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DEPARTMENTS OF BIOLOGY and BIOCHEMISTRY AND MOLECULAR BIOLOGY

DEADLY KISS: KISSPEPTIN 10 INTERACTION WITH OSTEOBLASTS AND BREAST  
CANCER METASTATIC CELLS

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## ABSTRACT

Metastasis is a multistep process with significant impact on survival. Current cancer research focuses on blockading metastases with the hope of increasing patient survival and prognosis. This research has spurred an interest in metastasis suppressor genes, such as Kisspeptin (KISS-1). KISS-1 is important in the reproductive axis and many cancer types, but its role in breast cancer is controversial. Because breast cancer frequently metastasizes to the skeleton, it is important to understand the interaction of bone with cancer metastases.

This study focuses on the interactions among one of the proteolytic products of the Kiss-1 protein (kisspeptin-10, KP10), its receptor KISS1R (GPR54, AXOR12), murine MC3T3-E1 osteoblasts (bone forming cells), and bone-metastatic breast cancer cells, MDA-MB-231. The aim of this study was to determine if KP10 affected the interaction of osteoblasts and bone-metastatic breast cancer cells. The Mastro laboratory had previously determined that osteoblasts exhibit an inflammatory response when treated with conditioned medium from breast cancer cells. This inflammatory response may contribute to cancer cell proliferation in the bone. This response has not yet been characterized in the presence of KP10. However, previous research suggests that treatment with KP10 reduces the ability of the cancer cells to invade a reconstituted basement membrane (Matrigel) and to migrate in response to osteosarcoma cells. I hypothesized that KP10 would down regulate the inflammatory response of osteoblasts to breast cancer cells. MDA-MB-231 breast cancer cells express KISS1R; therefore, these cells were treated with KP10 (1-500 nM, 4hr), the medium was collected, and the osteoblasts were treated with it. KP10 significantly decreased the inflammatory response of osteoblasts to breast cancer conditioned media indicated by decreased levels of IL-6 as determined by ELISA. In addition, KP10 did not affect the differentiation of osteoblasts over a variety of doses or in presence of breast cancer

conditioned media. This research has important implications because it provides insight into how KP10 may be acting on either the breast cancer cells or the bone cells. This project is important because it is focused on a controversial pathway that holds promise for metastatic breast cancer research and ultimately treatment.

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**LIST OF ABBREVIATIONS**

KISS – Kisspeptin

KP10 – Kisspeptin 10

IL-6- Interleukin 6

MCP-1- Monocyte Chemoattractant Protein 1

CM- Breast Cancer Conditioned Media

VM- Vehicle Media

KISS CM- Kisspeptin 10 Breast Cancer Conditioned Media

OB-osteoblast

OC-osteoclast

IL-6 – Interleukin 6

TNF- $\alpha$  – Tumor Necrosis Factor Alpha

IL-1 $\beta$  – Interleukin 1 Beta

PGE2 - Prostaglandin E2

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## **Chapter 1**

### **Introduction**

#### **Breast Cancer Overview**

Breast cancer is the most frequently diagnosed cancer in women, second only to skin cancer, and the second leading cancer-associated cause of death. [1] The American Cancer Society anticipated that in the year 2013, there would be 232,340 new cases of invasive breast cancer and 39,620 deaths from breast cancer. [1] Breast cancer is defined as a malignant mass or tumor of invasive cells that originate in the breast. These cells can alter their function to continue to grow in the breast tissue or travel to other areas of the body and form metastases. Metastasis or the movement of cancer cells to other areas of the body has been defined as one of the most harmful aspects of cancer. Breast cancer and other highly metastatic cancers often metastasize to the bone leading to serious complications. [2]

Overall the existence and severity of metastasis is an important tool in the diagnosis and classification of breast cancer. This is because the stage of cancer at diagnosis and during treatment has a high correlation to survival rate (Table 1.1). [3] During a study of the patients diagnosed with breast cancer between 2001 and 2007, patients with localized primary tumors had a 5-year survival rate of 98.6%, and those who had regional spread to surrounding lymph nodes had a 5-year survival rate of 83.8%. [3]

Stage	Description
0	Classified as ductal carcinoma in situ (DCIS), which is a precancer in the breast. Cancer cells have not spread outside the breast to lymph nodes or distant sites.
1	The tumor is 2cm or smaller if present. The tumor has barely if at all disseminated from the breast. Small areas of breast cancer cells could be found in the lymph nodes near the breast.
2	No tumor is found, the tumor is 2cm or smaller but not bigger than 5cm. Cancer cells could be found in 1 to 3 lymph nodes but not in distant sites of the body.
3	If a tumor is present it is larger than 5 cm and groups of breast cancer cells can be found in several auxiliary lymph nodes
4	Tumor can be any size. The lymph nodes may or may not contain cancer cells. The cancer has metastasized to other parts of the body like the bones, lungs, liver, or brain.

**Table 1** Summary of the Stages of Breast Cancer

This table includes an overview of the physiological characteristics of the five stages of cancer (Stage 0-4). Adapted from Cancer Research UK [4]

## Normal Physiology and Function of Bone

### Bone Structure and Microenvironment

Bone is a dense connective tissue with critical functions such as structural support, a layer of protection for vital organs, location for blood production, and storage for essential minerals [5]. It is comprised of a complex system of fibrous tissue called matrix, and important minerals such as calcium, which are responsible for its strength and rigidity [5]. The types of bone are classified into two categories known as cancellous or trabecular and cortical or compact [6]. The majority of long bones, which are also likely sites for metastasis, have a rigid outer layer of cortical bone and an inner spongy section of cancellous bone [6]. Overall, the bone consists of

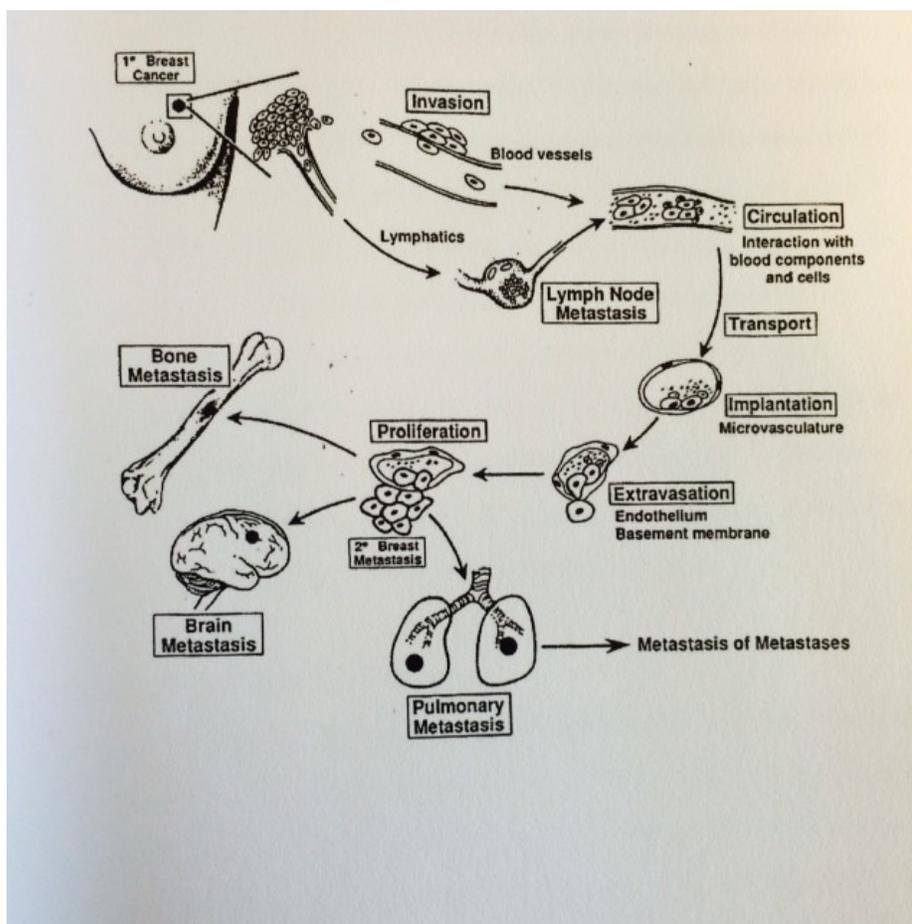
two types of cells, osteoblasts that create the bone and osteoclasts, which reabsorb the bone. Because bone is composed of several types of cells it is heterogeneous in nature. Cellular activities and interactions taking place in this bone microenvironment facilitate the process of bone remodeling. [5]

### **Bone Metastasis**

Bone is a dynamic tissue, which is always undergoing remodeling by reabsorption of old bone and the creation of new bone. Bone turnover occurs due to bone-reabsorbing osteoclasts and bone-depositing osteoblasts with no overall change. This strictly regulated balance between osteoblast and osteoclasts is interrupted in bone diseases such as osteoporosis, rheumatoid arthritis, and osteolytic metastasis. [7] Understanding the mechanisms that predispose tumor metastasis to bone will improve the therapeutic options for patients, maintain quality of life, and perhaps improve survival.

