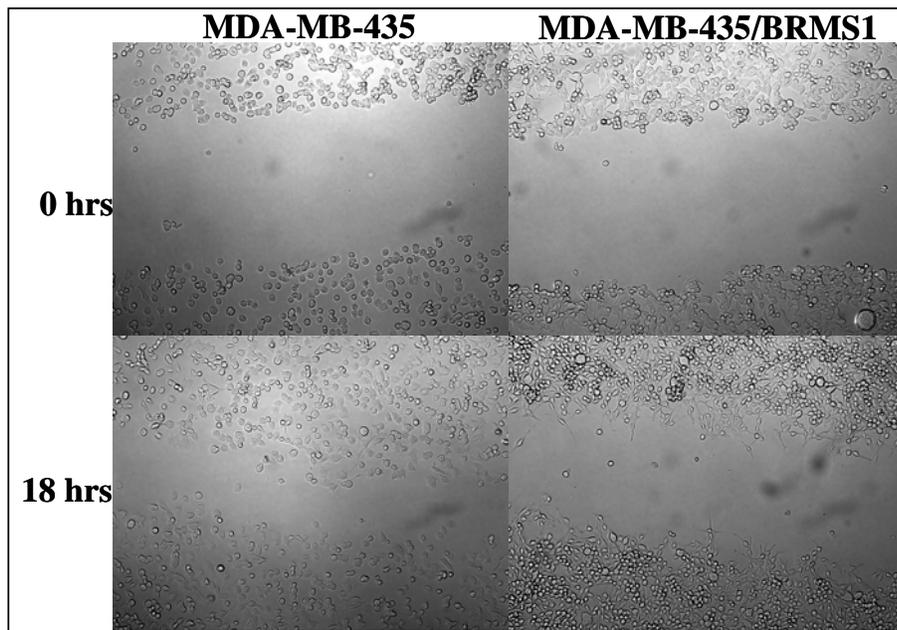


Figure 9. Wound-healing assay of MDA-MB-435 and MDA-MB-435/BRMS1 at 0 hr (top row) and at 18 hrs after wounding with pipet tip (bottom row).

tip and was measured at 0 hours and 18 hours. The relative proliferation was determined by subtracting the final average wound width from the initial average wound width. This relative proliferation was averaged with several replicates and was standardized as a percent of the maximum

proliferation by the cells. The MDA-MB-435 cells migrated more rapidly than the MDA-MB-435/BRMS1 cells (Fig. 9 and Fig. 10). After 18 hours of incubation, the wound created by scraping with a pipette tip was almost completely healed in the MDA-MB-435 cells. In contrast, the migration activity for the MDA-MB-435/BRMS1 cells was considerably slower.

