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KISS1R Signaling Promotes Breast Cancer Metastasis

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By

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ABSTRACT

Kisspeptins, peptide products of *KISS1*, are endogenous ligands for KISS1R, a G protein-coupled receptor. In numerous cancers, *KISS1* acts as a metastasis suppressor. However, studies have revealed that patients with elevated *KISS1* and *KISS1R* breast tumor expression have increased tumor grade, increased lymph node metastases and poor survival. We hypothesize that depletion of KISS1R inhibits breast cancer cell metastasis. In order to assess the role of KISS1R in breast cancer metastasis, we used a pre-clinical orthotopic xenograft mouse model using MDA-MB-231 breast cancer cells for breast tumor establishment. We discovered that depletion of KISS1R decreased primary tumor growth and reduced lung metastatic burden, suggesting that KISS1R plays a role in promoting breast cancer metastasis. Furthermore, we observed that kisspeptin-10 stimulation increased breast cancer cell invadopodia formation *via* a β-arrestin2 dependent mechanism. Overall, our results suggest that KISS1R may be a novel therapeutic target in the prevention of breast cancer metastasis.

Key Words: KISS1R, Breast Cancer, Metastasis, Angiogenesis, Cell Migration, Cell Invasion, Extravasation, Invadopodia, β-arrestin2

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ANOVA	Analysis of Variance
CAM	Chorioallantoic Membrane
CD31	Cluster of Differentiation 31
cDNA	Complementary Deoxyribonucleic Acid
DCIS	Ductal Carcinoma *in situ*
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Kinase
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FRET	Fluorescence Resonance Energy Transfer
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
G Protein	Guanosine Nucleotide-Binding Proteins
GPCR	G Protein-Coupled Receptor
GRKs	G Protein-Coupled Receptor Kinases
H & E	Hematoxylin and Eosin
HBSS	Hank's Balanced Salt Solution
HER2	Human Epidermal Growth Factor Receptor 2
HGF	Hepatocyte Growth Factor

HIF-1	Hypoxia Inducible Factor- 1
HPG	Hypothalamic-Pituitary-Gonadal
Il2rγ	Interleukin-2 Receptor γ
IQGAP	IQ motif containing GTPase activating protein
KISS1R	Kisspeptin Receptor
KP	Kisspeptin
LCIS	Lobular Carcinoma *in situ*
LH	Luteinizing Hormone
LPA	Lysophosphatidic Acid
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumor Virus
MT1-MMP	Membrane Type 1 Metalloproteinase
N-WASP	Neuronal Wiskott–Aldrich Syndrome Protein
NOD	Non-Obese Diabetic
PCR	Polymerase Chain Reaction
PI3K	Phosphatidyl-Inositol-3-Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol 12-Myristate 13-Acetate
PTP1B	Protein Tyrosine Phosphatase 1B
PyMT	Polyoma Virus Middle T Antigen
ROI	Region of Interest
RPMI	Roswell Park Memorial Institute Medium
SCID	Severe Combined Immunodeficiency

SEM	Standard Error of the Mean
shRNA	Small Hairpin Ribonucleic Acid
SMA	Smooth Muscle Actin
TIMP	Tissue Inhibitor of Metalloprotease
TRITC	Tetramethylrhodamine
uPA	Urokinase Plasminogen Activator
VEGF	Vascular Epidermal Growth Factor

Chapter 1- Introduction

1.1.1 Breast Cancer Staging and Classification

According to the Breast Cancer Society of Canada, breast cancer is the most diagnosed cancer in Canadian women over the age of 20 and the second leading cause of Canadian women cancer related deaths†. In 2013, 23 800 Canadian women were diagnosed with breast cancer representing 26% of all diagnosed cancers in Canadian women†. Additionally, approximately 5000 women in Canada died from breast cancer, accounting for 14% of all cancer related deaths†. Due to the high rates of incidence and death to Canadian women, it is clear that a better understanding of breast cancer is required in order to develop better therapeutics and clinical strategies to combat the disease.

Breast cancer staging assesses the extent cancer has developed within the body and whether the cancer has spread to other tissues (Figure 1.1) (1). The American Joint Committee on Cancer (AJCC) has developed a system of breast cancer classification based on tumor origin, cellular characteristics and metastatic extent. Stages I-III are classified based on the range of initial primary tumor size and the extent of breast cancer cell spread to lymph nodes that surround the breast (1). Most often, breast cancer arises from breast epithelium in either ductal or lobular tissue of the mammary glands and is termed ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS), respectively (2). *In situ* refers to non-invasive breast cancer in which the tumor is confined to the milk duct or lobules of the breast. At this point the tumor cells are confined to the tissue and are not a threat of undergoing metastasis and thus classified as benign hyperplasia. Cells in DCIS and LCIS demonstrate an increased number of

anaplastic and mitotic cells, pleomorphism, and central luminal necrosis due to lack of blood supply (3). As cancer progresses and begins to invade into the surrounding tissue, they are then classified as invasive and pose a great risk to metastasis. The most common invasive breast cancers are ductal carcinoma (IDC) and invasive lobular carcinoma. These cancers often demonstrate lymph node metastases. The final stage, Stage IV, represents breast cancer that has metastasized to distant organs, such as bone, lung and brain, at which point the cancer becomes inoperable and lethal (1, 4).

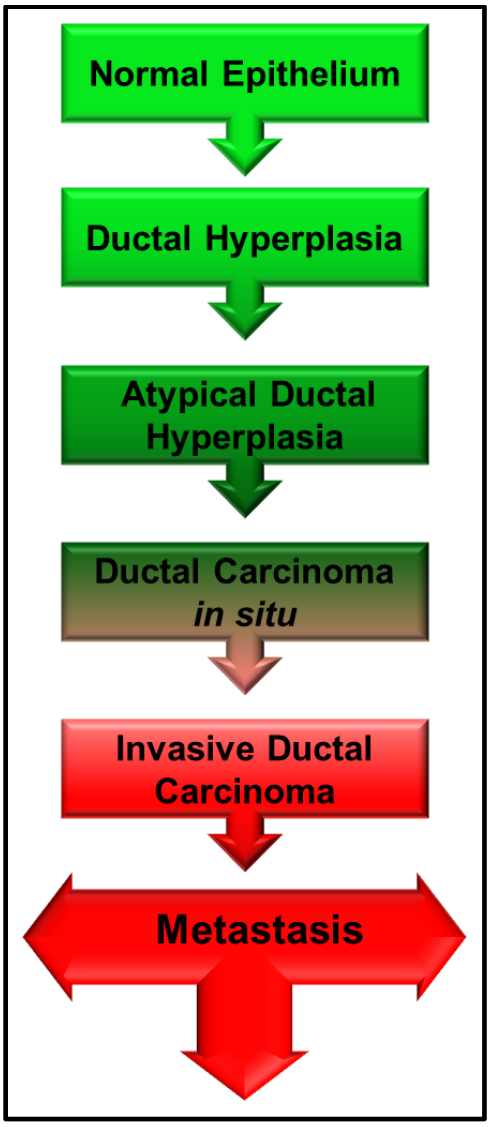

Figure 1.1. The steps of breast cancer progression. Non-malignant epithelial cells proliferate uninhibited leading to ductal hyperplasia. Epithelial cells lose their normal phenotype as well as morphology and begin to assume an expression profile of mesenchymal cells indicative of carcinoma advancement. Ultimately, cancer cells will acquire the ability to invade and migrate into the surrounding tissue initiating metastasis.

1.2.1 Metastatic Cascade

Metastasis is the process by which cancer cells leave the primary tumor and disseminate to secondary sites within the body either through the blood circulatory system, the lymphatic system or within body cavities (Figure 1.2)(5). When breast carcinomas are confined to breast tissue, 5-year survival rates exceed 90% (6). However, long-term survival is significantly decreased when cancer cells metastasize and survival time is adversely affected depending on the extent and sites of colonization (7). The initial step of the metastatic cascade involves a subpopulation of non-malignant cells accumulating mutations causing a malignant phenotype (8). In the context of breast cancer carcinoma, a tumor develops when epithelial cells undergo malignant transformation resulting in increased cellular proliferation and cell survival (9). Cancer cell survival is achieved through the development of anchorage independent growth and evasion of apoptotic signaling (5).

Angiogenesis, the process of new blood vessel formation from previous vasculature, is an important event that regulates tumor growth and survival (10). Under normal physiological conditions, angiogenesis plays an important role in wound healing, placentation during pregnancy and *in utero* cell proliferation during the female reproductive cycle (11). Angiogenesis is precisely controlled through a balance of pro- and anti-angiogenic factors. In disease, this balance of pro- and anti-angiogenic growth factors is lost. In cancer, a plethora of pro-angiogenic factors are released inducing tumor-associated microvasculature (12). This tumor-associated microvasculature is essential for tumor growth and

survival for the new blood vessels supply nutrients and oxygen that regulates tumor growth and size (13). Thus, the amount of tumor-associated microvasculature, or tumor microvessel density, is used as a prognostic marker for cancer stage and progression (14, 15). In these circumstances, blood vessel formation can arise from pre-existing vasculature from the host or originate from the cancers cells themselves in a process known as vascular mimicry (16). The molecular events underlying angiogenesis are stimulated under hypoxia conditions. As tumors increase in size, the availability of oxygen for each individual cell becomes exhausted and results in a hypoxic state. Under hypoxic conditions, the hypoxia inducible factor-1 (HIF-1) transcriptional complex becomes activated and induces promotion of pro-angiogenic factors, apoptotic resistance, anaerobic metabolism, and resistance to apoptosis (17). The vascular epithelial growth factor (VEGF) is a pro-angiogenic factor that has been demonstrated to play an important role in cancer induced angiogenesis. VEGF is created by tumor cells themselves or is released from the degradation of extracellular matrix (ECM) that surrounds the tumor (10). VEGF will then diffuse into the surrounding tissue and bind to VEGF receptors on the endothelial cells of pre-existing blood vessels, stimulating endothelial cell proliferation (10). The new endothelial cells form new networks of immature vessels within and surrounding the tumor. Often these immature vessels are fenestrated providing an opportunity for cancer cell metastasis (8).

Initial cell spreading or dissemination occurs in a process known as epithelial-to-mesenchymal transition (EMT), where epithelial cells lose cell-to-cell

adhesion, lose cellular polarity, and gain a mesenhymal phenotype of invasion and migration (18). Under normal physiological conditions, epithelial cells are organized in continuous sheets of polarized cells conveyed by cell-cell connections accomplished by gap and tight junctions, desmosomes, and adherens junctions (19). Furthermore, epithelial cells demonstrate an apical-basolateral polarity organization which prevents individual cells from migrating. During EMT, epithelial cells will lose cellular polarity and cell-cell contact via deceased expression of important adhesion molecules such as E-cadherin (20). Concurrently, an increase in mesenchymal markers is observed. These markers include matrix metalloproteases (MMP), N-cadherin, vimentin, β-catenin, and Snail/Slug conferring a highly mobile disorganized cellular morphology (18). Cells which undergo EMT migrate away from the primary tumor through cellular cycling of adhesion/ de-adhesion molecules regulated by integrins and the formation of F-actin stress fibers through which myosin cycling initiates movement (5).

In combination with cell migration, cancer dissemination requires cells to invade through the underlying basement membrane, that surrounds the tumor, and into the surrounding ECM (8). For invasion to occur, the tumors cells must induce ECM remodeling to allow movement of the cancer cell. Cancer cells secrete several proteases that confer ECM remodeling (21). The most common proteolytic enzymes involved in metastasis are MMPs, cathepsins and urokinase-type plasminogen activators (uPA) (21-23). Activation of these proteases during cancer metastasis results in uncontrolled degradation of the basement membrane and ECM. The degradation of ECM results in the release of growth

factors such as the epidermal growth factor (EGF) and VEGF, further contributing to local angiogenesis and metastatic progression (24).

After the tumor cells have invaded and migrated through the basement membrane and surrounding stromal compartment, they will reach the newly formed tumor-associated microvasculature formed through the angiogenesis process. Due to the highly fenestrated nature of these vessels, tumor cells can enter the blood vessels through the event known as intravasation (24). Tumors cells will bind to endothelial cells inducing endothelial cell retracting and tumor cell entrance into cardiovascular circulation (24). Additionally, tumor cells may enter circulation through the lymphatic system. Due to the lack of tight junctions between endothelial cells in the lymphatic system, as opposed to the cardiovascular system, tumor cells may pass more easily through lymphatic endothelial cells (8). Once in cardiovascular or lymphatic circulation, tumor cells must survive by evading immune system destruction. One mechanism of survival is achieved through tumor cell clumping with other cells to help to create a physical barrier from the immune system (25). This clumping can occur with other cancer cells or with cells of the circulation system, such as blood platelets (24). At distant sites within the body, the cancer cells arrest in capillary beds, bind to endothelial cells and enter surrounding tissue in the process of extravasation (26). Metastasized cells may undergo apoptosis, however the few that survive can lay dormant for an extended length of time or proliferate immediately establishing a secondary tumor site (27). Similar to primary tumor growth, the secondary tumor requires angiogenesis to supply the growing tumor

and may act as a barrier to secondary tumor proliferation if angiogenesis cannot be induced (27). Once secondary tumors are established successful medical intervention is limited and the lethal effects of cancer result. It is proposed that if a cancer cell cannot complete any step of the metastatic cascade, metastasis will be inhibited. However, due to the number of complementary molecular signaling pathways that are involved in each step of metastasis, identifying effective therapeutic targets remains a challenge (8).