Cancer cells are able to migrate from the original tumor and disperse throughout the body using lymph or blood vessels. Usually the cancer cells that break away from the primary tumor site will senesce without causing much damage to the patient; however if the cells are able to find a hospitable environment, they can settle in this new area and proliferate. [2] Breast cancer preferentially metastasizes to the skeleton, a supportive environment that attracts and allows breast cancer cells to thrive. [8] As a consequence of interactions between cancer cells and the native bone cells, cancer cells produce factors that activate osteoclasts and lead to osteoclastogenesis [2]. As a result osteoclasts become hyperactive and resorb and weaken the bones and in some cases cause holes in the bones known as osteolytic lesions [2]. The most

common detrimental signs and symptoms of osteolytic bone metastasis include bone pain, fracture, spinal cord compression, and hypercalcemia. [9] The bone microenvironment is able to widely support the metastasis of various tumor types due to the expression of specific chemokines that enhance tumor cell growth and invasion. In addition to resulting in a dire prognosis, bone metastases cause significant morbidity including bone pain, fractures, hypercalcemia, spinal cord compression, and other nerve compression syndromes.[2]

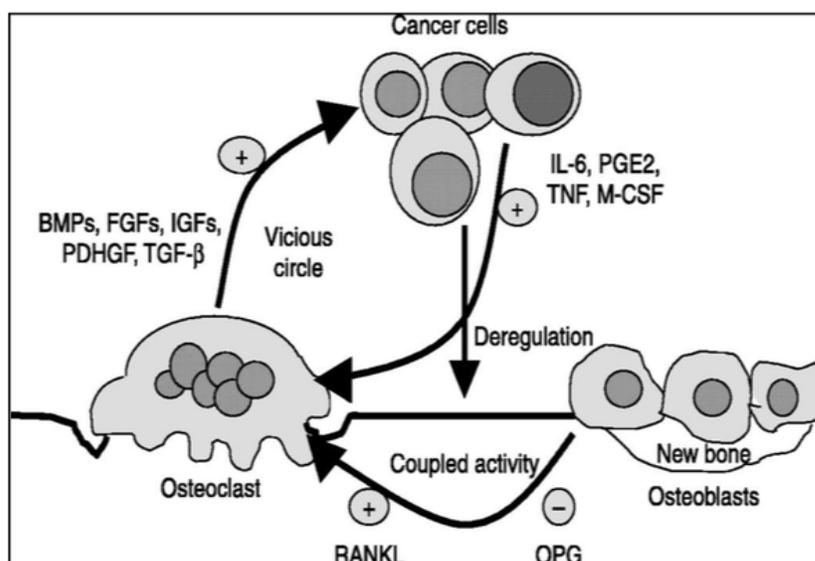


**Figure 1** Process of Breast Cancer Metastasis

Price, J.E. The Breast Comprehensive Management of Benign and Malignant Disorders [10]

## Osteoblasts

Growth factors released, during bone remodeling can result in tumor cell growth, and perpetuate osteolytic activity and bone degradation. This is characterized as a vicious circle between the osteoblasts, osteoclasts, and invading cancer cells. (Figure 2) While the osteoclasts have been characterized as the direct effectors of bone degradation, key studies have also found that osteoblasts can add to bone loss. As this study is directed specifically to the osteoblasts role in the bone microenvironment it is critical to understand how osteoblasts are networking with tumor cells and osteoclasts. It has also been found osteoblasts contribute through loss of function induced by metastatic breast cancer cells. Metastatic breast cancer cells suppress osteoblast differentiation, alter morphology, and increase apoptosis.[7] Additionally, studies have shown that osteoblasts are directed by breast cancer cells to release inflammatory cytokines involved in breast cancer cell migration, survival, and osteoclast activation.[11][12] In previous studies it was demonstrated that osteoblasts undergo an inflammatory stress response in the presence of human metastatic breast cancer cells. When cancer cells or their conditioned medium was added to human osteoblasts, the osteoblasts were induced to express increased levels of IL-6, IL-8, and MCP-1; cytokines known to attract, differentiate, and activate osteoclasts. This release of cytokines and chemokines can have significant effects in the bone.



**Figure 2** The Vicious Cycle of Bone Metastasis

Communication between bone cells and invading cancer cells leads to a cycle of increased bone resorption and cancer cell growth.[8]

### **Cytokines Contribute to Breast Cancer Metastasis to Bone**

There are many important regulatory molecules, which coordinate bone-remodeling activity. Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha (TNF- $\alpha$ ), Interleukin-1 Beta (IL-1 $\beta$ ), and Prostaglandin E2 (PGE2) are especially of interest as they stimulate bone remodeling, but more interestingly they may also activate cancer cells.[13], [14] The extent to which these cytokines work together and impact metastatic breast cancer cells remains to be fully understood, but has been an increasingly popular topic in cancer metastasis research. Additionally, cancer researchers are interested in how chemokines and cytokines secreted by breast cancer cells impact metastasis. [12]

The IL-6 family of cytokines has a variety of functions. These cytokines have been shown to cause differentiation of osteoblasts [15]. During differentiation there is an up regulation

of specific proteins such as alkaline phosphatase and osteocalcin. Increased expression of these factors results in bone nodule formation and mineralization of the extracellular matrix osteoblasts create. Literature studies have shown IL-6 interacts with osteoblasts predominantly through the STAT3 pathway, however this does not rule out the existence of other pathways.[15] Other possible effects of IL-6 on maturing osteoblasts are reduced proliferation and inhibition of apoptosis. These effects have been observed in the presence of IL-6 when the osteoblasts are less mature. Cancer cells release specific cytokines when they infiltrate the bone. One of these cytokines is IL-6, which in conjunction with others such as parathyroid hormone increases bone resorption by initiating osteoclastogenesis and decreasing osteoblastogenesis. [16] Thereby the vicious cycle of bone degradation and cancer cell growth commences.[17], [18] Thus depending on the conditions in the bone microenvironment, IL-6 induces differentiation of osteoblasts depending on the conditions, and alters their activity, and consequently the structure of the bone microenvironment. [17]

The role of IL-6 in breast cancer cells is much more complicated and paradoxical. For both the human metastatic MDA-MB 231 cell line and the human non-metastatic MCF7 cell line, IL-6 has been proven to decrease apoptosis and increase proliferation; while in other cases it promotes apoptosis and inhibits proliferation.[19] IL-6 has been shown to suppress proliferation of breast cancer cell lines by blocking the G1 phase of the cell cycle. The STAT pathway activation by IL-6 is predicted to promote p21, a cell cycle inhibitor. It is also thought to have a pro-apoptotic effect by mobilizing numerous death inducers and p53. [20] However, IL-6 has also been classified as pro-tumorigenic and operates via the STAT3 pathway. STAT3 is the major effector of IL-6. It was noted that when this pathway was blocked, cell growth was slowed in culture and in a xenograft [19], [21] This model is important because cancer cells can be

studied in the *in vivo* environment. Thus, IL-6 has been observed to affect cancer metastatic cells both *in vitro* and *in-vivo*. Furthermore, it has been noted that elevated levels of IL-6 are correlated with a poor prognosis for breast cancer patients. [22] Nonetheless, the role of IL-6 remains ambiguous because it may act differently depending on unique experimental conditions such as various concentrations and the specific cell lines studied. Overall, IL-6's dual role in the bone-remodeling pathway and cancer cell growth make it an excellent cytokine to study in the bone remodeling-metastatic niche.

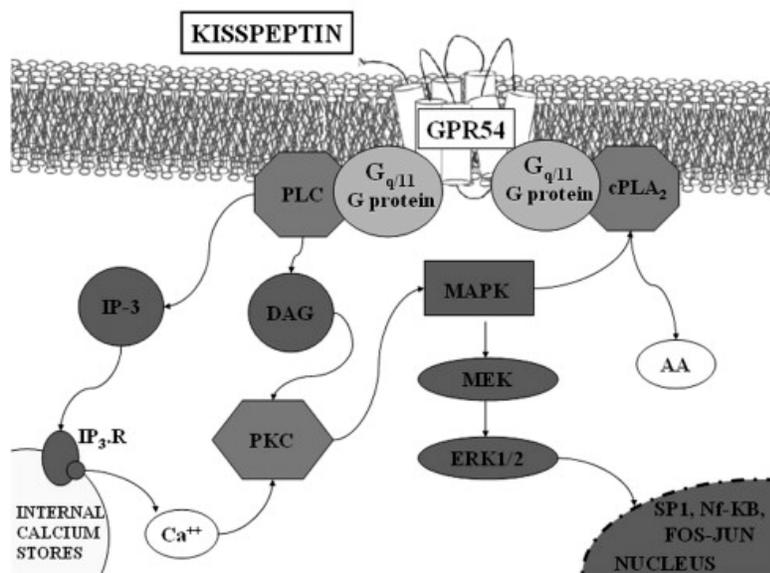
In addition to IL-6, monocyte chemoattractant protein-1 (MCP-1) is also a key molecule in the bone and is implicated in the inflammatory response. Various cells including monocytes, fibroblasts, endothelial cells, and tumor cells secrete MCP-1. [23] This cytokine has been reported to control the movement of macrophages into tumors. [24] Furthermore, transfection of this gene into cancer cells increased the metastatic activity of cancer cells. However, other studies report MCP-1 can stimulate monocytes to recognize and eliminate tumor cells. [23] In summary, MCP-1 has a key role in regulating both the activity of breast cancer cells and the immune system.