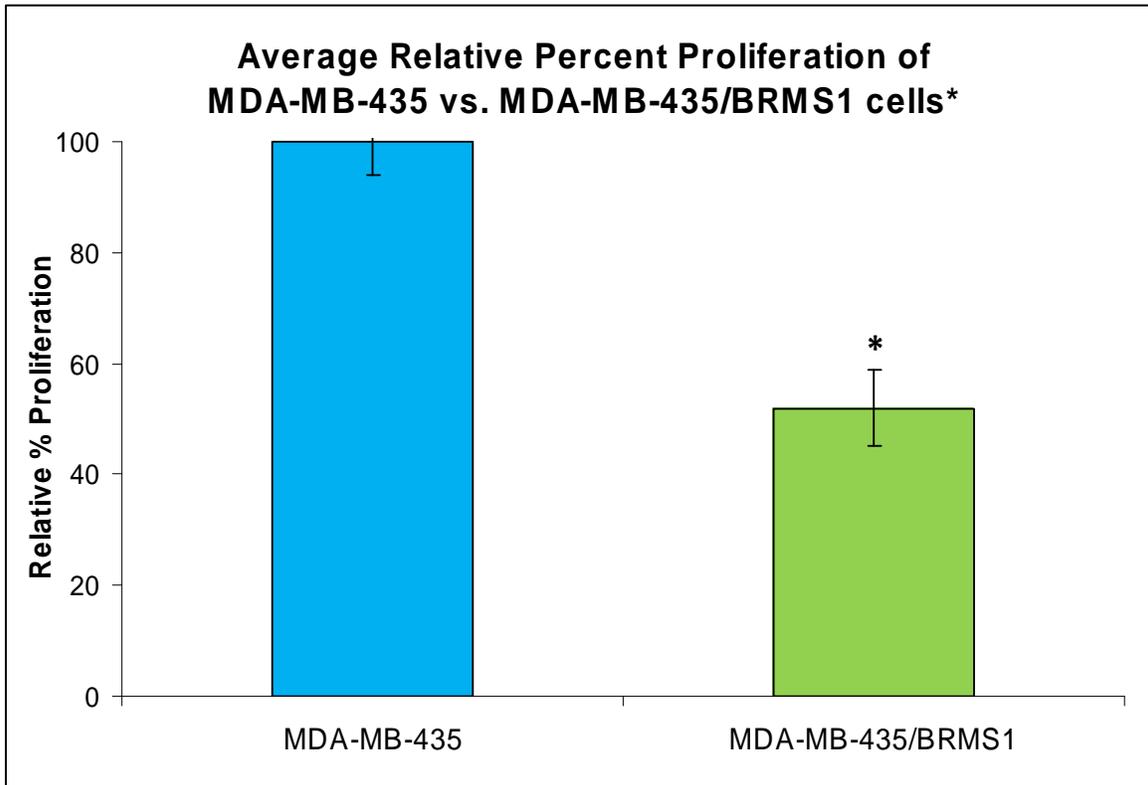


Figure 10. Average relative proliferation (mobility as a percent of the maximum) was measured between the MDA-MB-435 and MDA-MB-435/BRMS1 from the wound healing assay. *Two-sample t-test assuming unequal variance showed a statistically significant difference ($P = 5.811E-5$).

Th

e wound healing assay was repeated with the MDA-MB-231 cells and the MDA-MB-231/BRMS1 cells. The MDA-MB-231 cells showed a similar migration pattern relative to the MDA-MB-231/BRMS1 cells (Fig 11 and Fig. 12). After 18 hours of incubation, the wound created by scraping with a pipette tip was almost completely healed in the MDA-MB-231 cells as well as in the MDA-MB-231/BRMS1. This suggests that the MDA-MB-231/BRMS1 may inhibit invasiveness of the MDA-MB-231 cells by a different means than the MDA-MB-435 cells.

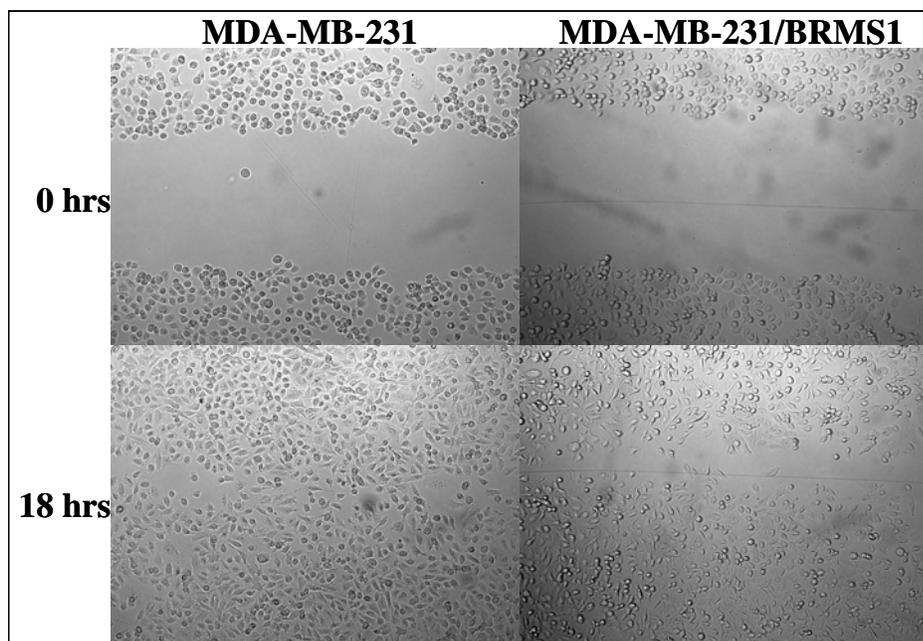


Figure 11. Wound-healing assay of MDA-MB-231 and MDA-MB-231/BRMS1 at 0 hr (top row) and at 18 hrs after wounding with pipet tip (bottom row).