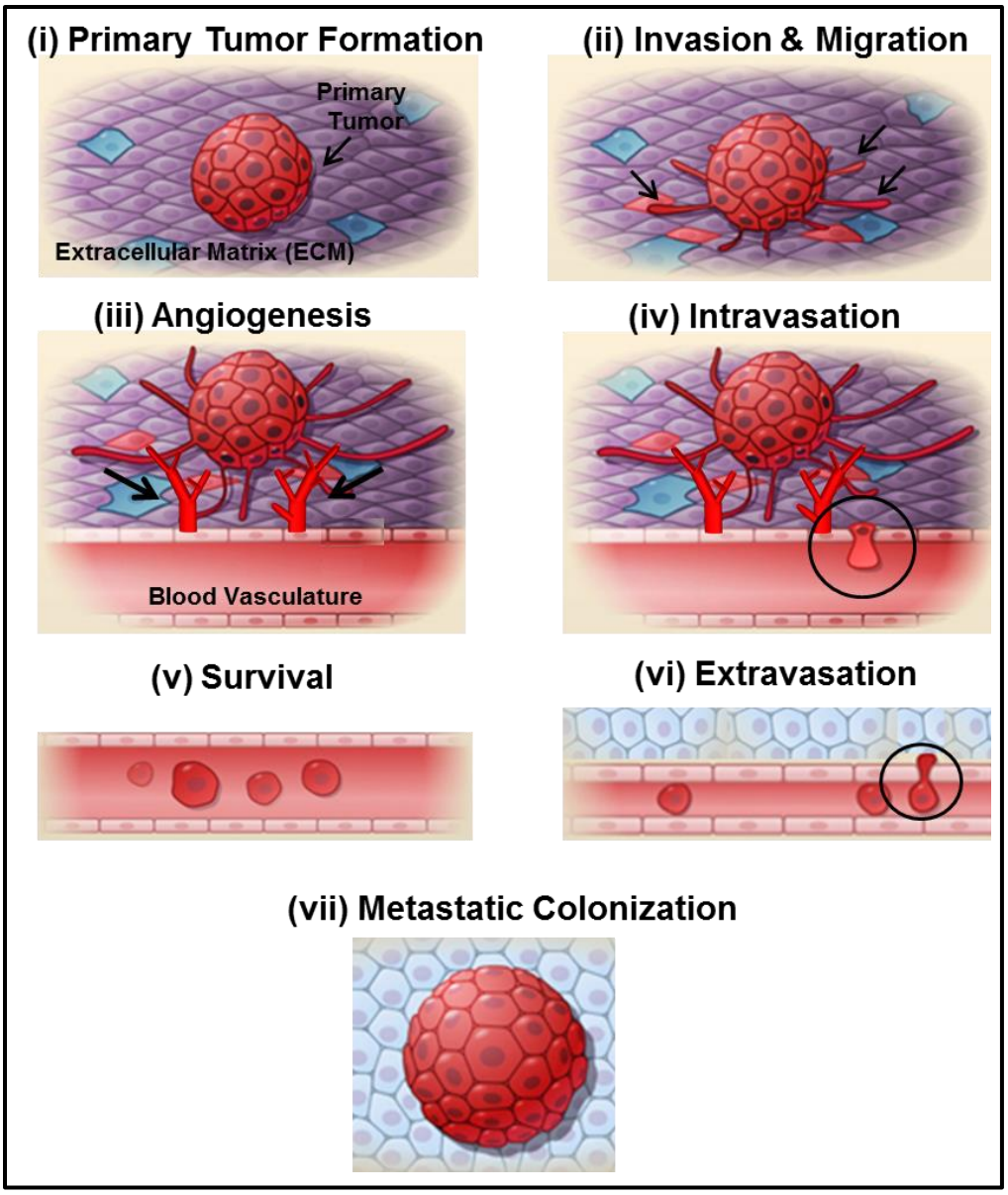

Figure 1.2. The metastatic cascade. In order for cancer cells to spread to distant tissues, they must undergo several events that make up the metastatic cascade. The initial step is the formation of the primary tumor through proliferation of cancer cells (i). As the tumor progresses, some cancer cells will leave the primary tumor through invasion and migration into the surrounding tissue (ii). Tumor cells stimulate the formation of new blood vessels enabling tumor growth (iii). Eventually, some cancer cells will enter blood vessels through intravasation (iv) and survive in circulation (v). At distant tissues some cancer cells will leave blood vessels through extravasation (vi) and colonize at secondary sites (vii). *Adapted from Valastyan et al. (2011)* (28)

1.2.2 Invadopodia

The ability of cancer cells to metastasize is dependent on the formation of the cellular structure known as invadopodia. Invadopodia are actin rich membrane protrusions formed on cancer cell surfaces that contribute to invasion through focal delivery of proteases (29). Structurally, invadopodia are recognized as punctate actin structures on the ventral surface of cells with co-localization of cortactin and associated underlying degradation (30). *In vivo* evidence demonstrates that invadopodia formation is directly involved in primary tumor invasion of the underlying basement membrane and ECM (31, 32). Furthermore, when invadopodia formation is inhibited, cancer metastasis is inhibited through the reduction of cancer cell intravasation and extravasation (31, 32).

Invadopodia formation occurs through several stages from initial formation to final maturity. Each stage of invadopodia is dependent on the coordinated activation and localization of several proteins (Figure 1.3). Invadopodia formation is stimulated by various growth factors, such as the epidermal growth factor, leading to the invadopodia precursor organization (29, 33, 34). The invadopodia precursors are involved with binding to actin and inducing actin polymerization forming the invadopodia structure (35). The first major invadopodia precursor involved is the actin binding protein known as cofilin, which functions to cleave actin filaments to create new barbed end actin formations (36). Theses actin barbed end formations provide sites for actin polymerization and invadopodia extension (36). Two primary mechanisms of cofilin regulation have been proposed. The first mechanism involves blocking the binding of cofilin to actin *via*

serine phosphorylation at residue 3 by LIM-kinase 1 (37). The second mechanism is blocking cofilin's ability to bind to actin through the binding of phosphorylated cortactin to cofilin (38). The second major invadopodia precursor is cortactin. Cortactin's function is to promote invadopodia formation through actin binding and complexing with Arp2/3-N-WASP-dynamin, to promote actin polymerization and invadopodia protrusion (32). Cortactin is a major Src substrate where several tyrosine residues act as on/off switches for cortactin activity. Src phosphorylation at residues Y421, Y466, and Y482 act as an inhibitory mechanism by inhibiting the interaction and activation of N-WASP (39, 40). When the mechanism of cofilin and cortactin control goes unchecked, invadopodia formation is favored, promoting cancer cell invasion and metastasis (41). Finally, invadopodia maturation occurs when the invadopodia acquire its matrix degradation ability.

The invasive ability of invadopodia is mediated by the membrane bound matrix degradation protein, MT1-MMP. An accumulation of MT1-MMP occurs in invadopodia which in turn degrades the extracellular matrix (ECM) and basement membrane components including fibronection, laminins 1 and 5 and collagen I, II, and III (42). *In vitro* studies have demonstrated that MT1-MMP is required for cancer cell migration and invasion through Matrigel, an ECM mimetic (43). Furthermore, MT1-MMP, as opposed to MMP-2 or MMP-9, is responsible for invasion of cancer cells through the basement membrane, establishing an essential role for MT1-MMP in the initiation of metastasis (44). Upon invadopodia stimulation and formation, newly synthesized or recycled MT1-MMP molecules

are delivered to the invadopodia tip. The scaffolding protein IQGAP1, which provides a link between microtubule and actin cytoskeleton networks, coordinates vesicle-tethering of the exocyst complex for MT1-MMP surface delivery (45, 46). Upon activation of small GTPase RhoA, IQGAP1 complexes with the exocyst vessel containing MT1-MMP providing focal degradation (45). Whether MT1-MMP activity can be stimulated by receptors is unknown and is a focus of this study.

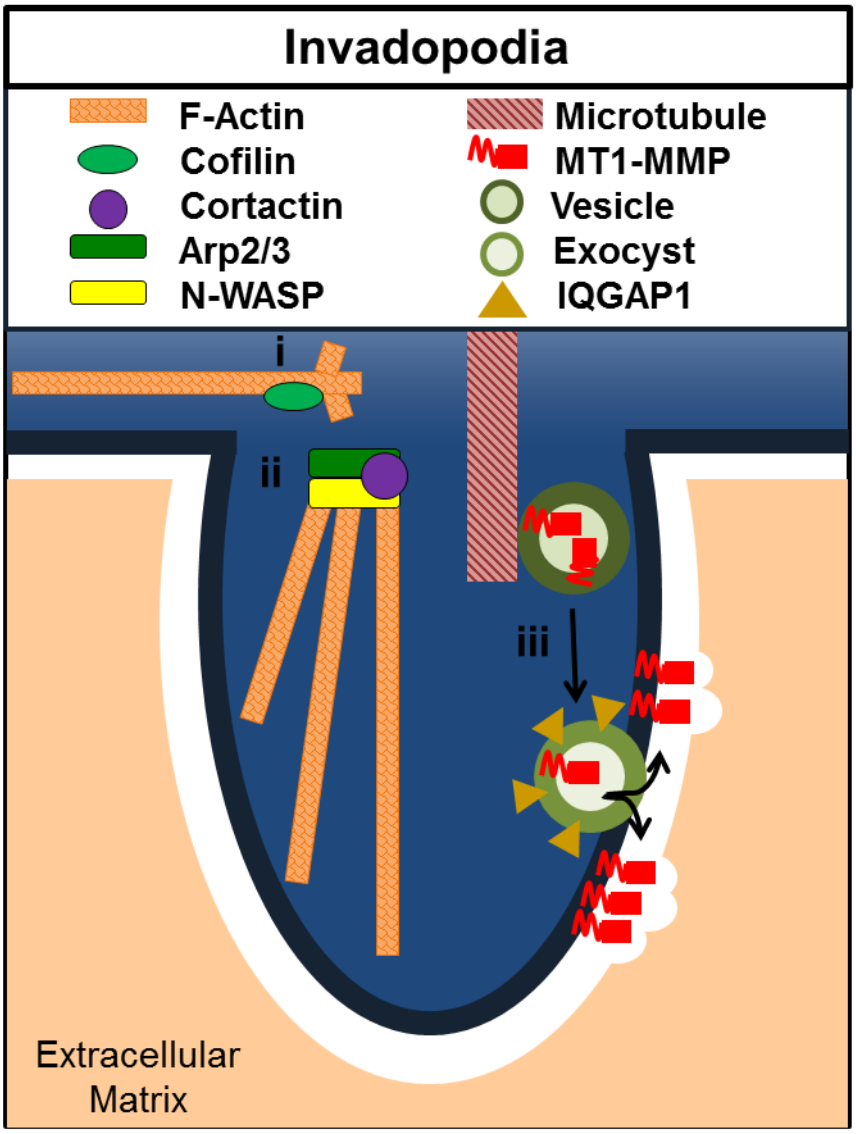

Figure 1.3. Invadopodia formation and activity. Growth factor stimulation induces invadopodia formation through invadopodia precursor organization. Cofilin initiates actin polymerization by severing actin filaments to generate free actin barbed ends (i). Cortactin binds to actin filaments at actin barbed end formations and favors actin polymerization by recruiting the N-WASP Arp2/3 complex (ii). MT1-MMP is transported to the invadopodia tip by microtubule vesicle trafficking and IQGAP1 coordinates exocyst release of MT1-MMP onto the invadopodia surface (iii). MT1-MMP causes extracellular matrix degradation facilitating cellular invasion.

1.3.1 Metastasis Suppressor Genes

Breast tumorgenesis, the transformation of non-malignant cells to cancer cells, begins in breast cells of varying types and due to either genetic or environmental factors inducing a malignant change (47). The cancer cells exhibit specific characteristics not seen in non-malignant cells such as loss of differentiation, loss of apoptotic signaling, promotion of angiogenesis, loss of cell-cell contact, evasion of host immune system, and immortalization (47). It has been proposed that cancer arises from the accumulation of mutations in genes that affect key cellular pathways involved in growth and development leading malignant capacity (48). These alterations can occur within malignant cells or within host cells that surround malignancy such as vascular or immune cells (47, 49). Recently, a new classification of genes have been discovered which are involved in tumor progression as opposed to tumor initiation. These genes are known as metastasis activator and metastasis suppressor genes. Unlike oncogenes or tumor suppressor genes, metastasis activator or suppressor genes do not affect the primary tumor tumorgenicity, but instead are involved in the ability of cancer cells to metastasize to distant tissue areas (50). Several metastatic suppressor genes have been identified whose expression have shown to disrupt the metastatic cascade, rendering metastatic cells non-metastatic. A few examples are *NME1, MKK4, KAI1, E-cadherin,* and *KISS1* (51). Due their ability to inhibit metastasis without blocking primary tumor growth, metastasis suppresser genes provide a promising option for the development of therapeutic strategies in the prevention of metastatic disease.