### **KISS1**

Cancer research is focused on the blockade of the metastatic process at its primitive stages. Hence, there continues to be an interest in finding metastasis suppressor genes, which may be involved in regulating this deleterious process. The KISS1 gene and its peptides are involved in multiple physiological mechanisms such as neuroendocrine regulation of reproduction, pregnancy, and tumor metastasis. The KISS1 gene was discovered as a metastasis

suppressor gene. [25] The antimetastatic effect was first identified in mice after injection of melanoma and breast cancer cell lines, which were transfected with KISS1. [25] The KISS1 gene encodes for a peptide of 145 amino acids, which is proteolytically cleaved into a number of peptides (KP54, KP14, KP13, KP10).[26], [27] These peptide fragments known as kisspeptins (KP) are classified by their common amidated C-terminal. The kisspeptins act as endogenous ligands to a G protein-coupled receptor known as KISS1 receptor or hOT7T175 or AXOR12 or GPR54.[28] Kisspeptin-10 (KP10) shows the highest potency upon the kisspeptins in receptor activation.

GPR54 is a G protein coupled receptor responsible for phospholipase C activation and phosphatidylinositol 4, 5-biphosphate hydrolysis. Ca<sup>2+</sup> mobilization, arachidonic acid release, extracellular signal regulated kinases 1 and 2 (ERK1/2) and p38 mitogen activated protein (MAP) kinase phosphorylation and stress fiber formation. [28] The receptor is involved in cell cycle arrest and apoptosis.



**Figure 3** Schematic representation of the intracellular signaling of GPR54

The binding of kisspeptin to the receptor results in multiple events via intracellular signaling[28]

## **KISS1 and Breast Cancer**

The role of KISS1 and GPR54 expression in cancer tissue is not yet elucidated. In normal tissue, KISS1 is expressed in the placenta, testis, pancreas and liver, while the receptor is predominantly expressed in brain, pancreas, and placenta.[28] KISS1 was originally hypothesized to suppress breast metastasis based on the map location of KISS1 (chromosome 1 bands q32-q41) and on the observation that chromosome 1q alterations are not common in most tumors except breast carcinomas.[25] The results of this initial hypothesis lead to a study that confirmed the metastatic potential of cells transfected with KISS1 cDNA was significantly reduced. [25] However as other studies have proceeded to analyze KISS1 and its receptor GPR54 some conflicting evidence has been reported. In one clinical study it was found that the expression of KISS1 was significantly higher in tumor compared to normal tissues in addition lymph node positive tumors compared to lymph node negative ones. [29] In other cancer types, KISS1 and GPR54 expression are associated with good overall survival. [30] Therefore, it is evident further research into the antimetastatic potential of KISS1 and its peptide products needs to be performed.

### **Statement of Objectives and Goals**

The main objective of this study was to determine if KP10 affected MC3T3-E1 osteoblasts and MDA-MB-231 breast cancer cells. Based on many previous studies it was hypothesized KP10 would interact with both of these cells. The first objective of this study was to determine if KP10 affected osteoblasts maturation. This objective was assessed by determining if various amounts of KP10 affected the alkaline phosphatase expression of

MC3T3-E1 osteoblasts. The next step to this experiment was to determine if introducing breast cancer conditioned media with KP10 affected osteoblast maturation. The subsequent objective of this study was to determine if KP10 downregulated the inflammatory response of MC3T3-E1 osteoblasts in the presence of breast cancer conditioned media. This objective was assessed by quantifying the presence of IL-6 and MCP-1. Finally, the last objective was to determine if osteoblast express KISS1R and KISS1. Overall, the goal of this experiment was to further understand if KP10 could have antimetastatic potential in the bone microenvironment.

## **Chapter 2**

### **Materials and Methods**

#### **Cell Lines**

##### **Breast Cancer Cell Lines**

The human metastatic breast cancer cell line, MDA-MB-231 originated from a pleural effusion from a 51 year-old white patient. MDA-MB-231 cells were cultured in growth medium consisting of 5% neonatal fetal bovine serum (FBS), penicillin (100 U/ml solution), streptomycin (100 µg/ml solution; Mediatech Inc., Manassas, VA) and 1X non-essential amino acid solution (NEAA; Mediatech Inc., Manassas, VA) in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech Inc., Manassas, VA). The culture was sustained in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

##### **Murine Osteoblastic Cell Line**

The murine pre-OB cell line, MC3T3-E1, was a gift from Dr. Norman Karin of Pacific Northwest National Laboratories. The cells were maintained in growth medium of 10% neonatal FBS, penicillin (100 U/ml), streptomycin (100 µg/ml; Mediatech Inc., Manassas, VA) in  $\alpha$ -MEM (Mediatech Inc., Herdon, VA). To yield mature matrix-producing OBs, the cells were cultured at an initial density of 10<sup>5</sup> cells/cm<sup>2</sup> for 4 weeks in differentiation medium consisting of 10%

neonatal FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma–Aldrich) in α-MEM. Differentiation media was changed every 3<sup>rd</sup> day.

### **Breast Cancer Conditioned Media**

Human metastatic breast cancer cell line, MDA-MB-231, were grown to 90% confluency. Breast cancer growth medium was removed and the cultures rinsed once with PBS. α-MEM (for later use with MC3T3-E1 cells) was added to the cancer cells (30 ml in a T-175 flask, ~ 1.3 x 10<sup>5</sup> cells/cm<sup>2</sup>). Cultures were incubated for 24 h. Breast cancer cell conditioned medium (CM) was collected, centrifuged 5,000xg, 10 min) to remove cellular debris, and stored at -20 C.

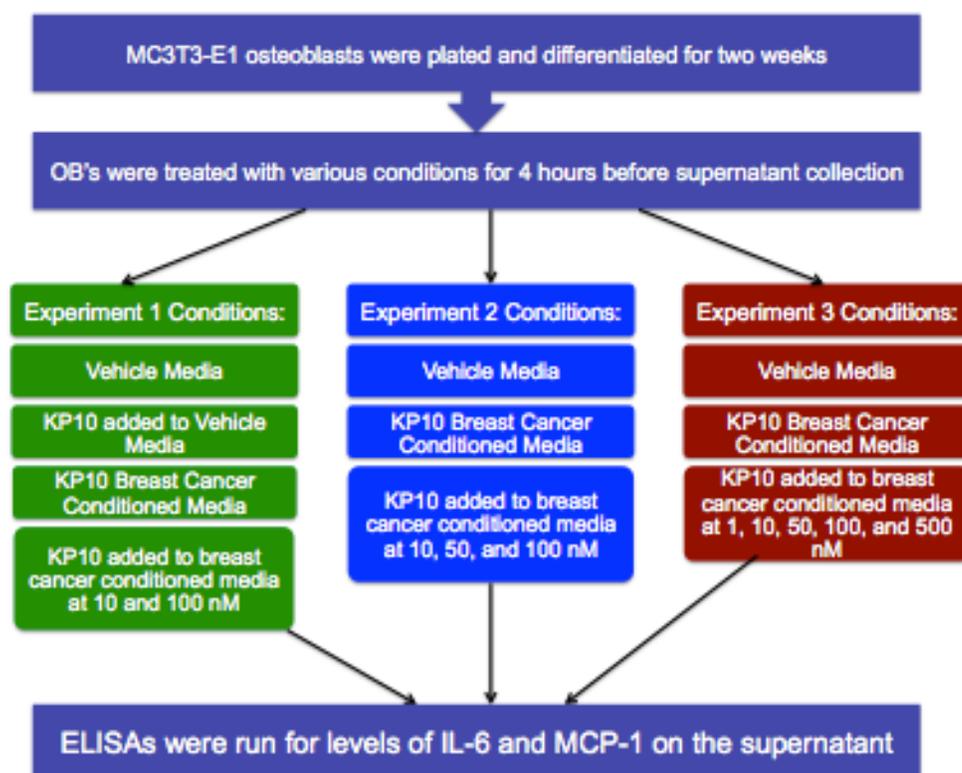
### **Kisspeptin Breast Cancer Conditioned Media**

Human metastatic breast cancer cell line, MDA-MB-231, were grown to 90% confluency. Breast cancer growth medium was removed and the cultures rinsed once with PBS. α-MEM (for later use with MC3T3-E1 cells) was added to the cancer cells (30 ml in a T-175 flask, ~ 1.3 x 10<sup>5</sup> cells/cm<sup>2</sup>). Additionally, 100 nM of Kisspeptin 10 (Tocris Bioscience) was added to the medium. Cultures were incubated for 24 h. The kisspeptin breast cancer cell conditioned medium (Kiss CM) was collected, centrifuged 5,000xg, 10 min) to remove cellular debris, and stored at -20 C.

## Conditioned Media Treatments on Osteoblasts

Vehicle media (VM) consisted of osteoblast differentiation media. A 2x differentiation medium was used for MC3T3-E1 osteoblasts and consisted of  $\alpha$ -MEM, 20% neonatal FBS, 100  $\mu$ g/ml ascorbic acid, 20 mM  $\beta$ -glycerophosphate, 200 IU/ml penicillin, and 200  $\mu$ g/ml streptomycin.