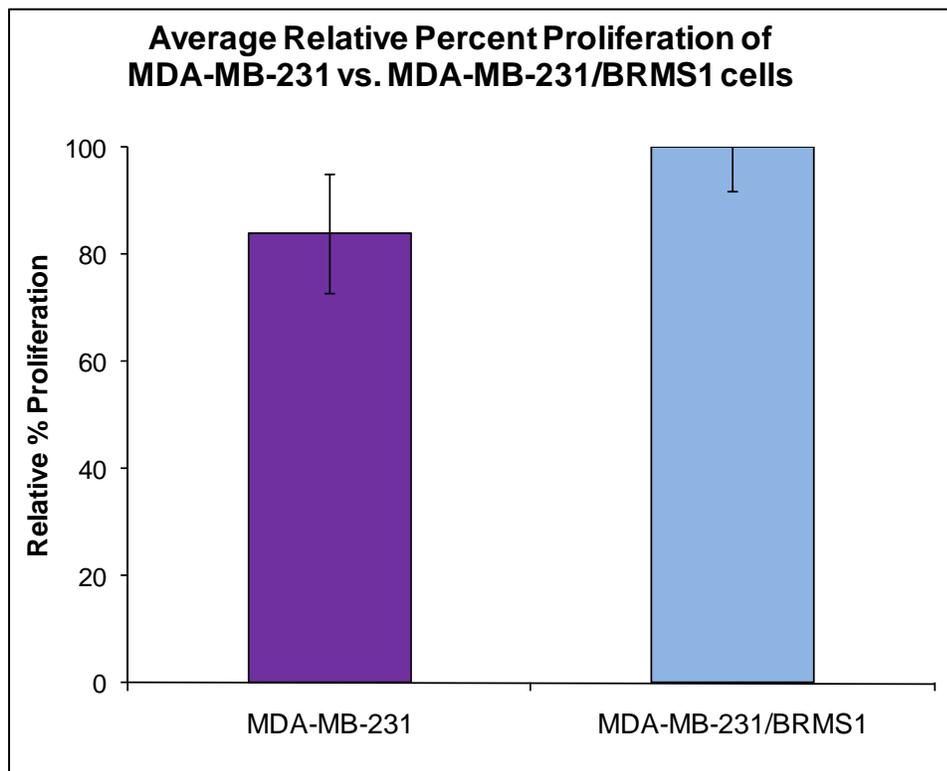


Figure 12. Average relative proliferation (mobility as a percent of the maximum) was measured between the MDA-MB-231 and MDA-MB-231/BRMS1 from the wound healing assay. Two sample t-test assuming unequal variance did not show a statistical difference ($P = 3.975E-2$).

FUTURE WORK

This work has shown that the expression of the metastasis suppressor gene BRMS1 in the MDA-MB-435 cells reduces secretion of MMP-9 and MMP-2. However, the expression of the metastasis suppressor gene BRMS1 in the MDA-MB-231 cells does not reduce secretion of these MMPs. The expression of metastasis suppressor gene *KiSS-1* in the C8161 cells reduces secretion of MMP-9 and MMP-2. Also, the MDA-MB-435 cells migrate faster than MDA-MB-435/BRMS1 cells, although the MDA-MB-231 cells migrate at a comparable rate as the MDA-MB-231/BRMS1 cells. These results imply that the reduction in specific MMP secretion in the MDA-MB-435 cells may reduce the invasive potential of the metastatic cancer cells. Additional work should be done to determine the mechanisms that regulate the differential MMP secretion to further the understanding of these metastasis suppressors. This understanding may allow the development of new therapeutics for the treatment and prevention of metastatic cancers.

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APPENDICES

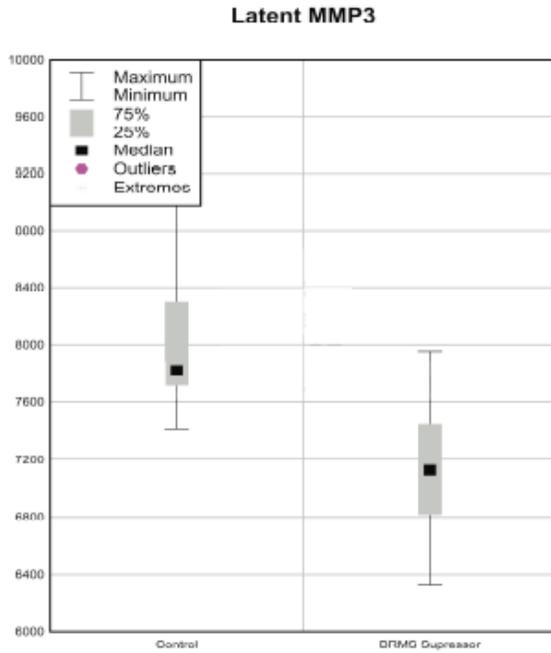


Figure 1. Differences in latent MMP3 secretion from control (MDA-MB-435) and BRMS suppressor (MDA-MB-435/BRMS1) cells.

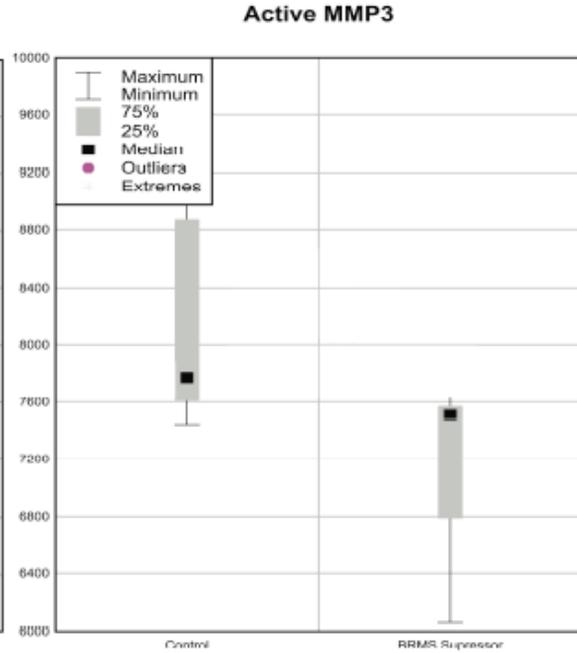


Figure 2. Differences in active MMP3 secretion from control (MDA-MB-435) and BRMS suppressor (MDA-MB-435/BRMS1) cells.