1.3.2 KISS1 and KISS1R Discovery

KISS1, a metastasis suppressor gene, was originally identified by Lee and colleagues in 1996 (50). To investigate what governs the ability of cells to undergo metastasis, Lee and colleagues used subtractive hybridization to determine the gene expression differences between two melanoma cell lines, the non-metastatic neo6/C8161.1 and metastatic cells C8161. Lee and colleagues discovered that an unidentified cDNA sequence encoding for a 164 amino acid protein was amplified in the non-metastatic neo6/C8161.1 cell line in comparison to the metastatic C8161 cell line (50). This unknown cDNA was termed *KISS1* gene. Since then, studies have shown *KISS1* mRNA expression in the placenta, testis, brain, pancreas, liver, ovaries, small intestine, heart, skeletal muscle, breasts, kidneys, and lungs (52, 53). *KISS1* gene encodes a 145 amino acid peptide that is cleaved to 10, 13, 14, or 54 amino acid lengths (53, 54). These secreted proteins are called kisspeptins (KP) and the smallest active form KP-10, is comprised of the amino acid sequence H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2 (55). This sequence is conserved over an assortment of species, differing from the human sequence by a single amino acid in sheep, mouse, rat, and cow (56). Using saturation binding experiments to kisspeptin receptor (KISS1R), KP-10 exhibits a K_D of 1.0 ± 0.1 nM (52, 53, 56). Additionally, KP-10 presents higher potency than KP-54 (EC_{50} 5.47 ± 0.03 nM), KP-14 (EC_{50} 7.22 ±0.07 nM), or KP-13 (EC_{50} 4.62 ± 0.02nM), with an EC_{50} of 4.13 ± 0.02 nM (52, 53, 56).

The kisspeptin receptor (KISS1R; formerly known as AXOR12, GPR54 or hOT7T175) was originally identified as orphan receptor GPR54 by three independent groups (52, 53, 57). Placental tissue extract was used to purify peptides to stimulate biological activity of GPR54 and mass spectrometry revealed these to be *KISS1* gene products, kisspeptin (53). *KISS1R* expression as determined using reverse transcription polymerase chain reaction was found in breast, ovary, placenta, pituitary, spinal cord, pancreas, thymus, stomach, kidney, small intestine, lung, testis, and brain regions (52, 53, 57).

1.3.3 KISS1R Signaling

KISS1R is a G protein-coupled receptor (GPCR) that signals through the $G_{q/11}$-coupled pathway leading to activation of phospholipase C (PLC) (Figure 1.4). PLC signaling in turns causes an increase in inositol 1,4,5-trisphosphate (IP3) and diacylglycerol formation, an increase in intracellular calcium, and activation of protein kinase C and extracellular signal-regulated kinase (ERK) 1/2 (52, 53, 57). Additionally, KISS1R has been shown to signal through a G-protein independent pathway via β-arrestin2, leading to activation of extracellular signal-regulated kinase 1/2 (ERK1/2) (58). KISS1R is able to constitutively associate with β-arrestin1 and β-arrestin2, through residues in the second intracellular loop and cytoplasmic tail of KISS1R (59). It has been shown that β-arrestin-1 can inhibit KISS1R signaling whereas β-arrestin-2 stimulates KISS1R signaling to ERK1/2 (60). With the diverse ability of KISS1R to induce cellular signaling, understanding the effects of KISS1R signaling is necessary to determine if KISS1R is a potential therapeutic target in breast cancer.

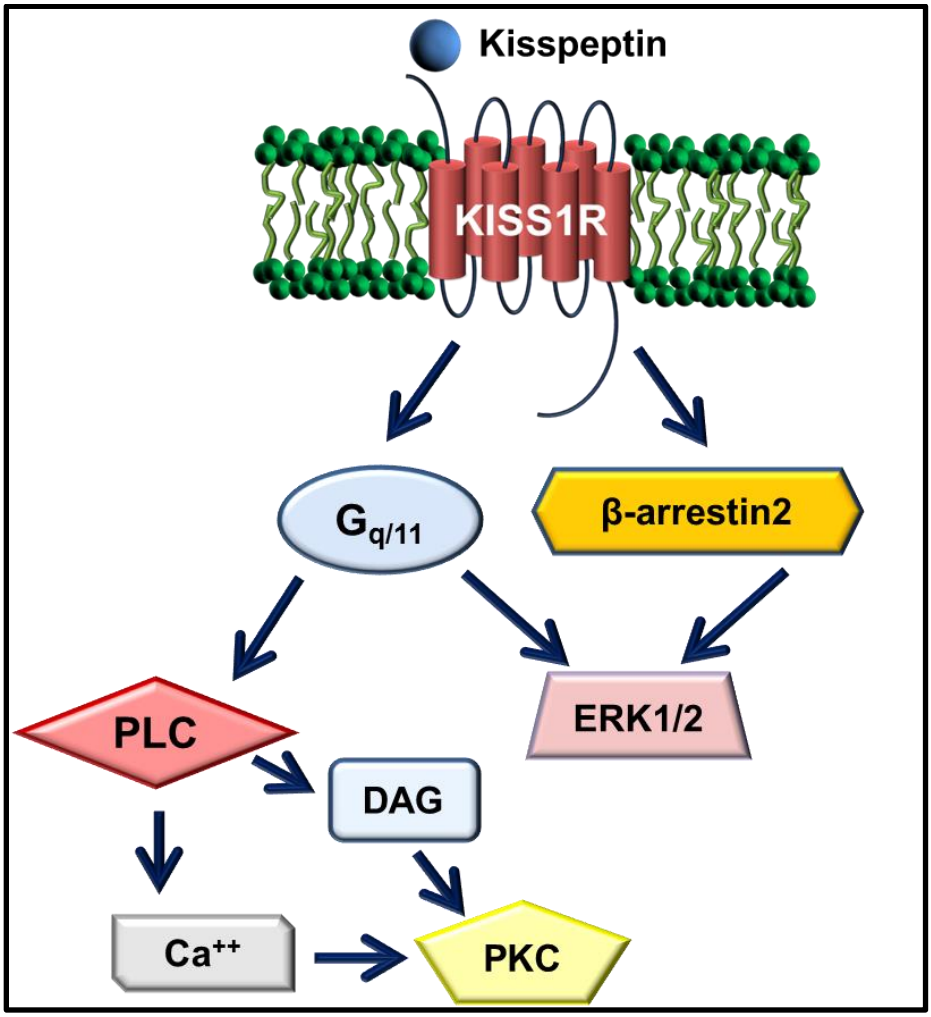

Figure 1.4. KISS1R signaling pathways. KISS1R is a $G_{q/11}$ coupled receptor which activation leads to extracellular signal-regulated kinases (ERK1/2) activation and phospholipase C (PLC) stimulation, calcium mobilization and protein kinase C (PKC) activity. KISS1R activation can also signal through a G protein independent pathway *via* β-arrestin2 mediated ERK1/2 activity.

1.3.4 Physiological Roles of KISS1R

KISS1R signaling plays a significant role in initiation of puberty as defined by sexual development, increased bodily growth, and adrenal maturation. Seminara and colleagues first discovered that a homozygous mutation, L148S, in KISS1R caused autosomal recessive idiopathic hypogonadotropic hypogonadism in humans (61). Neuroendocrine research has demonstrated that secretion of kisspeptin, resulting in KISS1R signaling, conveys control of the reproductive axis by regulating gonadotropin-releasing hormone (GnRH) release (61-66). Kisspeptin is secreted in a pulsatile fashion from the arcuate nucleus of the hypothalamus (67). The kisspeptins in turn act on KISS1R in the median eminence to cause the release of GnRH into the hypophyseal portal which is then delivered to and acts on anterior pituitary to cause the release of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (67). Mice with mutations of the *Kiss1r* or *Kiss1* gene fail to achieve puberty establishing an essential role for KISS1R signaling during puberty development (61). Furthermore, *Kiss1r*-null mice demonstrate a decrease in LH and FSH serum levels suggesting a crucial role for KISS1R in LH and FSH secretion (61-66). Further studies using *KISS1*-null mice demonstrated that LH and FSH serum levels could be recovered through subcutaneous injections of kisspeptin-54 which resulted in GnRH release and puberty initiation (66). Finally, studies using the KISS1R antagonist (P-234) in pre-pubertal rats demonstrated that central injection of the antagonist could inhibit kisspeptin mediated signaling and delay puberty (68). Thus, research in kisspeptin signaling has established that KISS1R

activity is the major regulator of pubertal onset and the major regular of the hypothalamic-pituitary-gonadal (HPG) axis in several species including humans.

Additionally, kisspeptin/ KISS1R signaling plays an important role in pregnancy and placentation. High levels of *KISS1* and *KISS1R* gene expression is found in the human placenta (50, 52, 53, 56, 57). During pregnancy, kisspeptin and KISS1R expression decreases from first trimester to the third, which correlates with decreased placental tissue invasion (69, 70). Conversely, circulating kisspeptin concentrations are increased 1000 fold during the first trimester and increases to 10,000 fold by the third trimester (69). The varying expression levels during pregnancy suggest a regulatory role of trophoblastic invasion during placentation. *In vitro* studies have demonstrated that kisspeptin-10, the kisspeptin splice variant found in the placenta, inhibits migration of trophoblastic cells and decreases MMP expression which is required from placental invasion into the uterine walls (71). Furthermore, studies have revealed an important role for kisspeptin/ KISS1R signaling in maternal artery formation, required for proper fetal development (71).

1.3.5 KISS1R Signaling In Cancer

The *KISS1* gene was originally identified by Lee and colleagues in 1996 as metastasis suppressor gene in melanoma (50). Since this original discovery, kisspeptin/KISS1R signaling has been further identified to suppress metastasis in bladder (72), esophageal (73), lung (74), thyroid (75, 76), gastric (77, 78), ovarian (79-82), and pancreatic (83) cancers. In these cancers, *KISS1R*

expression was found to decrease in cancer cells compared to non-malignant tissue which correlates with poor prognosis in patients. The first study which examined *KISS1* signaling in melanoma found that *KISS1* mRNA expression was found only in non-metastatic melanoma cell lines and absent in metastatic melanoma cells. Furthermore, when *KISS1* was overexpressed, melanoma cell motility was decreased and metastasis was suppressed in an expression-dependent manner (50). A second study investigated *KISS1* expression in various stages of melanocytic tumors found an inverse correlation of *KISS1* expression and melanoma metastases (60). When examination of pancreatic cancer tissues were performed, a lower expression *KISS1* mRNA was observed in pancreatic cancer as compared to non-malignant tissue (83). Likewise, when exogenous *KISS1* gene was added to pancreatic cancer cells, a reduced migration of pancreatic cancer cells occurred while having no effect on cellular proliferation (83). In gastric cancer, *KISS1* mRNA expression was reduced in patients that had lymph node and liver metastases (77). Additionally, the lymph node and liver metastases demonstrated a reduction of kisspeptin protein expression as compared to the primary gastric tumor (78). Furthermore, KISS1R activity in gastric cancer decreases MMP-9 activity, inhibiting invasion, as well as increasing the tissue inhibitor of metalloprotease (TIMP)-1 (53, 77, 78). In ovarian carcinoma (79-82), esophageal squamous cell carcinoma (73), and bladder carcinoma (72), decrease in *KISS1* and *KISS1R* expression was determined to be a strong prognostic factor for lymph node metastasis.

1.3.6 KISS1R Signaling in Breast Cancer

In contrast to its metastasis suppressor role in many cancers, studies indicate that kisspeptin/KISS1R signaling induces a detrimental outcome in breast cancer. In 2005, the first clinical study focusing on kisspeptin signaling in breast cancer was carried out. Using quantitative-PCR analysis, Martin and colleagues measured *KISS1* and *KISS1R* mRNA expression in breast tumor tissue. An observation of *KISS1* mRNA expression was seen to be increased in breast cancer tissue compared to non-malignant mammary tissue (7). Furthermore, the breast tumors that had lymph node metastasis demonstrated an increased *KISS1* mRNA levels in comparison to lymph node negative breast tumors (7). However, *KISS1R* mRNA expression was not significantly different between breast tumors and non-malignant tissue or breast tumors that were lymph node positive or negative for metastases (7). Additionally, overexpression of *KISS1* gene in the human breast cancer MDA-MB-231 cells increased cell invasiveness and decreased cellular adhesion (7). Finally, this study reported that *KISS1* mRNA expression correlated with poorer prognosis suggesting that *KISS1* gene expression could be used as a prognostic marker in breast cancer patients (7).

In 2007, Marot and colleagues reported that estrogen status of breast tumors was correlated to *KISS1* mRNA expression. This study observed that estrogen receptor (ER) α-positive primary breast tumors expressed sevenfold less *KISS1* levels as compared to ERα negative breast tumors (84). Furthermore, post-menopausal women with ERα positive breast tumors that were treated with

the estrogen antagonist, tamoxifen, had higher expression of both *KISS1* and *KISS1R* mRNA tumor levels (84). This elevation in *KISS1* and *KISS1R* mRNA tumor levels correlated with reduced relapse-free survival (84). Moreover, *KISS1* mRNA expression was found to increase with breast tumor grade (84). To further evaluate the links between ERα status and *KISS1* expression, this study used ectopic re-expression ERα in the ERα-negative MDA-MB-231 breast cancer cell line and a corresponding reduction in *KISS1* mRNA expression was observed (84). Likewise, tamoxifen treatment in the ERα-positive MCF7 and T47D breast cancer cells resulted in an increased expression of *KISS1* and *KISS1R* (84). This study concluded that *KISS1* and *KISS1R* mRNA levels in breast tumors could be a marker of tumoral resistance to anti-estrogen treatment. Thus, clinical results report that kisspeptin/KISS1R signaling may play an important role in breast cancer progression; however the mechanism by which this occurs remains elusive.