Conditioned media (CM) was made up of one half volume BCCM and one half volume 2x osteoblast differentiation medium. Kiss conditioned media (Kiss CM) was made up of one half volume Kiss BCCM and one half volume 2x differentiation medium. Finally, CM + KP10 was made up of one half BCCM and one half volume of 2x differentiation medium. Additionally, varying doses (1, 10, 50, 100, 500 nM) of KP10 were added to the medium. This design was used to ensure the concentrations of serum and differentiation factors were identical for VM and CM. Osteoblasts were differentiated with 1x differentiation medium consisting of  $\alpha$ -MEM, 10% neonatal FBS, 50  $\mu$ g/ml ascorbic acid, 10 mM  $\beta$ -glycerophosphate, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin for 2 weeks before all of the conditions were changed to different experimental conditions. Refer to Figure 4 below for summary of experimental design.



**Figure 4** Experimental Design of Testing the Effect of KP10 and Breast Cancer Conditioned Media on Osteoblasts

Three different experiments were conducted to test the effect of conditioned media from MDA-MB-231 metastatic breast cancer cells treated with Kisspeptin 10 on MC3T3-E1 osteoblasts. Levels of IL-6 and MCP-1 were quantified by ELISA to quantify their inflammatory response. Each experiment followed similar procedures but the medias tested were different. Each box in the figure describes a different type of medium. Vehicle media was the control for each experiment. It should be noted experiment 2 and 3 include a dose expansion to determine if KP10 had a dose response. Refer to Experiment 1 (Figure 7), Experiment 2 (Figure 8), and Experiment 3 (Figure 9)

### **Alkaline Phosphatase Staining**

MC3T3-E1 osteoblasts were plated at  $2 \times 10^4$  cells/ml, 2mls/well into three- 24 well plate multi-cell culture dishes in MC3T3-E1 growth medium and were allowed to incubate overnight. The following day, medium was changed to 1ml 1x differentiation medium and specific concentrations of KP-10  $1 \times (10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, \text{ and } 10^{-13})$  M or no KP-10M and no KP-10. On days 7, 9 and 11 after the addition of KP, cultures were fixed with 10% formaldehyde at room temperature and rinsed 3 times with PBS. Alkaline phosphatase stain was mixed as follows: 0.0013g Naphthol AS-BI Phosphate, 0.2M Tris, pH 8.5, and 0.00075 g Fast Blue RR Salt. The stain was filtered with Whatman paper and incubated with the cells at 37 C for 30 minutes. Cells were rinsed several times with water and left to air-dry overnight.

### **ELISA**

IL-6 and MCP-1 were quantitated using a sandwich ELISA. Flat-bottom 96-well plates were coated with IL-6 or MCP-1 antibody and incubated overnight at 4 C. The plates were washed four times with PBS with .05% Tween 20 and blocked for 2 hours with PBS and 1% BSA. After the plates were washed three times, samples and standards were added and incubated overnight at 4 C. All experimental samples were tested in triplicate. The plates were then washed four times and incubated with IL-6 or MCP-1 detection antibody for 2 hours at room temperature. The remaining steps were carried out at room temperature. The plates were washed 6 times and incubated with NeutrAvidinn horseradish peroxidase conjugate (Pierce #31001) for 30 minutes; and washed 8 times and incubated with ABTS peroxidase substrate at for 90 minutes. Plates were read at 405 nm in an ELISA plate reader.

## **Quantitative PCR**

This assay was performed and quantified at the University of Kansas Medical Center by one of our collaborators Carolyn Vivian in Dr. Danny Welch's research laboratory. Quantitative PCR was used to analyze differentiated MC3T3-E1 osteoblasts transcriptional regulation of KISS1 and KISS1R over a period of 4 weeks. All samples were run in triplicate. The final results depicted are average  $C_t$  values of the samples (threshold cycle values). Beta actin was used for normalization.

## **Statistical analysis**

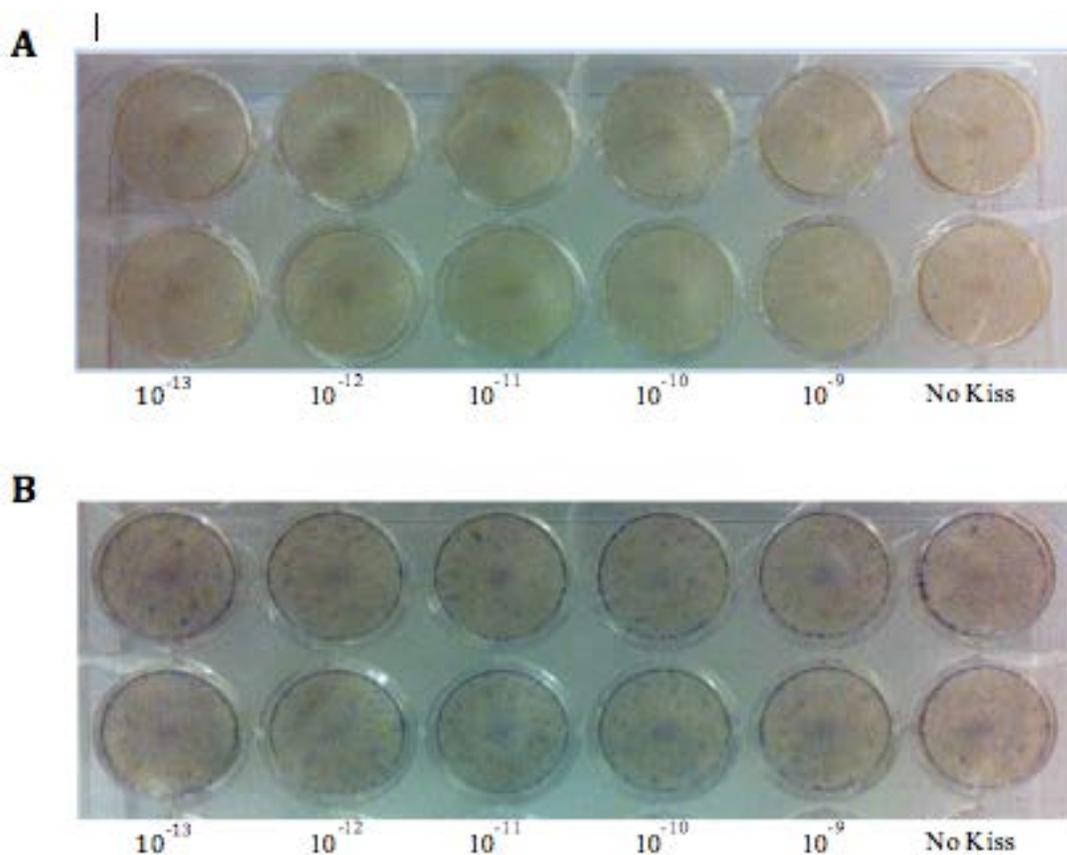
Average concentration values from the ELISA assays were analyzed using the Graph Pad 4-Prism. A two way ANOVA was performed in order to determine statistical significance. (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ )

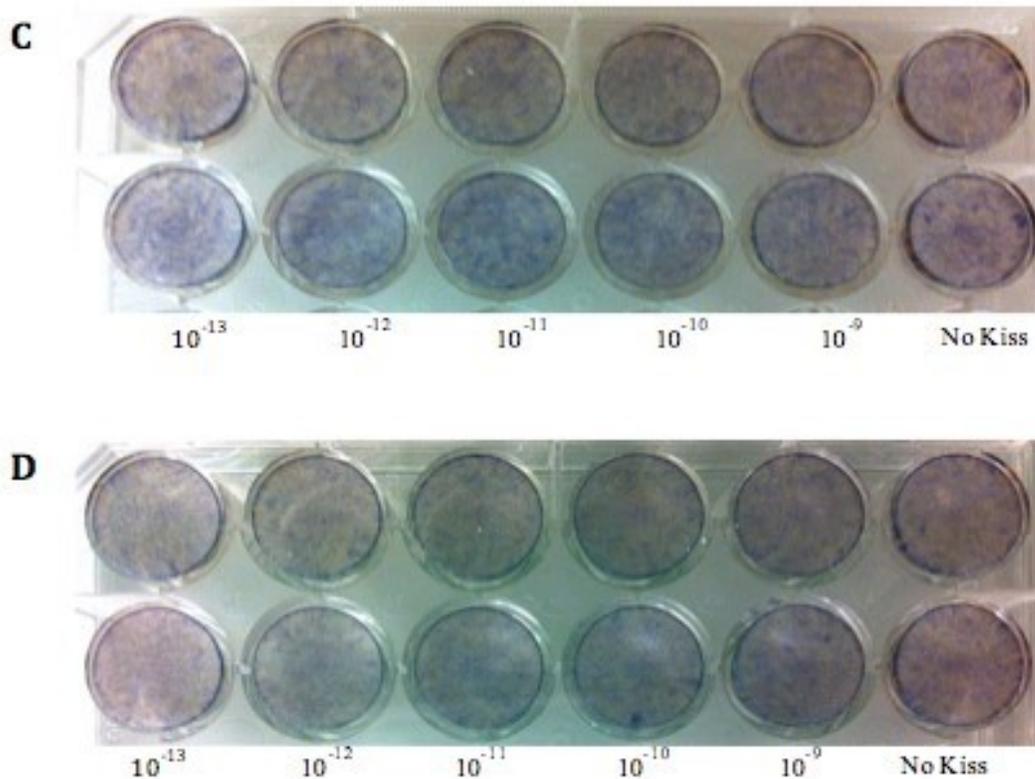
## Chapter 3 Results

### Differentiation of Osteoblasts with Kisspeptin-10

#### Experimental Rationale

Few previous studies have looked at the effect of KP10 on the bone microenvironment and none have studied specifically osteoblasts. Therefore, this study was carried out to determine if KP10 would have any effect on osteoblast differentiation. The rationale behind this experiment was KP10 is an important protein in development. Additionally, this experiment was necessary to determine appropriate dosing concentrations. Differentiation was measured by osteoblast alkaline phosphatase expression (Figure 4), which was confirmed by the presence of a dark purple stain.