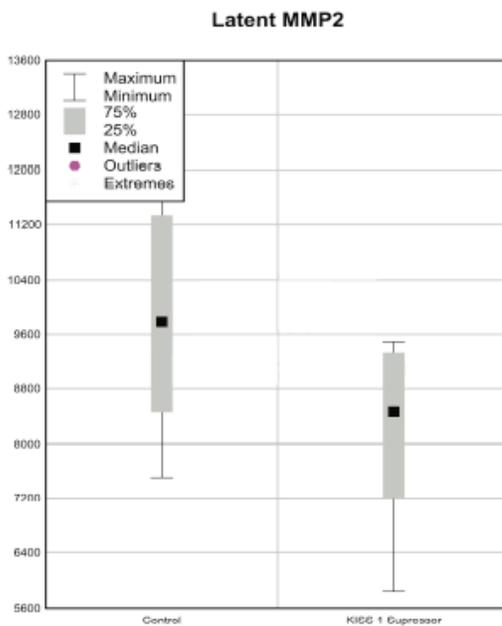


Figure 3. Differences in latent MMP2 secretion from control (C8161) and KISS1 suppressor (C8161 *KiSS-1*) cells.

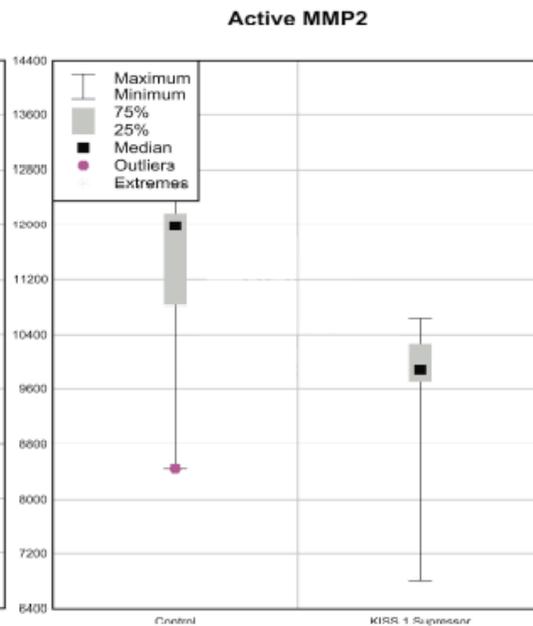


Figure 4. Differences in active MMP2 secretion from control (C8161) and KISS1 suppressor (C8161 *KiSS-1*) cells.

AUTHOR'S BIOGRAPHY

Sherry Baker, raised in Smithfield, Utah, graduated in 2005 from Sky View High School. A Dean's Scholarship recipient, she entered Utah State University in the fall as a Public Health Education major with interests in pursuing medical school. In the Spring of her Freshman year, she heard about Dr. Daryll DeWald's research in her Honors Inquiry Seminar class, and her interest was piqued. While an Aggie, Sherry kept herself busy as an undergraduate researcher completing an American Heart Association Western States Affiliate Undergraduate Research Fellowship, a Willard L. Eccles Undergraduate Research Fellowship, a Research Fellowship from the Center for Integrated BioSystems at USU, a Multicultural Research Fellowship, and an Undergraduate Research and Creative Opportunities Grant.

She served as an Ambassador for the College of Science, as President and Vice President of Alpha Epsilon Delta Prehealth Professional Honor Society, as an officer in Women in Medicine and the Health Education Association of USU, and as a Peer Advisor for the USU Biology Department. She also volunteered her time with Community Nursing Services Hospice and the Logan Regional Hospital Emergency Room. In the summer of 2008, Sherry traveled on a study abroad through the John M. Huntsman School of Business to China, South Korea, and Vietnam learning how business and biotechnology work together and earning a business minor along the way.

After she graduates in May 2010, Sherry plans to pursue medical school. Following medical school, she is interested in pursuing clinical research in preventative medicine with an international and infectious disease focus. She hopes to further her research experience through a Doctorate or Masters in Public Health following medical school. She is interested in eventually working for an epidemiologic or clinical based research group within an international health organization such as the CDC or WHO.