Presently, a single study has attempted to investigate a role for kisspeptin/ KISS1R signaling in the regulation of breast cancer metastasis. Cho and colleagues (2011) used a *Kiss1r* haploinsufficiency mouse model with the polyoma virus middle T antigen (PyMT) under the control of the mouse mammary tumor virus (MMTV) promoter (MMTV-PyMT) to investigate the effects of *Kiss1r* expression on breast cancer establishment and metastasis. The MMTV-PyMT mice model enables mammary epithelium transformation into multifocal mammary adenocarcinoma and lymphatic/lung metastasis (85). This study demonstrated that loss of Kiss1r expression in *Kiss1r* heterozygotic PyMT-

Kiss1r$^{+/-}$ mice attenuated breast tumor initiation, growth, latency, multiplicity and lung metastasis as compared to the homozygotic PyMT-*Kiss1r*$^{+/+}$ mice (86). To assess the mechanisms by which Kiss1r regulated metastasis, the authors extracted cells from the primary breast tumors of the PyMT-*Kiss1r*$^{+/-}$ and PyMT-*Kiss1r*$^{+/+}$ mice for *in vitro* analysis. Kisspeptin-10 stimulation of Kiss1r in the extracted cell cultures resulted in small GTPAse RhoA activation through a Gαq pathway (86). Interestingly, this study also observed that decreased *Kiss1r* expression correlated with a significant reduction in *VEGF* and *MMP-9* mRNA suggesting a possible role for Kiss1r in both angiogenesis and breast cancer invasion (86). In conclusion, this study suggests kisspeptin/Kiss1r signaling via RhoA is required for breast cancer metastasis in mouse models.

Studies from our laboratory have provided some insight on the mechanism by which KISS1R stimulates breast cancer cell migration and invasion, two processes required for metastasis (87, 88). Our first study found that kisspeptin-10 stimulation led to significant increase in MMP-9 secretion and activity in MDA-MB-231 breast cancer cells suggesting a mechanism of breast cancer invasion (88). Most interestingly, we found that kisspeptin-10 induced EGFR transactivation in a β-arrestin2 dependent mechanism in ERα-negative breast cancer cells (88). Furthermore, FRET analysis revealed that KISS1R interacts directly with EGFR under basal conditions (88). We also found that KISS1R expression or kisspeptin-10 treatment stimulates EMT of ERα negative MCF10 breast epithelial cells through the increase in N-cadherin and Snail/Slug expression resulting in increased cell invasiveness (87). Furthermore,

overexpression of exogenous KISS1R in ERα-negative SKBR3 breast cancer cells induced extravasation *in vivo* using the chick chorioallantoic membrane (CAM) assay (87). However, kisspeptin-10 treatment failed to induce invasiveness or EGFR transactivation in the ERα-positive MCF7 and T47D breast cancer cells suggesting that ERα negatively regulates KISS1R signaling in breast cancer (87). To confirm this, exogenous ERα was expressed in MDA-MB-231 cells which resulted in decreased KISS1R expression and inhibited kisspepin-10 mediated effects on EGFR transactivation and cell invasion (87). Finally, the actin cytoskeletal binding protein, IQGAP1, was found to be a novel binding partner of KISS1R which regulated kisspeptin-10 induced EGFR transactivation (Figure 1.5) (87). However, whether human KISS1R signaling regulates breast cancer metastasis is unknown and is a focus of this thesis.

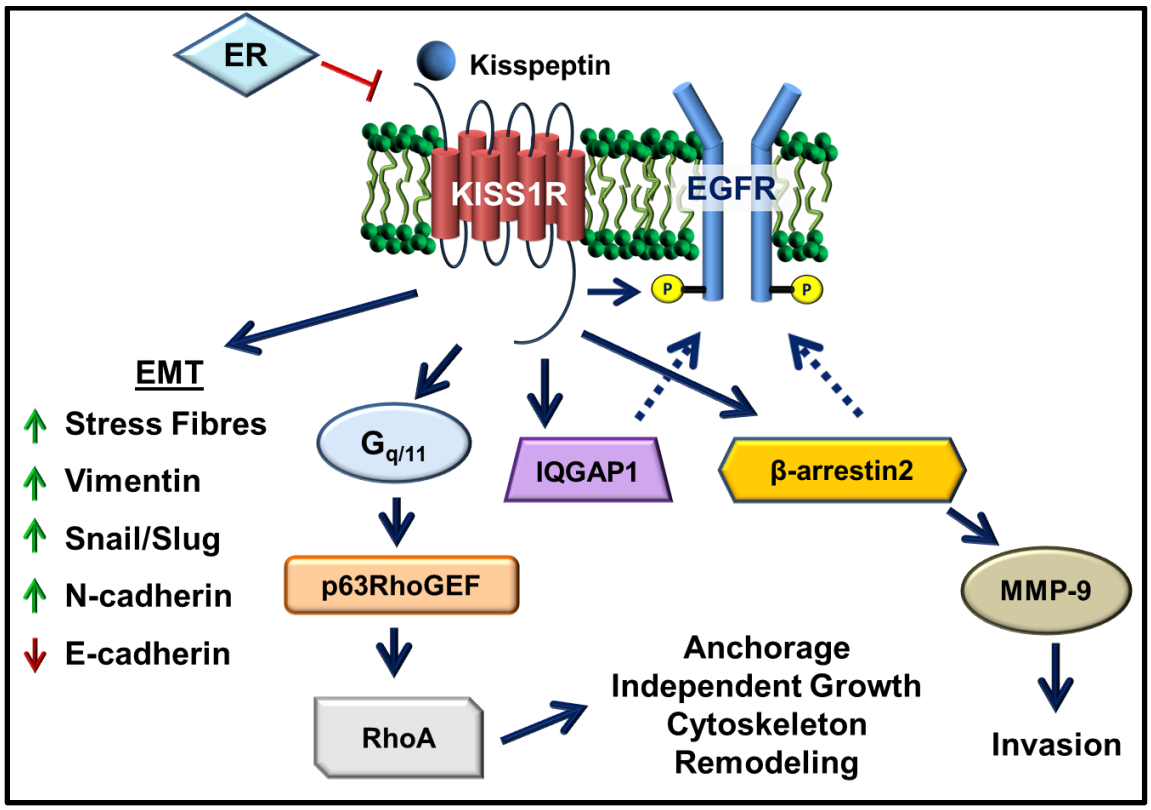

Figure 1.5. KISS1R signaling pathways in breast cancer. (i) Kisspeptin leads to KISS1R activation causing transactivation of epidermal growth factor (EGFR) *via* β-arrestin2 and IQGAP1 mediated mechanisms. (ii) KISS1R activation leads to epithelial-mesenchymal transition (EMT) causing a mesenchymal phenotype. (iii) Likewise, KISS1R activation leads to RhoA anchorage independent growth and cytoskeletal remodeling. (iv) Estrogen receptor, ERα, down regulates KISS1R expression in breast cancer cells. *Adapted from Cvetkovic et al. 2013* (89).

1.4.1 β-arrestins

β-arrestin1 and β-arrestin2 are two isoforms of 78% identical amino acid sequence which have demonstrated a diverse set of roles in cells (90). Studies have demonstrated abundant expression of β-arrestins in numerous cell types and are involved in regulating receptor desensitization, internalization and signaling (91-95). The β-arrestin structure consists of two antiparallel β-sheet domains with a connecting hinge region and an α-helix amino-terminal tail which conveys cellular localization (96, 97). Additionally, the hydrophobic core interacts with phosphorylated residues, important in the interactions of β-arrestins and GPCRs. Under basal conditions, β-arrestins are phosphorylated on serine 412. Upon activation, dephosphorylation of serine 412 enables β-arrestin binding to other proteins such as Src (99).

Originally, β-arrestins were discovered to be a regulator of GPCR desensitization and internalization. Desensitization and internalization are mechanisms by which receptor signaling leads to receptor internalization and directed to either the endosome for receptor recycling or to the lysosome for destruction (98, 99). During receptor internalization, β-arrestins function in coordinated effort with GPCR kinases (GRKs) to bind to the phosphorylated receptor being internalized (100). Desensitization is thought to be an adaptive response in order to prevent potential detrimental effects from continuous receptor stimulation (101). By dictating receptor recycling or destruction, internalization provides control of the duration and intensity of GPCR signaling by regulating the number of receptors available on the cellular surface for ligand

binding. Furthermore, the binding of β-arrestin to GPCRs inhibits receptor coupling to heterotrimeric G proteins, thus limiting G protein mediated signaling. However, receptor bound β-arrestin can mediate signaling, providing a G protein independent signaling cascade (102). β-arrestin acts as molecular scaffold by bringing signaling molecules in proximity to each other; these signaling proteins include, but are not limited to, Src, ERK1/2, and AKT (97, 103).

1.4.2 β-arrestin and Breast Cancer

β-arrestins are emerging as key regulators of tumorgenesis and metastasis. In breast cancer, β-arrestins have been shown to regulate breast cancer growth and proliferation (104). Depletion of β-arrestin1 expression in MCF-7 breast cancer cells inhibits ERK1/2 and c-JUN activity resulting in decreased cellular proliferation; thus, implicating a role for β-arrestins in the regulation of breast cancer cell growth (104). Additionally, β-arrestins regulate breast cancer migration and invasion (105-107). For instance, protease activated receptor 2 (PAR2) signaling in MDA-MB-231 human breast cancer cells induced cell migration through a β-arrestin dependent ERK1/2 mechanism (107). Likewise, β-arrestin expression is required for LPA_1-stimulated migration of MDA-MB-231 cells via small GTPase Ral and Rap (106). One proposed mechanism by which β-arrestin regulates cancer cell migration involves the interaction of β-arrestin with the actin filament-severing protein, cofilin. Activation of PAR2 in MDA-MB-468 breast cancer cells leads to β-arrestin1 and β-arrestin2 binding to cofilin and co-localization to the leading edge of migrating cells (108). Furthermore, PAR2 signaling through cofilin is necessary for actin cytoskeleton reorganization and

chemotaxis (109). Additionally, β-arrestins inhibit LIM kinase, the kinase that inhibites cofilin activation through serine phosphorylation (108). Moreover, global proteomics analysis has demonstrated that β-arrestins interact directly with cofilin and cortactin, two important proteins that regulate invadopodia formation (110).

Beyond primary growth, β-arrestins have also been implicated in the regulation of breast cancer metastasis. In 2011, Lundgren and colleagues investigated the expression of β-arrestin1 in clinical breast cancer samples. This study reported that elevated stromal β-arrestin1 correlated with poor patient survival (111). Further analysis revealed β-arrestin1 expression to positively correlate with tumor size, lymph node status, proliferation and distant metastases (111). Furthermore, this study showed that elevated stromal β-arrestin1 was linked to increased HIF-1α expression, suggesting a role for β-arrestin1 in angiogenesis (111). Thus, this study suggests that β-arrestin1 expression is a negative prognostic marker of patient outcome. A second study by Shenoy and colleagues (2012), examined β-arrestin1 protein levels in non-malignant and breast cancer tissues. Infiltrating ductal carcinoma tissue demonstrated elevated levels of β-arrestin1 in comparison to non-malignant breast tissue suggesting elevated β-arrestin1 correlates with breast cancer progression (112). Furthermore, this study used tail vein injection of MDA-MB-231 breast cancer cells in nude mice to examine the roles of β-arrestin1 and β-arrestin2 in breast cancer metastasis. Upon depletion of both β-arrestin1 and β-arrestin2, tumor growth was delayed and mouse survival increased, suggesting a role for β-arrestins in breast cancer metastasis (112). Moreover, this study found that β-

arrestin1 interacts with HIF-1α and controls HIF-1α nuclear trafficking suggesting a role for β-arrestin1 regulation of nuclear signaling under hypoxic conditions (112). Likewise, analysis of invasive human breast cancer samples revealed that increased β-arrestin1 expression correlates with increased levels of VEGF expression (112). Hence, this study suggests that β-arrestin1 regulates breast cancer survival and metastatic progression through regulation of pro-angiogenic factor expression and nuclear signalling.

1.5.1 Rationale

Clinical studies have established a potential role for KISS1R in breast cancer progression and metastasis (7, 84). Examination of *KISS1* and *KISS1R* expression in breast cancer has revealed that *KISS1* expression is elevated in human breast cancer tissues and correlates with increased tumor grade and lymph node metastasis (7). Furthermore, patients with high *KISS1* and *KISS1R* mRNA expression had shorter relapse-free survival (84). Currently, the only *in vivo* study examining the role of KISS1R in breast cancer demonstrated that decreased mouse Kiss1r expression attenuated breast tumor initiation, growth, latency and metastasis in MMTV-PyMT/Kiss1r mouse models (86). *However, a role for KISS1R signaling in regulation of human breast cancer metastasis remains unknown and will be investigated in this study.* In our laboratory, our earlier studies have shown that overexpression of exogenous KISS1R in ERα-negative SKBR3 breast cancer cells increased cellular invasion and induced extravasation as revealed using the CAM assay (87). Additionally, we have found that kisspeptin stimulation of ER-negative breast cancer cells leads to increased

breast cancer cell invasion through kisspeptin-10 EGFR transactivation and increased MMP-9 activity via a β-arrestin2 dependent mechanism (88). β-arrestins have been demonstrated to play a substantial role in breast cancer cell migration and invasion (88, 105, 106). Furthermore, β-arrestin has been established to regulate cofilin activity and bind to cortactin, two important proteins that regulate invadopodia formation. Nevertheless, whether β-arrestins regulate invadopodia formation and whether KISS1R signaling can promote invadopodia is unknown and will be investigated here.