**Figure 5** MC3T3 E1 osteoblast differentiation following the addition of KP10 at varying concentrations

The concentrations tested were 1 x ( $10^{-9}$ ,  $10^{-10}$ ,  $10^{-11}$ ,  $10^{-12}$ , and  $10^{-13}$ ) M or no KP-10. Cells were cultured with osteoblast differentiation medium and KP10 for up to 29 days. At times indicated (12, 19, 26 and 29) osteoblasts, were fixed and then stained for alkaline phosphatase expression, indicated by the blue color. A = Day 12, B = Day 19, C = Day 26, and D= Day 29

## Results

In the first experiment (Figure 4) varying the concentration of KP10 did not have an effect on osteoblast differentiation over a three-week period. This was quantified through the presence of alkaline phosphatase (presence of purple stain). Osteoblasts undergo a distinct differentiation mechanism, starting with the secretion of a variety of non-collagenous matrix

proteins and concluding with mineralization.[8] Three phases of osteoblast differentiation can be detected, which include rapid proliferation, matrix maturation, and mineralization. None of the five concentrations had a significant effect on the amount of alkaline phosphatase activity when compared to the vehicle media control. All of the samples showed a steady increase in alkaline phosphatase activity as they matured. The results of this experiment were important because they indicated that osteoblasts have normal maturation when treated with KP10 and continue to be able to create a bone matrix. Because kisspeptin is a molecule involved in development it was unknown if treating maturing osteoblasts with KP10 would have an effect on maturation. Once this principle was established the next experiment was designed to determine if adding breast cancer conditioned media would have an effect on osteoblast differentiation in the presence of KP10.

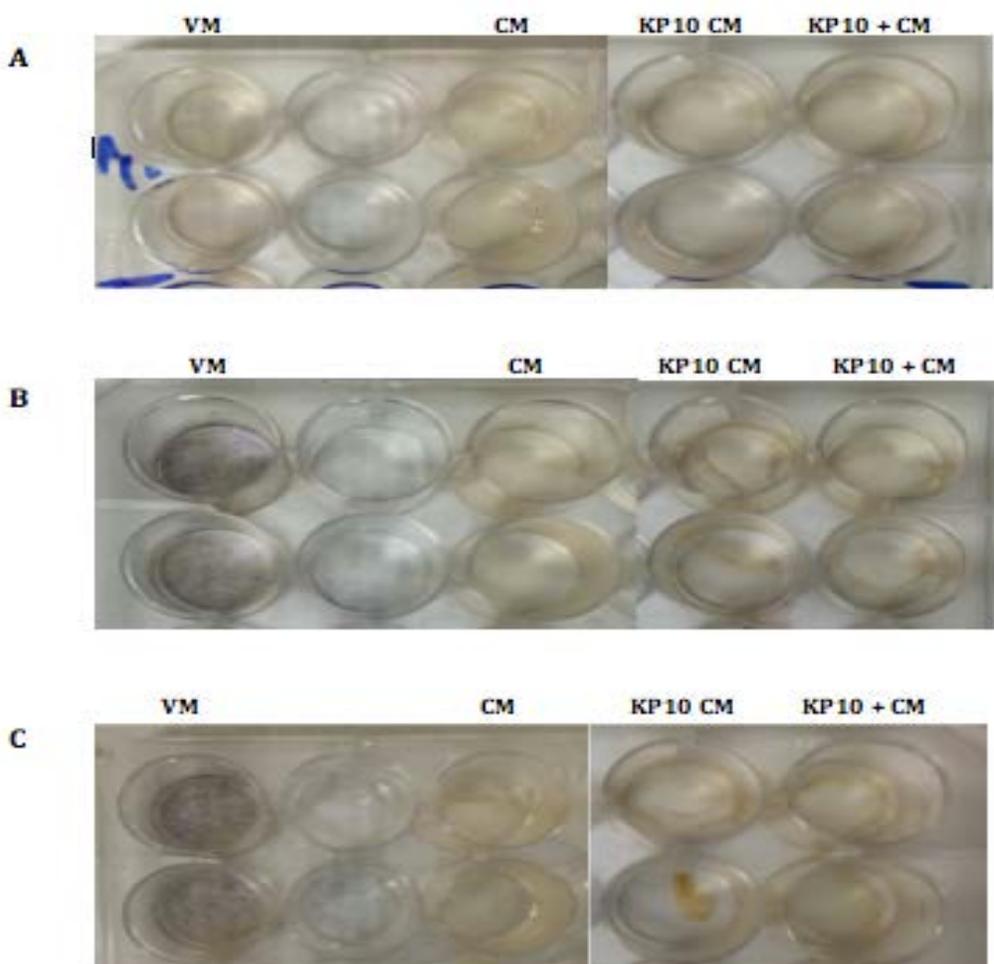
### **Osteoblast Differentiation In the Presence of Breast Cancer Conditioned Media and Kisspeptin 10**

#### **Experimental Rationale**

In the subsequent experiment (Figure 6) the aim was to determine if KP10 would have an effect on osteoblast differentiation in the presence of breast cancer conditioned medium. Previous studies done in our laboratory resulted in MDA-MB-231 conditioned medium blocking MCT3-E1 differentiation leading to no alkaline phosphatase expression. Therefore, it was hypothesized that KP10 could alter the effect of breast cancer conditioned media on osteoblast differentiation.

#### **Results**

Similar to the first experiment in Figure 5, the presence of KP10 did not affect osteoblast differentiation. Normal differentiation was indicated by the presence of a dark purple stain. (Figure 5) As seen in Figure 6 below, the conditions with KP10 looked similar to the breast cancer conditioned media condition versus the control or vehicle media condition. Therefore, KP10 did not change the effect of breast cancer conditioned medium on osteoblast differentiation.



**Figure 6** MC3T3-E1 osteoblast differentiation in the presence of breast cancer conditioned media and KP10.

Two types of breast cancer conditioned media were prepared by treating MDA-MB-231 breast cancer cells with serum-free media for 24 hours. One conditioned medium was serum-free medium  $\alpha$ MEM (CM) and the other conditioned medium contained serum-free medium  $\alpha$ MEM and 100nM of kisspeptin10 (K10 CM). After 24 hours, the media were collected and added at 50% to MC3T3-E1 osteoblasts. Combining CM with 100nM of KP10 created an additional conditioned medium (KP10+CM). All conditions were tested for a period of three weeks. A = Day 7, B= Day 14, C=Day 21

### **The effect of MDA-MB-231 metastatic breast cancer conditioned media treated with Kisspeptin 10 on MC3T3-E1 osteoblasts**

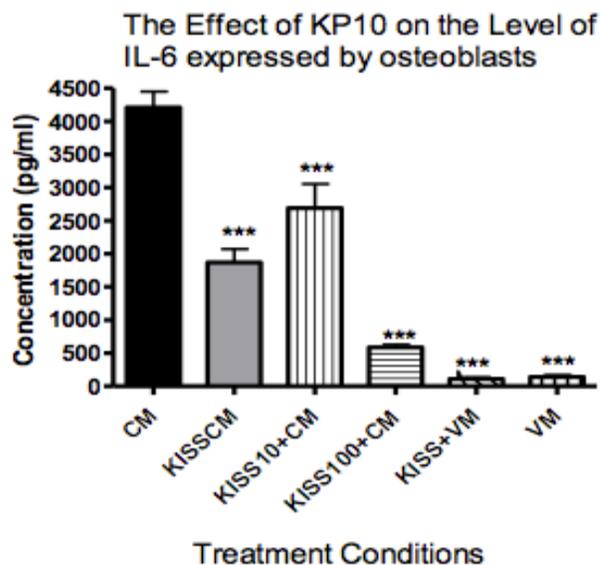
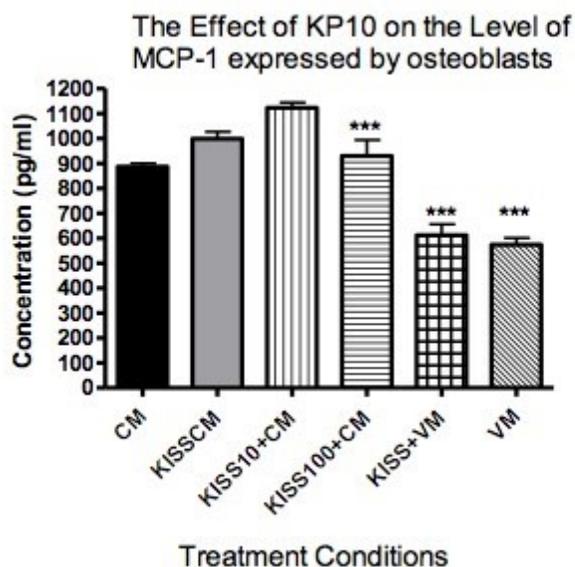
#### **Experimental Rationale**

Because there was no observable change in the differentiation of osteoblasts when they were treated with KP10 the question posed was do breast cancer cells need to be present in the bone microenvironment in order for osteoblasts to be affected by KP10? Additionally, I asked if breast cancer cells were affected by KP10 because MDA-MB-231 cells express KISS1R. Therefore, the subsequent experiments in Chapters 5 and 6 were designed and carried out to test these questions. In order to test both KP10's effect on breast cancer cells and osteoblasts two conditioned medias were prepared and tested. The two conditioned medias are defined as KISSCM media and KP10+CM. Once this conditioned media was prepared it was added to differentiated osteoblasts and their inflammatory response was measured through ELISA.