1.5.2 Hypothesis

The depletion of KISS1R expression will inhibit human breast cancer cell metastasis.

1.5.3 Objectives

1) To investigate if KISS1R depletion inhibits human breast cancer metastasis using an orthotopic xenograft mouse model.
2) To determine the underlying mechanisms of KISS1R signaling in breast cancer metastasis and invadopodia formation.

1.6.1 References

† http://www.cancer.ca/en/cancer-information/cancer-type/breast/statistics/?region=bc

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Chapter 2- Methods and Results

2.1 Materials and Methods

Human Cell Culture. Two breast adenocarcinoma cancer cell lines were used for this study; the highly metastatic MDA-MB-231 and moderately invasive Hs578T cell lines (1). The MDA-MB-231 and Hs578T cell lines are estrogen receptor-α negative and endogenously express epidermal growth factor receptor and KISS1R (2, 3). Cell lines (all human, all female) were purchased from ATCC (Manassas, VA) and maintained at 37°C with 5% CO_2. Breast cancer cells, MDA-MB-231, MDA-MB-231 Scrambled control, MDA-MB-231 KISS1R shRNA #1, MDA-MB-231 KISS1R shRNA #2, MDA-MB-231 β-arrestin2 shRNA #1, MDA-MB-231 β-arrestin2 shRNA #2, Hs578T, Hs578T Scrambled control, Hs578T KISS1R shRNA #1, and Hs578T KISS1R shRNA #2 are cultured in RPMI 1640 (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma).

Stable Transfections and Gene Knockdowns. Gene knockdown of KISS1R, β-arrestin 1, β-arrestin 2 in MDA-MB-231 cells and Hs578T cells was achieved as previously described (2-4). HuSH vectors containing gene specific shRNA and scrambled controls (OriGene Technologies) were transfected into cells by electroporation (Gene Pulser Xcell, Bio-Rad), according to the instructions of the manufacturer (250 V, 950 μF). Heterogeneous populations of stable transfectants were selected using media containing puromycin (1.5 ng/mL) and expression of proteins was verified by Western blot analysis.

Spontaneous Metastasis Assay in NOD/SCID/IL2 receptor γ null Mice. To assess the role of KISS1R in regulating breast cancer metastasis, we used an established orthotopic spontaneous metastasis model using NOD/SCID/IL2 receptor γ null immunocompromised mice as described previously (5). MDA-MB-231 cells expressing scrambled control or KISS1R shRNA were resuspended in growth factor-reduced Matrigel (1:1) (1 x 10^6 cells/mice) and injected into the right thoracic mammary fat pad of 6 week old female NOD/SCID-IL2Rγ null mice (5 mice/group). Tumor growth was evaluated by caliper measurement bi-weekly, in three dimensions. At 5 weeks post-injection, mice were sacrificed and primary tumor and the lungs, liver and mammary fat pads harvested. Tissues were collected and fixed in 10% neutral-buffered formalin and embedded in paraffin as performed by the London Laboratory Services Group at the London Health Sciences Centre. Each tissue was sectioned 10 times at 4 μm thickness and the first and final section stained with hematoxylin and eosin for histological analysis and each adjacent section stained using anti-human Ki67 (1:100, Fisher) to measure human cell proliferation or with anti-mouse CD-31 (1:100, BD Bioscience) to measure blood vessel density. Lung metastatic tumor burden was quantified by measuring the number of metastatic tumors per mm^2 and percent of lung area with metastases as assessed in each hematoxylin and eosin stained lung section using Aperio ImageScope software. Primary tumor blood vessel density was determined by counting the total number of blood vessels with CD-31 positive staining and dividing by total tumor cross section area as determined using Aperio ImageScope software. All animal procedures were conducted in

accordance with the recommendations of the Canadian Council on Animal Care, under a protocol approved by the University of Western Ontario Council on Animal Care.

Immunofluorescence Microscopy. Immunostaining was conducted as described previously (2, 3, 6). MDA-MB-231 and Hs578T cells expressing scrambled control or KISS1R shRNA were washed with Hank's Balanced Salt Solution, fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton-X (room temperature, 20min). Phalloidin (1:100, Invitrogen) conjugated to Alexa Fluor (AF) 555 was incubated for 1 h. Nuclei were stained with 0.01% Hoechst 33258 (Invitrogen). Cells were then washed with Hank's Balanced Salt Solution (HBSS) and slides mounted using Immuno Mount (Fisher) and #1 glass coverslip (VWR). Images were acquired using an LSM-510 META laser scanning microscope (Zeiss, Germany).

Immunoblot Assays. MDA-MB-231 and Hs578T cells stably expressing scrambled control, KISS1R shRNA, β-arrestin2 shRNA, or β-arrestin1/2 shRNA were lysed using RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 5 mM EDTA, 1% NP40, 0.1% SDS, 10 mM NaF, 0.5% deoxycholate, 10 mM sodium pyrophosphate, protease inhibitors). Protein (50 µg) was separated by SDS-PAGE and protein expression was examined using either: goat anti-KISS1R (N-20) sc-48220 (1:1000, Santa Cruz Biotechnology), rabbit anti-Snail/Slug (1:500,

Santa Cruz), mouse anti-β-catenin (1:1000, BD), rabbit smooth muscle actin (1:1000, Invitrogen), rabbit anti-laminin V (1:1000 Millipore), anti-β-arrestin1 (1: 300, abcam), anti-β-arrestin2 (1:500, abcam), anti-VEGF (1:200, Santa Cruz), anti-HIF-α (1:1000 Cell Signaling) and visualized by chemiluminescence. Densitometric analysis of protein bands was performed using VersaDoc Imaging System (Bio-Rad). Rabbit β-actin (polyclonal antibody, 1:2000; Sigma) expression was used to control for equal loading of samples.

Scratch Assays for Cell Motility. MDA-MB-231 and Hs578T cells expressing shRNA targeting KISS1R or scrambled control were grown to 100% confluence in a 12-well plate, serum starved in RPMI 1640 media for 24 h, and scratched by a sterile pipette tip as described previously (2, 3). Cells in fetal bovine serum supplemented media were allowed to migrate into the scratch for 18 hours and imaged every 15 minutes using an automated Olympus IX-81 microscope. Distance travelled was measured and analyzed using ImagePro software.

Cell Migration and Invasion Assays. Transwell chamber migration and Matrigel-invasion assays were conducted as described (2-4). Transwell filters (8 μm pores) were placed into a 24-well plate containing either serum-free RPMI 1640 media or media supplemented with 10% fetal bovine serum. Cells were serum-starved for 24 hours. MDA-MB-231 cells and Hs578T (4.0×10^4 cells for migration assay, 1.0×10^5 cells for invasion assay) expressing scrambled shRNA

control or KISS1R shRNA were plated in the upper chamber in either serum-free media or serum-free media supplemented with 100 nM kisspeptin-10 (Phoenix Pharmaceuticals) and incubated for 20 hours. All experiments were conducted with kisspeptin-10 concentration of 100 nM based on dose response studies conducted as described previously (3). For invasion assays, cells were suspended in 1 in 10 dilution of Matrigel (8.0 mg/mL, BD Biosciences) dissolved in serum-free RPMI 1640. Cells were then fixed with a 20% acetone: 80% methanol solution and nuclei stained with 0.1% Hoechst 33258 (Invitrogen). Two replicates were conducted for each condition and 12 fields (migration assay) or 24 fields (invasion assay) in each replicate were chosen and counted using an Olympus IX-71 inverted microscope. Results are presented as a ratio of cells that migrated relative to cells that migrated or invaded in control conditions (cells seeded in serum-free media and migrating towards media with 10% (v/v) fetal bovine serum).

Three-Dimensional Invasion Assays. These experiments were conducted as described previously (2, 3). Matrigel is effective for establishing both malignant and non-malignant breast cell growth, resembling what is occurring in the *in vivo* tumor micro-environment (2, 3, 6, 7). MDA-MB-231 and Hs578T expressing scrambled control or KISS1R shRNA (2.5×10^4 cells/mL) were seeded in a 1:1 dilution of phenol red-free Matrigel and culture media on Matrigel-coated glass-bottomed culture dishes (35 mm, MatTek). Cultures were overlaid with culture media and maintained for five days. Cell colonies were scored blindly as being

either stellate or spheroidal. A colony is deemed to be stellate if one or more projections from the central sphere of a colony of cells are observed. Images were taken with an Olympus IX-81 microscope (Olympus), using InVivo Analyzer Suite (Media Cybernetics).

Anchorage-Independent Growth Assay. This assay was conducted as described (8). One to one dilution of 1.2% agarose (in media) was layered over 3.5 mm wells to form a base. MDA-MB-231 cells (5.0×10^3) expressing scrambled control or KISS1R shRNA were suspended in 1 to 1 dilution of 0.6 % agarose in 10% (v/v) fetal bovine serum supplemented with RPMI 1640 medium and allowed to grow for five weeks to form colonies. Colonies were stained with crystal violet, imaged with Olympus IX-71 inverted microscope and analyzed using ImagePro software (Media Cybernetics).

Invadopodia Formation Assay. To assess the ability of cells to form invadopodia, a gelatin degradation assay was performed using QCM™ Gelatin Invadopodia Assay kit (Millipore), as described by the manufacturer. Briefly, an 8-well chamber slide was coated with poly-L-lysine, followed by glutaraldehyde, and then fluorescent-gelatin. Following gelatin incubation, each well was incubated with RPMI 1640 growth media for 30 minutes to quench residual free aldehydes. Finally, MDA-MB-231 (75,000 cells/cm^2) and Hs578T (60,000 cells/cm^2) cells were plated in RPMI 1640 growth media and incubated at 37°C

with 5% CO_2 for 24 hours. After incubation, cells were treated with 100 nM kisspeptin-10 and/or 1 μM P-234 (KISS1R antagonist) for 30 minutes, or left unstimulated. Following treatment, cells were washed with HBSS, fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton-X (room temperature, 20min) and blocked with 3% bovine serum albumin. The primary antibody for cortactin (anti-cortactin antibody, ab33333, abcam) was incubated for 1 hour. After primary antibody incubation, the following secondary antibodies anti-mouse DyLight 350 (Thermo/Pierce) and TRITC-phalloidin (2 μg/mL) were incubated with the cells for 1 hour. Cells were washed with HBSS and slides mounted using Immuno Mount (Fisher) and #0 coverslip (VWR). Images were acquired using an LSM-510 META laser scanning microscope (Zeiss, Germany).

Co-localization of Proteins in Invadopodia. To assess the distribution and localization of endogenous proteins within invadopodia, the gelatin degradation assays were performed as described above, using fluorescein free gelatin. Briefly, after 30 minutes of 100 nM kisspeptin-10 stimulation, cells were washed with HBSS, fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton-X (room temperature, 20min) and blocked with 3% BSA. The following primary antibodies were incubated for 1 hour; anti-KISS1R (N-20) sc-48220 (1:100, Santa Cruz Biotechnology), anti-cortactin (1:100, ab33333, Abcam), anti-MT1-MMP (1:100, Abcam), anti-β-arrestin 2 (1:50, Santa Cruz Biotechnology), anti-IQGAP1 (1:50, Santa Cruz Biotechnology), or anti-EGFR (1:50, Abcam). Subsequently the cells were incubated for 1 hour with the following secondary

antibodies: anti-rabbit Alexa Fluor (AF) 488 (1:250, Invitrogen), anti-goat AF 555 (1:1000, Invitrogen), anti-mouse DyLight 350 (1:50, Thermo/Pierce) were incubated with the cells for 1 hour. Cells were washed with HBSS and slides mounted using Immuno Mount (Fisher) and #0 coverslip (VWR). Images were acquired using an LSM-510 META laser scanning microscope (Zeiss, Germany).

Cortactin and Cofilin Phosphorylation. MDA-MB-231 cells expressing shRNA targeting either KISS1R or β-arrestin 2 or scrambled control were grown to 70% confluence, serum starved for 24 hours and then treated with 100 nM kisspeptin-10 for either 5 or 15 minutes. Cells were lysed with RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH8, 5 mM EDTA, 1% NP40, 0.1% SDS, 10 mM NaF, 0.5% deoxycholate, 10 mM sodium pyrophosphate, and protease inhibitors) and proteins (50 µg) were separated by SDS-PAGE. The phosphorylation of cortactin (anti-cortactin (phospho Y421) antibody ab47768, Abcam) and cofilin (anti-cofilin (phospho S3 antibody, ab47281, Abcam) was visualized by chemiluminescence. Total cofilin (cofilin 1 (H-12) sc-32158, Santa Cruz) or total cortactin (anti-cortactin antibody ab33333, abcam) expression was used to control for equal loading of samples.

MT1-MMP Phosphorylation. MDA-MB-231 cells were grown to 70% confluence, serum starved for 24 hours, and then treated with 100 nM kisspeptin-10 for either 5 or 15 minutes. Cells were lysed with RIPA-modified lysis buffer

(150 mM NaCl, 50 mM Tris ph8, 5 mM EDTA, 1% NP40, 0.1% SDS, 10 mM NaF, 0.5% deoxycholate, 10 mM sodium pyrophosphate, and protease inhibitors). To immunoprecipitate MT1-MMP, cell lysates (800 µg) were incubated with anti-MT1-MMP (1:200, Abcam) over-night at 4°C. Following antibody incubation, Dynabeads (Life Technologies) were added to lysates with the antibody for 30 minutes at 4°C. Immunoprecipitated proteins were separated by SDS-PAGE and MT1-MMP phosphorylation was determined using a mouse monoclonal anti-phosphotyrosine antibody (PY-20, Santa Cruz). MT1-MMP phosphorylation was normalized to total MT1-MMP expression as determined using a rabbit monoclonal anti-MT1-MMP antibody (1:100, Abcam).

Extravasation In Chick Chorioallantoic Membrane (CAM) Assay. These experiments were performed as previously described (9-12). On day 12 of embryonic development, MDA-MB-231 cells expressing shRNA for either KISS1R, β-arrestin 2, or scrambled control were fluorescently labeled with CellTracker Green CMFDA (Life Technologies) and injected into a vein within an intact chorioallantoic membrane. Cells were pre-treated with 10 nM or 100 nM kisspeptin-10, 1 µM KISS1R antagonist P-234, 1 µM EGFR antagonist AG-1478, or vehicle (acetonitrile) treatment. Immediately after injection, aluminum foil with an inner square (1-cm^2 region of interest (ROI)) was placed on the surface of the CAM for macroscopic imaging. The number of cells that arrested within the capillaries of the ROI were visualized using fluorescence microscopy and enumerated (t=0). At least 150 cells for each ROI at T=0 was analyzed and

enumerated. At 24 hours post injection, the ROI was examined again and cells that extravasated to the stroma were counted allowing percent cellular extravasation to be determined.

MTT cell viability assays. These assays were conducted using cell proliferation kit I (MTT) (Roche) as previously described (2). MDA-MB-231 and Hs578T expressing scrambled control or depleted of KISS1R were seeded in triplicates in a 96 well plate and grown to 70% confluence. MTT labeling reagent (10 µl) was added to each well and cells were incubated for 4 h at 37°C and 5.0% CO_2. Following incubation, 100 µl of solubilization reagent was added to each well and cells were incubated for 24 hours at 37 °C and 5.0% CO_2. Absorbance was then measured at 550 nm using a SpectraMaxM5MultiMode Microplate Reader (Molecular Devices) with a background subtraction at 700 nm.

Cell Growth Assay. To determine the rate of cell growth in monolayer culture, 2.0 x 10^5 MDA-MB-231 and Hs578T cells expressing either scrambled control shRNA or KISS1R shRNA were plated in 6-well culture plates. At 24 hour intervals (24, 48, and 72 hours) cell samples were dissociated using trypsin and the number of cells were determined using a hemocytometer.

Statistical Analysis

One-way analysis of variance (ANOVA) with a Dunnett's post-hoc test or two-way ANOVA followed by Bonferroni post-hoc test was performed using GraphPad Prism 5 (GraphPad Software, Inc.). Differences were considered statistically significant at $p<0.05$.

2.2 Results

KISS1R depletion decreases tumor formation and lung metastasis in xenograft transplantation of human breast cancer cells in mice

The orthotopic xenograft model has been used to effectively study spontaneous metastasis, allowing tumor cells to undergo all the steps of the metastatic cascade (5, 13, 14). Hence, to examine the role of KISS1R in breast cancer metastasis, we used an *in vivo* orthotopic xenograft spontaneous metastasis model in NOD/SCID/IL2 receptor γ null mice. For xenograft transplantation, the human adenocarcinoma breast cancer line MDA-MB-231 was selected due to its highly metastatic characteristics and its endogenous expression of KISS1R (1). Using two different KISS1R shRNA constructs, KISS1R expression was depleted in MDA-MB-231 cells, as verified through western blot (Figure 2.1A) and did not affect cell viability (Figure 2.1B). The three MDA-MB-231 cell populations of scrambled control, KISS1R shRNA #1, and KISS1R shRNA #2 were injected into the right mammary fat pad of 6 week mice (5 mice per cell population). At 5 weeks post-injection, mice were sacrificed and the primary tumor, lung, liver and brain tissues were removed and examined. We

observed that upon depletion of KISS1R, primary tumor volume was reduced in the tumors with cells depleted of KISS1R compared to scrambled control (Figure 2.1C). Furthermore, using hematoxylin and eosin (H&E) staining we observed a reduction of breast cancer metastasis to lung, where area of metastatic burden and the number of metastases was reduced in mice injected with cells depleted of KISS1R compared to control mice (Figure 2.2 A-C). Using anti-human Ki67 antibody, human tumor cell proliferation was visualized within tumors in the lung sections adjacent to the H&E stained lung sections thus confirming the presence of human cell metastases (Figure 2.2A). Hence, our data indicates that KISS1R signaling promotes human breast cancer metastasis *in vivo.*

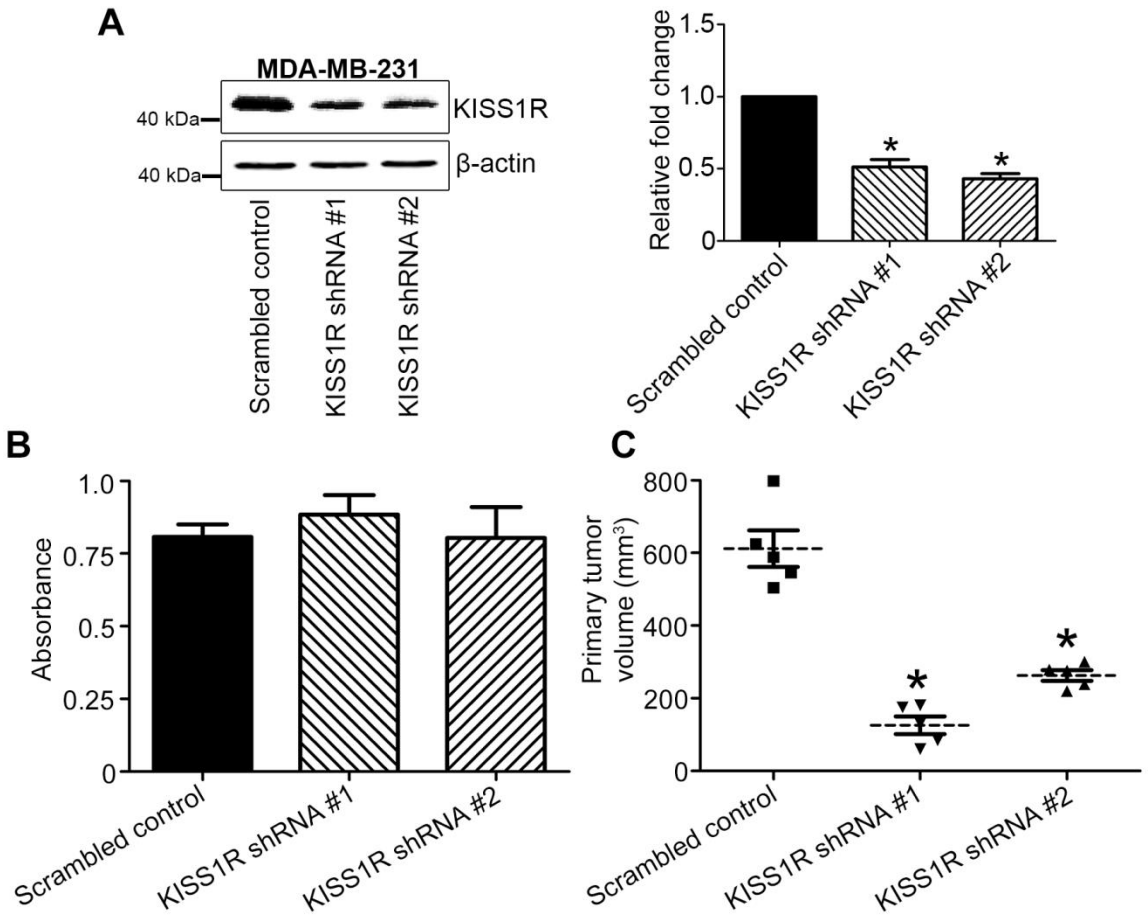

Figure 2.1. KISS1R depletion reduces MDA-MB-231 primary tumor formation *in vivo*. (A) Representative Western blots showing expression levels of endogenous KISS1R MDA-MB-231 in cell populations used for injections. Western blot analysis with goat polyclonal anti-KISS1R (N-20) antibody. Quantification of Western blot expression normalized to β-actin (n=4). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent protein expression ± SEM. (B) MDA-MB-231 cells expressing KISS1R shRNA or scrambled control where cultured in 96 well plate and viability was determined by MTT assay (n=4). Columns represent absorbance measured at 550nm subtract background reading at 700nm, ± SEM. (C) Primary tumors formed over 5 weeks formed within the mammary fat pad of NOD/SCID/IL2Rγ null mice using MDA-MB-231 breast cancer cells expressing scrambled control shRNA or KISS1R shRNA (10^6 cells/mouse; 5 mice/cell population). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Points represent each mouse tumor volume and bars represent mean volume ± SEM.

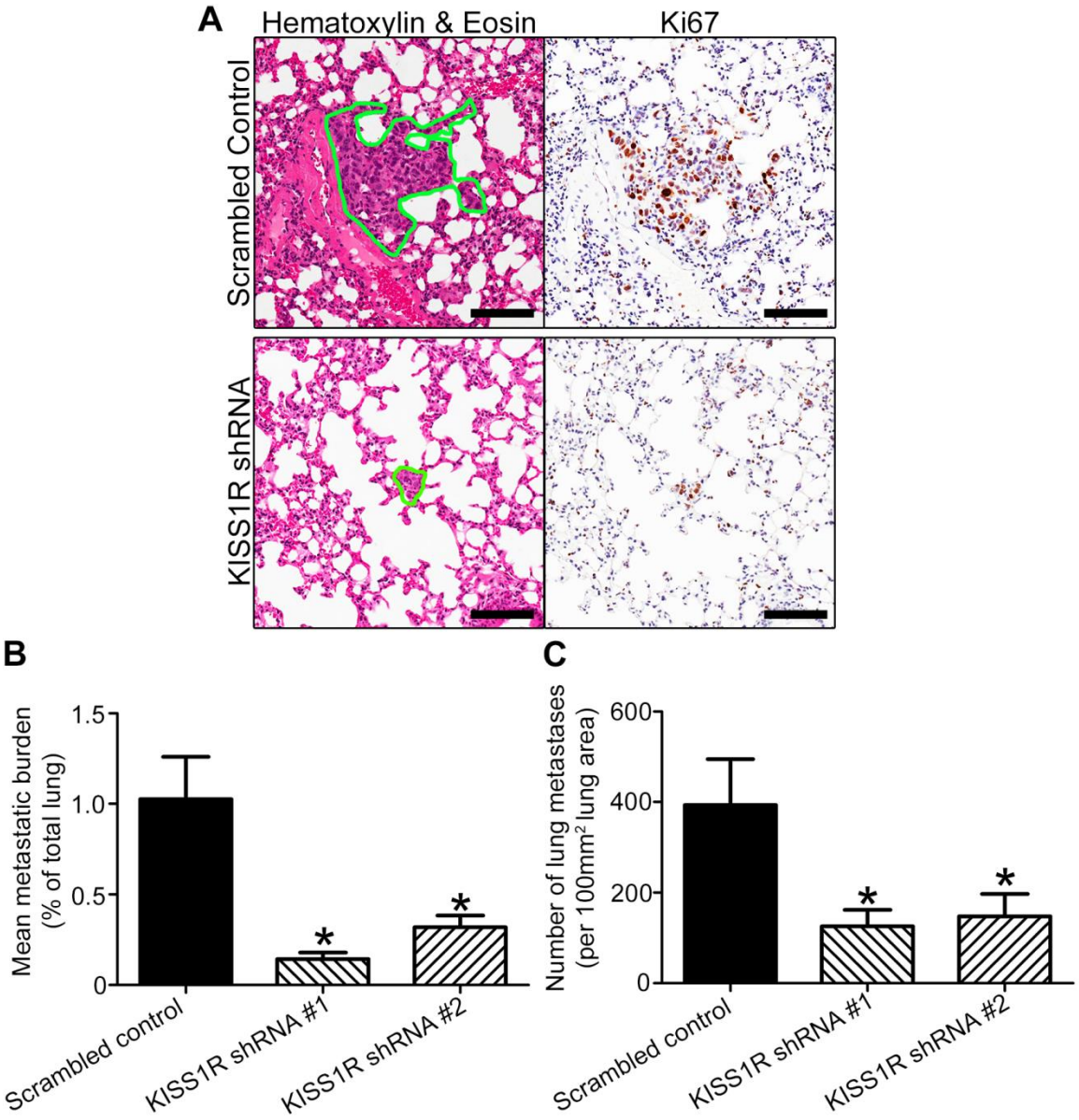

Figure 2.2. KISS1R depletion reduces lung metastatic burden. (A) At 5 weeks post-inject, mice were sacrificed and assessed for metastatic burden in the lungs. Representative images of lung tissue subjected to either hematoxylin and eosin or Ki67 staining detected with rabbit monoclonal anti-Ki67 antibody followed by anti-rabbit HRP conjugate secondary antibody. Lung metastasis outlined with green. Scale bar, 100 μm. The extent of lung metastasis was determined by area of lung (B) and number of lung metastases (C) (4 sections/mouse, 5 mice per cell population). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent lung area and number of metastases ± SEM , respectively.