#### **Results**

It was found that KP10 downregulated the osteoblasts inflammatory response in the presence of breast cancer conditioned media. This was quantified based on the levels of IL-6 and MCP-1 in the media using ELISA. This downregulation was only consistently and

significantly found in the levels of IL6. Both the KP10 CM and KISS CM downregulated the level of IL6 consistently across 4 separate experiments. Although here was not a dose response; the presence of KP10 significantly affected the levels of IL6 from a wide dose range of 1-500nM.

**A****B**

**Figure 7** The effect of conditioned media from MDA-MB-231 metastatic breast cancer cells treated with Kisspeptin 10 on MC3T3-E1 osteoblasts

Two types of breast cancer conditioned media were prepared by treating MDA-MB-231 breast cancer cells with serum-free media for 24 hours. One conditioned medium was serum-free  $\alpha$ MEM (CM) and the other conditioned medium contained serum-free  $\alpha$ MEM and 100nM of kisspeptin10 (KISSCM). After 24 hours, the media were collected and added at 50% to MC3T3-E1 osteoblasts that had been differentiated for 2 wks. Various concentrations of KP10 (10-100nM, CM+KISS) were also added to the conditioned media (CM) and used to treat the osteoblasts. Osteoblast differentiation medium (VM) was included as a negative control. After four hours, the different media were collected from the osteoblasts. The levels of IL-6 (C) and MCP-1 (D) present in their media used ELISA to measure the response of the osteoblasts after treatment. The samples were tested in triplicate. Shown is the averages  $\pm$  SEM. Statistical significance was determined by one-way ANOVA (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ).

## **Dose Expansion of Kisspeptin 10 Part I**

### **Experimental Rationale**

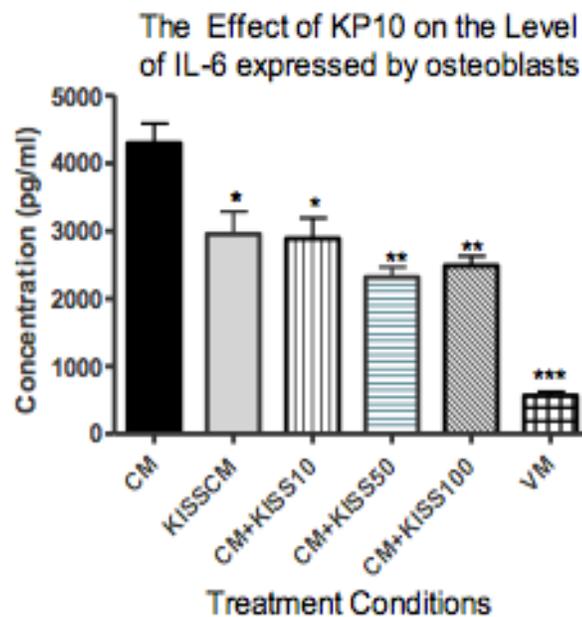
After the initial experiment testing the effect of breast cancer conditioned media treated with KP10 on osteoblasts, it was decided to expand the concentration of doses to include 10, 50, and 100 nM of KP10 combined with breast cancer conditioned media. This was after the observation that the amount of IL-6 in the breast cancer conditioned media made with KP10 (KISS CM) was approximately half of the amount in the other two conditions (CM+KP10). However, it should be noted there should not have been remaining KP10 in the conditioned media as KP10 has a short half-life in vitro.[31] Because the KISS CM was diluted by 50% and had an initial KP10 concentration of 100 nM when added to breast cancer cells, we deduced that

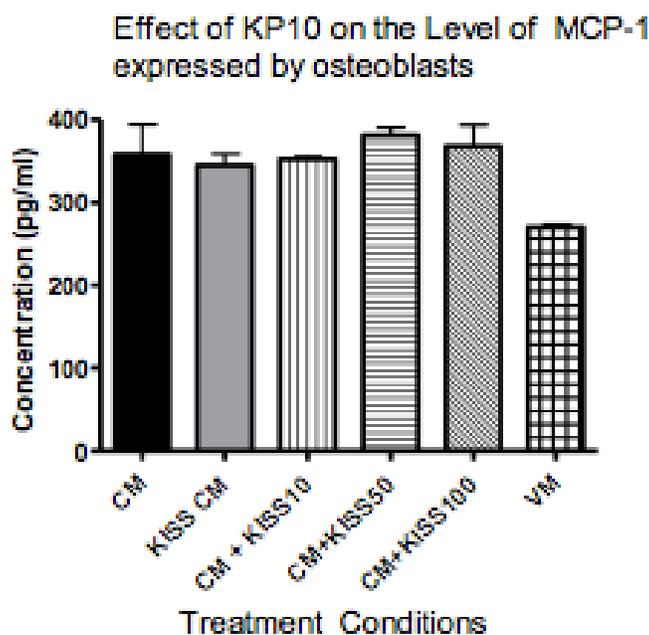
the KISS CM could have the same potency as adding 50 nM of KP10 to breast cancer conditioned media. Therefore, this study was carried out to test this hypothesis as well as to further confirm the downregulation of the osteoblasts inflammatory response. Another goal of this experiment was to test the levels of MCP-1

## Results

During this experiment it was evident when breast cancer conditioned media with the addition of KP10 this decreased the osteoblasts inflammatory response. This finding confirmed the results reported in the previous experiment in Section 4.2. IL-6 continued to be significantly decreased in relation to breast cancer conditioned media. There was no significant effect on the levels of MCP-1 expressed by the osteoblast when treated with KP10.

A



**B**

**Figure 8** Primary dose expansion of the effect of conditioned media from MDA-MB-231 metastatic breast cancer cells treated with Kisspeptin 10 on MC3T3-E1 osteoblasts

Two types of breast cancer conditioned media were prepared by treating MDA-MB-231 breast cancer cells with serum-free media for 24 hours. One conditioned medium was serum-free  $\alpha$ MEM (CM) and the other conditioned medium contained serum-free  $\alpha$ MEM and 100nM of kisspeptin10 (KISSCM). After 24 hours, the media were collected and added at 50% to MC3T3-E1 osteoblasts that had been differentiated for 2 wks. Various concentrations of KP10 (10-100nM, CM+KISS) were also added to the conditioned media (CM) and used to treat the osteoblasts. Osteoblast differentiation medium (VM) was included as a negative control. After four hours, the different media were collected from the osteoblasts. ELISA was used to measure the response of the osteoblasts by the levels of IL-6 (A) and MCP-1 (B) present in their media after treatment. The samples were tested in triplicate. Shown is the averages  $\pm$  SEM. Statistical significance was determined by one-way ANOVA (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ).

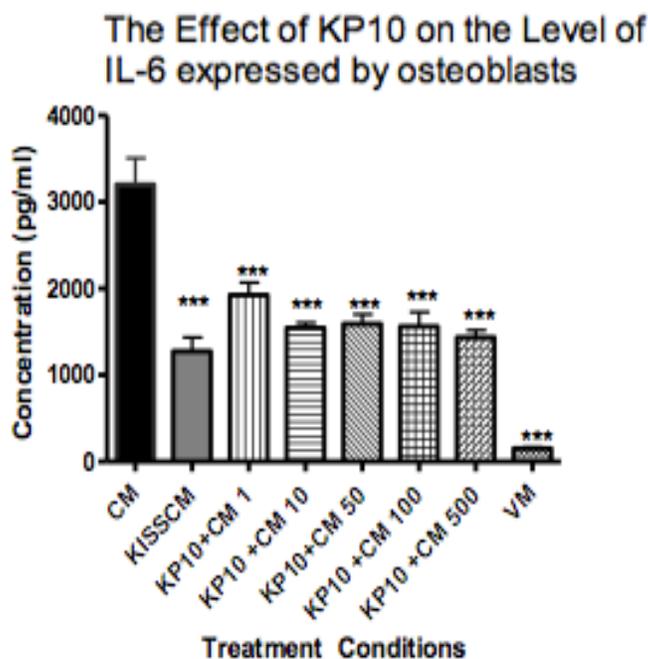
### Dose Expansion of Kisspeptin 10 Part II

#### Experimental Rationale

Based on the results from the previous study, it was decided to continue to expand the dose of KP10 added to osteoblasts to determine if we could establish a significant dose response and if the findings would remain consistent. It was decided because the data for the expression of MCP-1 remained inconsistent to only continue to measure the levels of IL6 using the previously describe experimental design. This study was repeated twice in order to replicate the results of the experiment.