KISS1R depletion reduces breast cancer angiogenesis

To determine the mechanism by which KISS1R depletion reduced breast cancer metastasis, we investigated the role of KISS1R in the promotion of angiogenesis. Using the primary tumor sections from the orthotopic xenograft experiments, we stained for blood vessel formation using CD31, a marker for endothelial cells (Figure 2.3A). In the primary tumors depleted of KISS1R, we observed a reduction in the blood vessel density in tumors depleted of KISS1R (Figure 2.3B). Vascular endothelial growth factor (VEGF) and transcription factor hypoxia-inducible factor-1(HIF-1α) are two proteins whose expression has been demonstrated to increase with breast cancer progression, leading to the promotion of angiogenesis (15, 16). Hence, we assessed whether KISS1R regulated the expression of VEGF and HIF-1α in MDA-MB-231 cells. We observed that a reduction in KISS1R expression in MDA-MB-231 cells was associated with a reduction in both VEGF and HIF-1α (Figure 2.4). Hence, our results implicate a possible mechanism by which KISS1R promotes breast cancer angiogenesis.

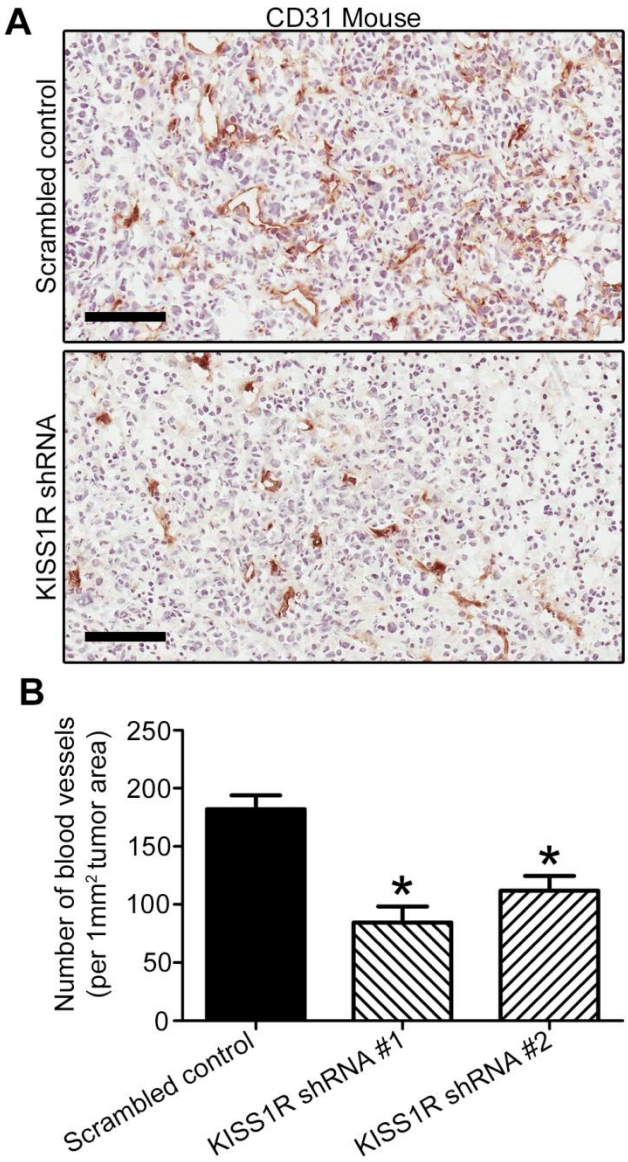

Figure 2.3. Depletion of KISS1R reduces breast cancer angiogenesis *in vivo*. (A) Representative images of blood vessels stained with CD31 mouse in primary tumors subjected to hematoxylin counter stain. Mouse CD31 was detected with rat monoclonal anti-mouse CD31 antibody followed by anti-rat HRP conjugate secondary antibody. Scale bar, 100 μm. (B) Number blood vessels in primary tumors formed within the mammary fat pad of NOD/SCID/IL2rγ mice injected with MDA-MB-231 breast cancer cells expressing scrambled control shRNA or KISS1R shRNA (5 mice/cell population). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent number of blood vessels ± SEM.

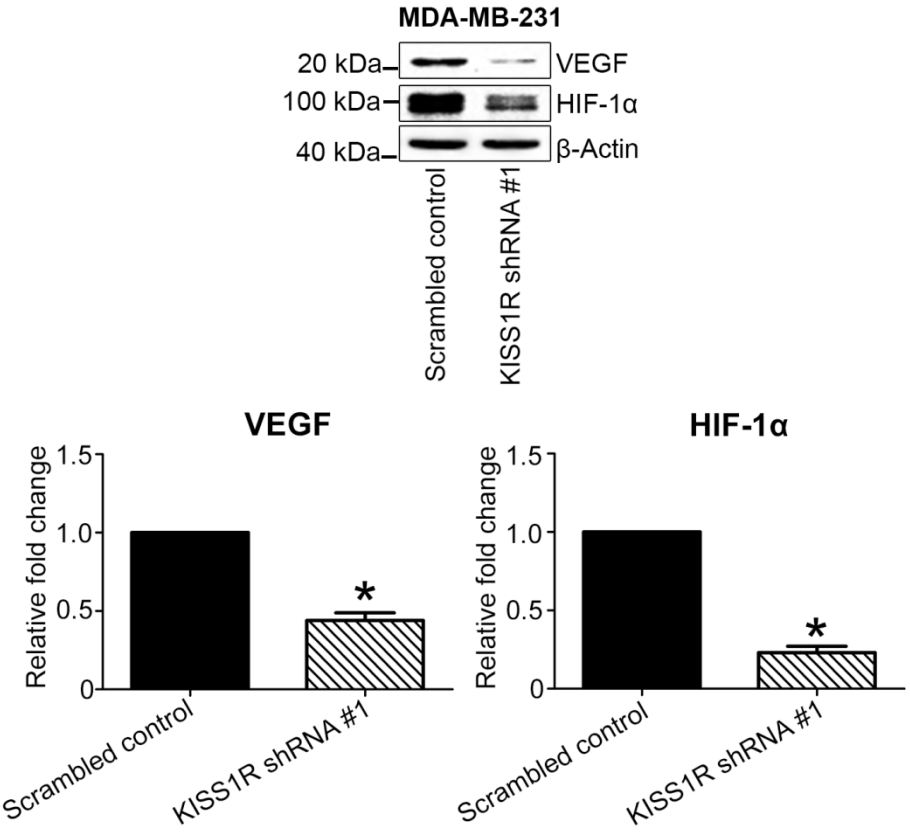

Figure 2.4. KISS1R expression regulates the pro-angiogenic factors in breast cancer cells. Representative Western blots showing expression levels of endogenous VEGF and HIF-1α in MDA-MB-231 cells depleted of KISS1R. Western blot analysis with a rabbit polyclonal anti-HIF-1α antibody and rabbit polyclonal anti-VEGF antibody (n=4). Quantification of Western blot expression normalized to β-Actin. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent protein expression ± SEM.

KISS1R depletion reduces the mesenchymal phenotype of human breast cancer cell lines MDA-MB-231 and Hs578T

To better understand the mechanism by which KISS1R signaling regulates metastasis, we investigated the effect of KISS1R depletion on breast cancer mesenchymal phenotype. Hence we depleted KISS1R in two invasive human adenocarcinoma breast cancer cell lines, MDA-MB-231 and Hs578T. Expression of two different KISS1R shRNA constructs in Hs578T cells depleted KISS1R expression as verified through western blot (Figure 2.5A) and did not affect cell viability (Figure 2.5B). Upon depletion of KISS1R we observed a reduction in mesenchymal morphology as characterized by a loss of long, spindle shaped morphology of invasive breast cancers cells (Figure 2.5C). Furthermore, a reduction of KISS1R expression decreased actin stress fiber formation in breast cancer cells (Figure 2.6). We have previously reported that overexpression of KISS1R stimulates epithelial-to-mesenchymal transition in the non-malignant human MCF10A mammary epithelial cells and the human breast cancer SKBR3 cells (2). Hence, we assessed whether depletion of KISS1R regulated the expression of mesenchymal markers smooth muscle actin, β-catenin, and Snail/Slug. We found that the depletion of KISS1R resulted in the loss of expression of these markers in MDA-MB-231 and Hs578T breast cancer cells in comparison to scrambled control (Figure 2.7, 2.8). Hence, our results reveal that KISS1R expression regulates the mesenchymal phenotype of breast cancer cells.

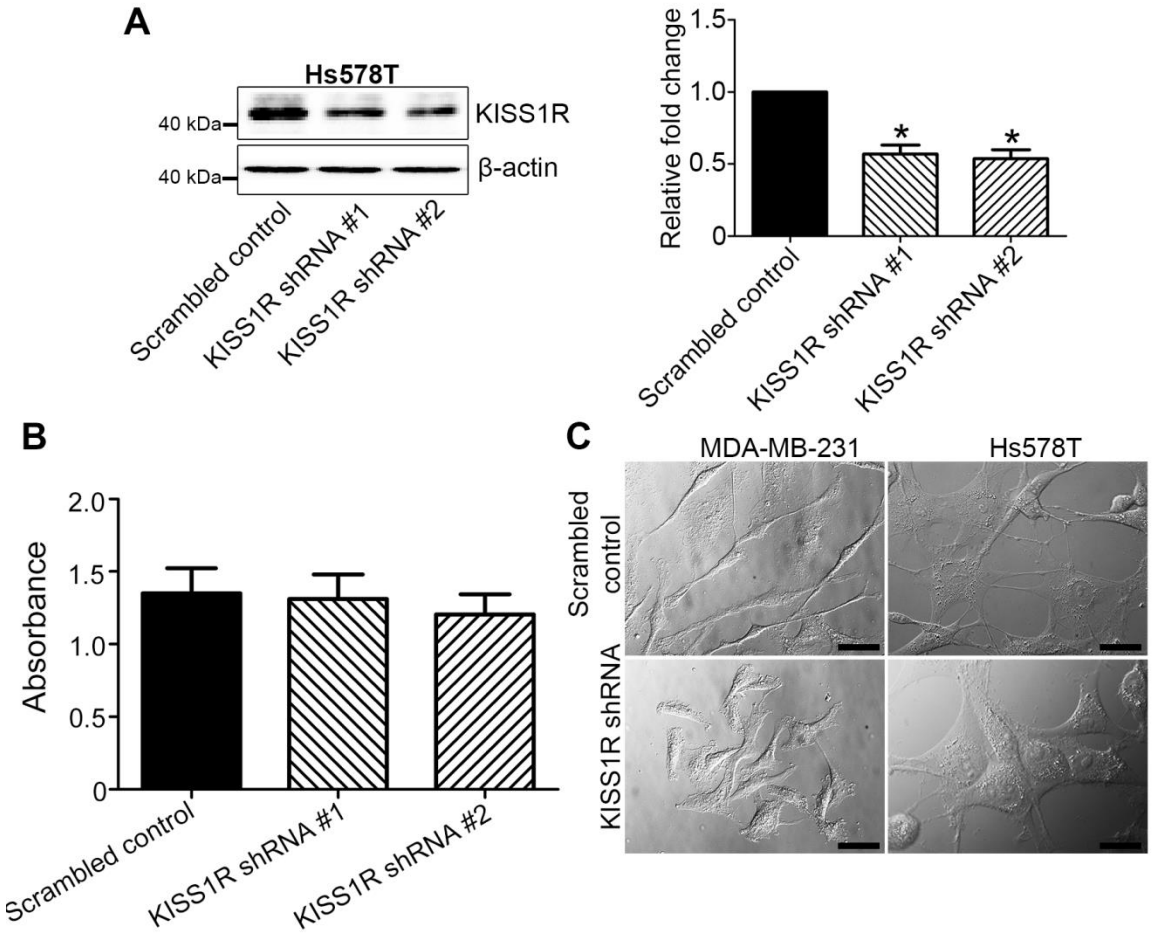

Figure 2.5. Depletion of KISS1R expression reduces the mesenchymal phenotype of MDA-MB-231 and Hs578T breast cancer cells. (A) Representative Western blots showing expression levels of endogenous KISS1R in Hs578T breast cancer cells expressing KISS1R shRNA or scrambled control. Western blot analysis with goat polyclonal anti-KISS1R (N-20) antibody. Quantification of Western blot expression normalized to β-actin (n=3). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent protein expression ± SEM. (B) Hs578T cells expressing KISS1R shRNA or scrambled control where cultured in 96 well plate and viability was determined by MTT assay (n=3-4). Columns represent absorbance measured at 550nm subtract background reading at 700nm, ± SEM. (C) Representative DIC images of MDA-MB-231 and Hs578T breast cancer cells expressing scrambled control shRNA or KISS1R shRNA (n=3). 40x. Scale bar, 100 μm.