## Results

In accordance with the other experiments performed it was evident when breast cancer conditioned media with the addition of KP10 decreased the osteoblasts inflammatory response. This conclusion was based on a decreased level of IL-6 present, which is quantitated by ELISA. This experiment did demonstrate that there is a dose response for KP10. However, all samples with KP10 showed significant decreases in the expression of IL-6



**Figure 9** Secondary dose expansion of the effect of conditioned media from MDA-MB-231 metastatic breast cancer cells treated with various concentrations of Kisspeptin 10 on MC3T3-E1 osteoblasts.

Two trials were completed and the data combined in Figure 9. Two types of breast cancer conditioned media were prepared by treating MDA-MB-231 breast cancer cells with serum-free media for 24 hours. One conditioned medium was serum-free  $\alpha$ MEM (CM) and the other conditioned medium contained serum-free  $\alpha$ MEM and 100nM of kisspeptin10 (KISSCM). After 24 hours, the media were collected and added at 50% to MC3T3-E1 osteoblasts that had been differentiated for 2 wks. Various concentrations of KP10 (1-500nM, CM+KISS) were also added to the conditioned media (CM) and used to treat the osteoblasts. Osteoblast differentiation medium (VM) was included as a negative control. After four hours, the different media were collected from the osteoblasts. ELISA was used to measure the response of the osteoblasts by the levels of IL-6 (C) and MCP-1 (D) present in their media after treatment. The samples were tested in triplicate. Shown is the averages +/- SEM. Statistical significance was determined by one-way ANOVA (\*P<. 05, \*\*P<. 01, \*\*\*P<. 001).

### **Osteoblast Expression of KISS1 and KISS1R**

#### **Experimental Rationale:**

Because little research has been done on the role of KISS1 in the bone an experiment was designed to determine if MC 3T3-E1 osteoblasts expressed the KISS1 gene and KISS1R receptor. It was previously confirmed that MD-MBA 231 cells express both the gene and receptor.[30] [32] Additionally, there was a published report that an osteosarcoma cell line MG63 and the MCF-7 breast cancer cell line showed high expression of GPR54. [33] However other studies had not reported high levels of KISS1 or GPR54 tissue specific expression. The presence of these entities and their level of expression were confirmed using qPCR. This experiment was completed over four weeks to determine if the expression of the gene or receptor changed.

**Table 2** Expression of KISS1R in Osteoblasts

	$C_t$ values
5 days	30.93
2 weeks	30.39
4 weeks	29.89

**Table 3** Expression of KISS1 in Osteoblasts

	$C_t$ values
5 days	35.13
2 weeks	33.23
4 weeks	32.88

This unpublished data was provided by one of our collaborators Carolyn Vivian of Dr. Welch's Laboratory at the University of Kansas Medical Center. Quantitative PCR was performed on differentiated MC3T3E1 osteoblasts to determine the expression of KISS1R and KISS1 over a period of four weeks. Shown in Table 2 and 3 are the average  $C_t$  or cycle threshold values from this assay. All samples were run in triplicate. The data was normalized to beta actin.

## Results

The average CTs obtained from qPCR for the KISS and KISSR indicate that MC 3T3E1 osteoblasts express both the KISS1 gene and KISS1R. It was evident (Table 2) that the receptor

was expressed at a moderate level. Then KISS1R increases expression at Week 4. It was evident (Table 3) KISS is weakly expressed at day 5 of differentiation but increased slightly on day 14 with no change between day 14 and 28. Overall, these data are important because they confirm the expression of both KISS1 and KISS1R. Thus KP10 can be acting on the osteoblasts by binding KISS1R.

## Chapter 4 Discussion

The rationale behind this research was breast cancer frequently metastasizes to the bone and KISS1 has been classified as a metastasis suppressor gene. KP10, which is a product of the KISS1 gene, could potentially downregulate the deleterious activity of breast cancer cells in the bone microenvironment. This study was designed to investigate the interactions among one of the proteolytic processing products of the KISS1 protein (KP10), its receptor KISS1R, osteoblasts, and metastatic breast cancer cells. The aim was to determine if KP10 affected the interaction of osteoblasts and breast cancer cells and elucidate KP10's possible role in breast cancer metastasis. In this study, the impact of KP10 on osteoblasts *in vitro* was analyzed and quantified through multiple methods. Because little research has been done on the effect of KP10 in the bone, it was unknown if KP10 would have an effect on the breast cancer cells or the osteoblasts. Hence it was hypothesized that KP10 would affect the differentiation of osteoblasts and downregulate their inflammatory response when in the presence of breast cancer conditioned media.

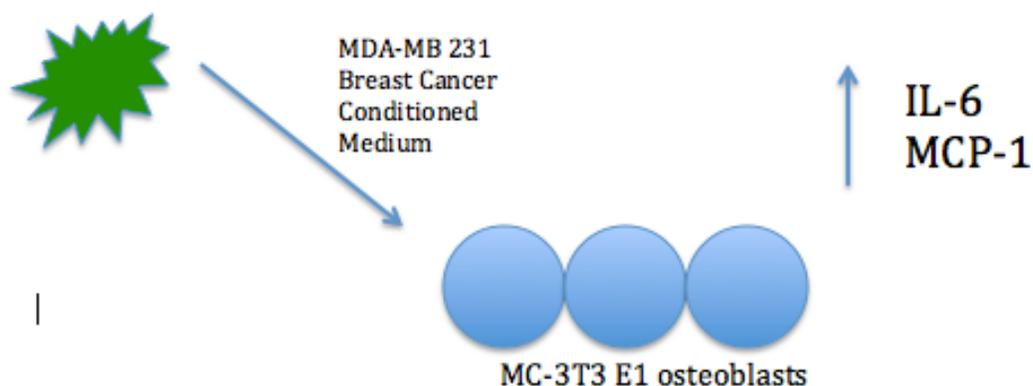
In the initial experiment it was found that osteoblasts did not directly interact with KP10 in a way that affects differentiation (Figure 5). Also the concentration of KP10 did not have an effect on the interaction as every test concentration  $1 \times 10^{-9}$ ,  $10^{-10}$ ,  $10^{-11}$ ,  $10^{-12}$ , and  $10^{-13}$  M had similar alkaline phosphatase expression. Initially, KP10 was postulated to have an effect in the bone because of its important role as a developmental molecule.[25] However because there was no observable effect on osteoblast differentiation when treated with KP10, the next step in this study was to test if in the presence of breast cancer conditioned media, would osteoblasts be affected by the addition of KP10.

Because there was no observable effect on differentiation in osteoblasts, other studies were designed to see if KP10 had an effect on the inflammatory response of breast cancer cells to osteoblasts. (Figures 7-9) It was found that KP10 downregulated the osteoblast inflammatory response in the presence of breast cancer conditioned media. Multiple types of conditioned media were used in this experiment to test the effect of KP10 on both osteoblasts and breast cancer cells. Kisspeptin conditioned media (KISS CM) was created to test the effect of KP10 on breast cancer cells when conditioned media was collected. Then KP10 was added to normal breast cancer conditioned media to test the effect of KP10 on osteoblasts (KP10 + CM). KP10 was tested at various doses (1-500nM) in order to determine if there would be a dose response. The response was quantified based on the levels of IL-6 and MCP-1 in the media using ELISA. This downregulation was only consistently and significantly found in the levels of IL6 but not MCP1. One possible explanation for why MCP-1 was not significantly or consistently affected is the osteoblasts were not exposed to the conditioned media for a long enough amount of time for a response to be observed. In addition, the concentrations of MCP-1 detected were in picomolar range, near the limits of detection of the assay. It was also extremely difficult to keep variables such as breast cancer conditioned media preparation for each individual trial consistent as new batches of conditioned media were used for each experiment. However, cell counts were collected after the conditioned media was harvested and cell counts were very consistent between batches. Interestingly, the levels of IL6 decreased for both KISSCM and KP+CM suggesting KP10 is acting on both the breast cancer cells and osteoblasts. In conjunction to determining if KP10 in the presence of breast cancer conditioned media had an effect on osteoblasts, an additional experiment was devised to discover if KP10 would affect osteoblast differentiation in the presence of breast cancer conditioned media. In this experiment osteoblasts

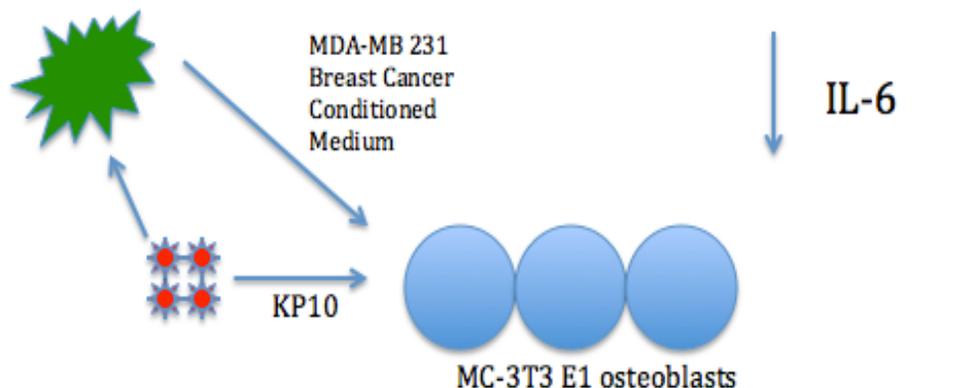
were differentiated in the presence of CM, KISS CM, and KP10+CM. Similar to the first differentiation experiment, there was not an observable effect on the differentiation of osteoblasts in the presence of breast cancer conditioned media and KP10 (Figure 6). Hence, it was observed in these experiments that KP10 had an effect on osteoblasts inflammatory response to breast cancer conditioned media, but KP10 did not have an effect on osteoblast differentiation in the presence of breast cancer conditioned media.

These results pose the possibility KP10 is acting both on the breast cancer cells and osteoblasts, which is supported by the final experiment conducted. Studies have shown that 231 breast cancer cells express KISS1R. The final experiments conducted showed that differentiated osteoblasts express both KISS1 and KISS1R. (Table 2 and 3) Because the breast cancer cells and the osteoblasts express KISS1R, it is possible KP10 is having an effect on both of these cells. However, there is also the possibility KP10 could be binding another molecule in the breast cancer conditioned media which is affecting the osteoblasts inflammatory response. Faced with the uncertainty of a definitive mechanism of action we propose a preliminary model of what is happening in this experimental system. As has been reported IL-6 and MCP-1 secretion by osteoblasts increases in the presence of breast cancer conditioned medium (Figure 10). This could lead to homing of breast cancer cells to the bone microenvironment or further activation and proliferation of breast cancer cells. Therefore, in order to elucidate if KP10 could have an effect on breast cancer cells or osteoblasts, this study was conducted (Figure 11). Overall because all of the conditions with KP10 present in them saw a significant decrease in the production of the inflammatory cytokine IL-6 it is reasonable to say that KP10 is having an effect on osteoblasts. However, it is still not definitive if KP10 is having an effect on breast cancer cells when the KISS CM is being made. There also exists the possibility that KP10 is

binding something in the breast cancer conditioned media, which could be producing this response. Therefore, more studies need to be done to further analyze this relationship.



**Figure 10** A schematic representation of the inflammatory response of MC 3T3-E1 osteoblasts in presence of MD-MBA 231 breast cancer cells



**Figure 11** A schematic representation of the two models in which KP10 interacts with both osteoblasts and breast cancer cells

As a result, the precise mechanism of the antimetastatic function of KP10 remains unclear in breast cancer. Although there has yet to be a consensus on the role of KP10 in breast

cancer, there are a multitude of studies focused on possible antimetastatic mechanisms for how KISS1 and specifically KP10 works. Some studies have implied KP10 decreased breast cancer invasion of the bone through the downregulation of TGF- $\beta$ . Downregulation of TGF- $\beta$  could be a possible connection to why KP10 is able to decrease the levels of IL6 during the inflammatory response, as TGF- $\beta$  is partially responsible for the expression of IL-6 by MC3T3-E1. Additionally, TGF- $\beta$  is a key mediator of the EMT [8] If KP-10 is interfering with the activation of TGF- $\beta$  this could not only be responsible for the lower levels of IL6 being observed but also other antimetastatic effects.

Additionally, published studies on bone-directed invasion identified the CXCL12/CXCR4 system as a potent regulating system. CXCL12 was identified in bone marrow and its protein extract, and CXCL12 treatment led to increased invasion and migration of CXCR4-expressing MDA-MB-231 cells. [34] Another study found high expression of CXCR4 in tumor cells caused them to migrate to the bone microenvironment. One group has found that KP-10 treatment significantly reduced expression of CXCR4 by breast cancer cells.[33] Because KP10 plays a role in regulating EMT in breast cancer cells that preventing this interaction could be a potential strategy for the treatment of breast cancer. Some studies have also shown that KP10 induced metastatic suppression is related to the suppression of matrix metalloproteinase (MMP)-9 activity and subsequent inhibition of cancer cell migration and invasion. [35] Results of one study showed that KP-10 increased MMP-9 and MMP-2 activity and subsequently inhibited cancer cell migration and invasion. [28] Overall, there are many explanations of how KISS1 and KP10 can have antimetastatic effects and from the literature it seems encouraging this activity can be seen in the bone.

Another important observation in this current study was there was not a significant dose response. This was an interesting observation as literature sources have reported statistically significant dose responses when cells were treated with KP10. [36] One explanation may be that other components in the conditioned medium are synergizing or antagonizing the KP10 and blunting the dose response to KP10. The possibility of a dose response effect of KP10 could be studied further. An influence of KP10 should be further investigated to determine if KP10 could affect proliferation or activation of breast cancer metastases in the bone. Other breast cancer cell lines could be used to determine if there is a difference between estrogen receptor status and interaction with KP10. Additionally, further studies could be done to analyze KP10s interaction with the bone microenvironment by analyzing its effect on osteoclasts.

## **Chapter 5**

### **Conclusion**

The results of these experiments suggest KP10 is interacting with both the osteoblasts and breast cancer cells. Overall, the presence of KP10 did not affect osteoblast differentiation; i.e. there was no change in the osteoblasts alkaline phosphatase expression over 29 days. KP10 also did not significantly decrease the levels of MCP-1 expressed by the osteoblasts. However, the presence of KP10 downregulated the levels of IL-6 expressed by MC3T3-E1 osteoblasts when exposed to both types of MDA-MB-231 breast cancer conditioned media. These preliminary observations suggest KP10s affected both the osteoblasts and breast cancer cells, but the precise mechanism remains unknown. We propose two different models for how this decrease may be occurring. One model is the KISSCM model and KP10+CM model (Figure 11). One future study that could be done to test this construct would be to use a neutralizing antibody to KISS1R; and repeat the same procedure and determine the levels of inflammatory cytokines that would be secreted. Additionally, further research must be completed to determine how KP10 can be used to prevent proliferation or activation of breast cancer metastases in the bone. KISS1 and its peptides are an interesting area of research as kisspeptins may have applications in the treatment of metastatic disease especially in patients suffering from breast cancer. Overall, it is evident KP10 has an effect on osteoblasts and breast cancer cells, and looking further into this relationship could provide important answers about KP10s role in breast cancer metastasis.

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# Academic Vita

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## Kaitlyn E. Leahey

[Kleahey15@gmail.com](mailto:Kleahey15@gmail.com)

### EDUCATION:

**Schreyer Honors College, Pennsylvania State University, University Park, PA**  
B.S. in Biology, Vertebrate Physiology Option, Bioethics and Medical Humanities Minor

Graduation: May 2015  
August 2007 - May 2011

**Presentation High School, Summa Cum Laude, San Jose, CA**

**RELEVANT SKILLS:** Scientific writing, project development, tissue culture, immunohistochemistry, ELISA, Polymerase Chain Reaction (PCR), gel electrophoresis, column chromatography, thin layer chromatography, recrystallization

### WORK EXPERIENCE:

**Pennsylvania State University, University Park, PA** January 2012 - Present

Research Assistant, Breast Cancer Research Lab, Dr. Andrea Mastro

- Design and execute experimental studies of the interactions between metastatic breast cancer cells, osteoblasts, and kisspeptin-10 using ELISA and immunohistochemistry
- Awarded a research grant from the National Academy of Sciences, Sigma Xi, The Scientific Research Society
- Published abstract in *Cancer Research*

**AbbVie Biotherapeutics, Redwood City, CA** May 2014 – August 2014

Oncology Research and Development Intern

- Engineered novel antibody structures to improve binding and specificity to key targets
- Interpreted Next Generation sequencing data and preformed protein modeling using PyMOL

**MedImmune, Gaithersburg, MD** June 2013 – August 2013

Oncology Clinical Development Intern

- Assisted in designing and monitoring clinical trials for immune mediated therapies for cancer
- Developed a clinical analysis and database of competitor immunotherapy trails
- Presented a formal presentation and poster on PD-1/PD-L1 pathway inhibitors

### CONFERENCES:

Leahey, K., Welch, D., Chen, Y., Bussard, K., Mastro, A., (2014, April). Deadly KISS, Kisspeptin10 interaction with osteoblast and breast cancer metastatic cells. Presented at American Association for Cancer Research conference. San Diego, CA.

### AWARDS/ HONORS:

Pennsylvania State University Society of Distinguished Alumni Protégé August 2013 - Present

Eberly College of Science Dean's List May 2012 - December 2013

NASA Women in Science and Engineering Research Fellowship January 2012 - December 2012

Silicon Valley Pennsylvania State University Alumni Scholarship July 2011-May 2015

### STUDENT VOLUNTEER SERVICE:

Fundraising and Special Events Captain, Penn State Relay for Life October 2013 – Present

Rules and Regulations Committee Member, Penn State Dance Marathon September 2012 – February 2013

### LEADERSHIP AND EXTRACURRICULAR ACTIVITIES:

Future of Healthcare Think Tank at Radboud University in Nijmegen, Netherlands September 2014-Present

- International consortium of students from the Schreyer Honors College at PSU and the Honors Academy at Radboud University to research and answer questions posed by the Dutch Ministry of Health on improvements to geriatric healthcare
- Collaborated with other students to develop a formal presentation and publication for the Dutch Ministry of Health, The Hague, Netherlands

Atlas THON August 2011 – Present

- Raised \$390,366 for Pennsylvania State University Dance Marathon to support Hershey Medical Center's Four Diamonds Fund for Pediatric Cancer

Science LionPride January 2012 – Present

- Ambassador to prospective students for the Eberly College of Science
- Led tours, chaired student panels, and delivered presentations

Alpha Xi Delta

- Solicitations Captain for Alpha Xi Delta's annual Autism Speaks 5k Walk/Run

September 2012 - Present