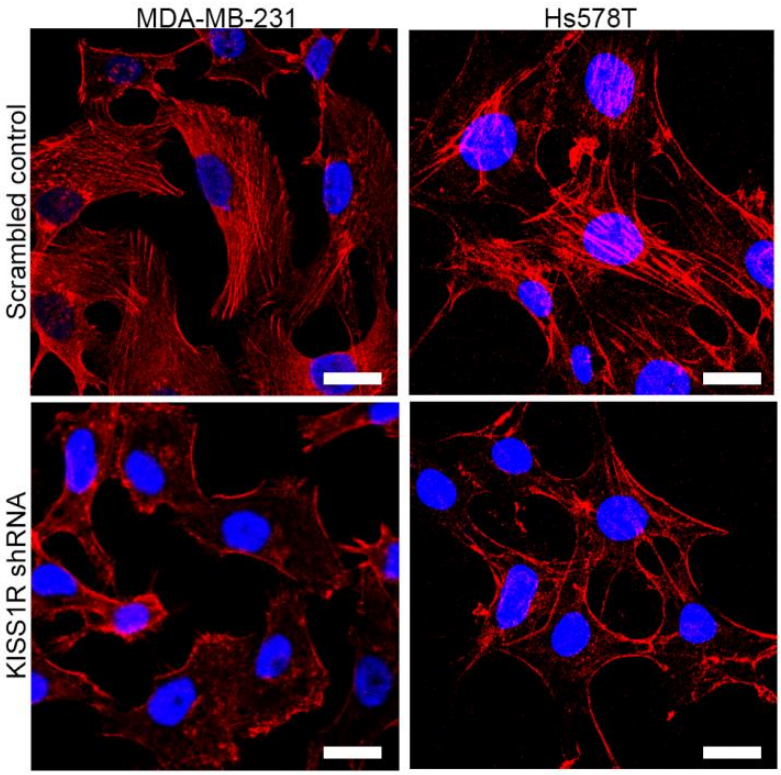

Figure 2.6. Reduction in KISS1R expression reduces stress fiber formation in MDA-MB-231 and Hs578T cells. F-actin stained with phalloidin -Alexa Fluor 555 (red) and nuclei stained using Hoechst 33258 (blue) and visualized by confocal microscopy. Images representative of three independent experiments. Scale bar, 20 μm.

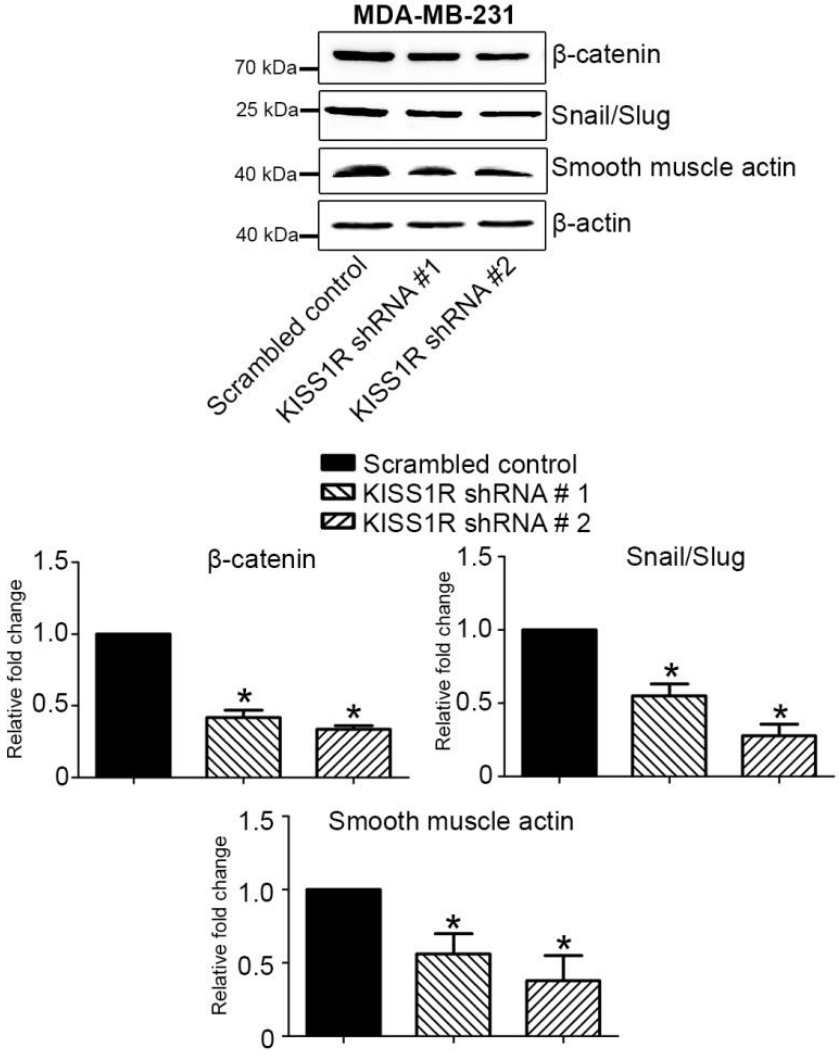

Figure 2.7. Decreased expression of KISS1R reduces mesenchymal markers in MDA-MB-231 cells. Representative Western blots showing expression levels of KISS1R and mesenchymal markers β-catenin (n=4), Snail/Slug (n=3) and smooth muscle actin (SMA) (n=3) in MDA-MB-231 breast cancer cells expressing scrambled shRNA or KISS1R shRNA. Western blot analysis performed with mouse monoclonal anti-β-catenin, rabbit polyclonal anti-Snail/Slug, and mouse monoclonal anti-smooth muscle actin. Quantification of Western blot expression normalized to β-actin. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent protein expression ± SEM.

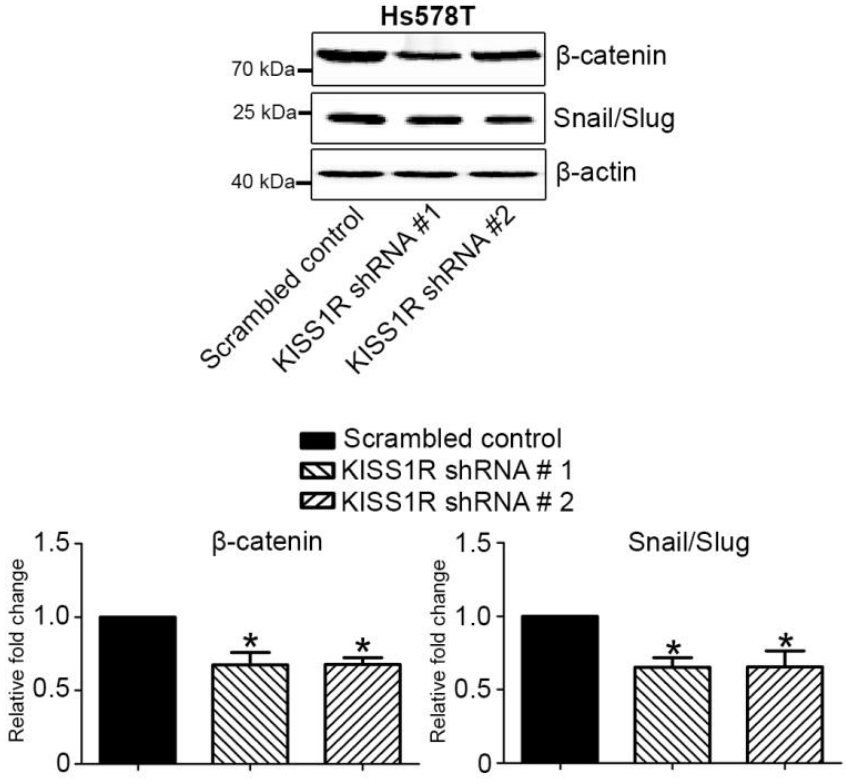

Figure 2.8. Decreased expression of KISS1R reduces mesenchymal markers in Hs578T cells. Representative Western blots showing expression levels of KISS1R and mesenchymal markers β-catenin (n=3) and Snail/Slug (n=3) in Hs578T breast cancer cells expressing scrambled shRNA or KISS1R shRNA. Western blot analysis performed with mouse monoclonal anti-β-catenin and rabbit polyclonal anti-Snail/Slug. Quantification of Western blot expression normalized to β-actin. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent protein expression ± SEM.

KISS1R depletion reduces breast cancer cell migration.

The ability of cancer cells to metastasize is dependent on two important cellular functions, the cell's capacity to invade or degrade the surrounding tissue as well as the cell's ability to migrate through the space, away from the primary tumor (17-19). To assess a role of KISS1R in promoting migration, we first used scratch migration assays. We found that reduction of KISS1R expression decreased the distance migrated in comparison to cells expressing scrambled control (Figure 2.9). We have previously reported that kisspeptin-10 increases migration and invasion at maximal response observed using 100 nM concentration, thus all of our following experiments were conducted using 100 nM kisspeptin-10 (2, 3, 12, 20, 21). Migration was also measured using the transwell chamber migration assays. MDA-MB-231 or Hs578T cells expressing scrambled control or KISS1R shRNA were seeded in serum-free media or serum free media containing 100 nM kisspeptin-10 and allowed to migrate for 20 hours. The depletion of KISS1R reduced basal migration as well as kisspeptin-10 stimulated migration (Figure 2.10). Moreover, we saw no differences in cell proliferation suggesting that the observed differences in scratch closure and transwell migration assays are due to changes in cell migration and not increase in cellular proliferation (Figure 2.11). Hence, these results suggest that KISS1R signaling regulates breast cancer cell migration.

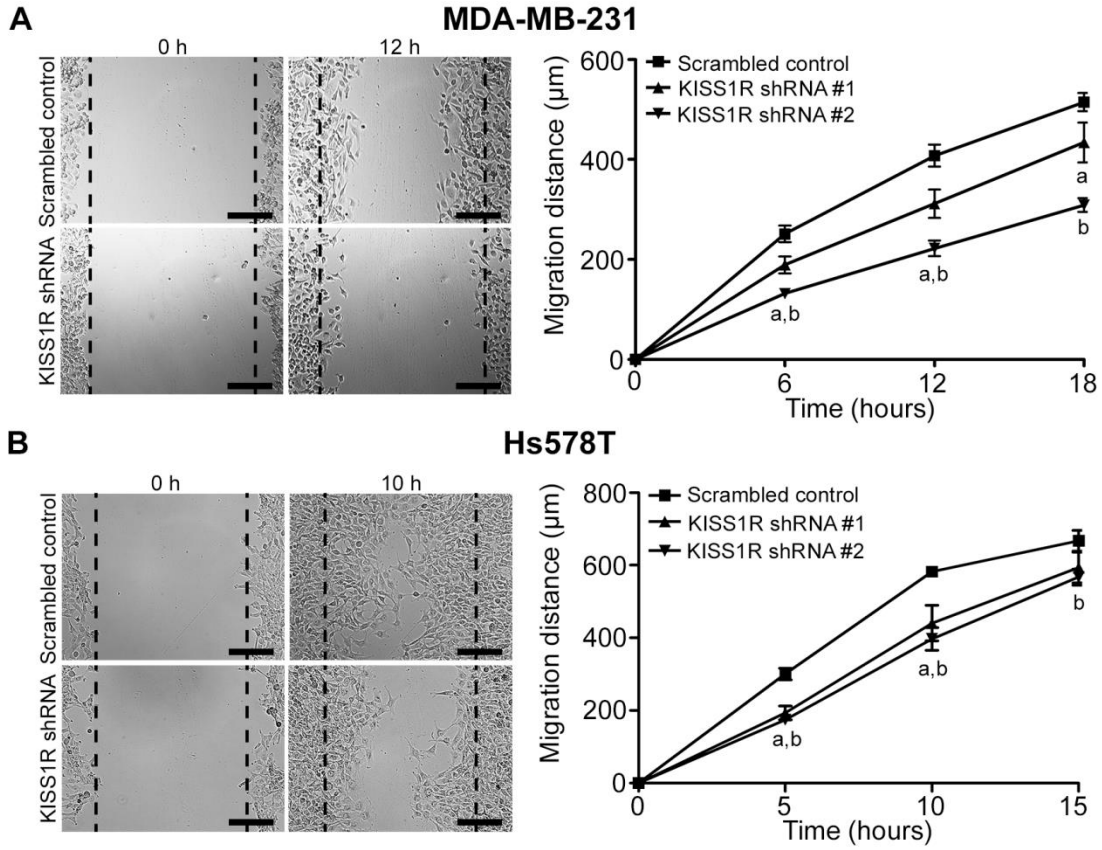

Figure 2.9. Depletion of KISS1R inhibits breast cancer scratch wound closure. Decreased expression of KISS1R reduces the distance closed by (A) MDA-MB-231 and (B) Hs578T cells in a scratch assay over an 18 hour and 15 hour period, respectively. Two-way ANOVA followed by Bonferroni post hoc test: a, $P < 0.05$ for KISS1R shRNA construct #1 vs. scrambled control; b, $P < 0.05$ KISS1R shRNA construct #2 vs. scrambled control. Scale bar, 100 μm.

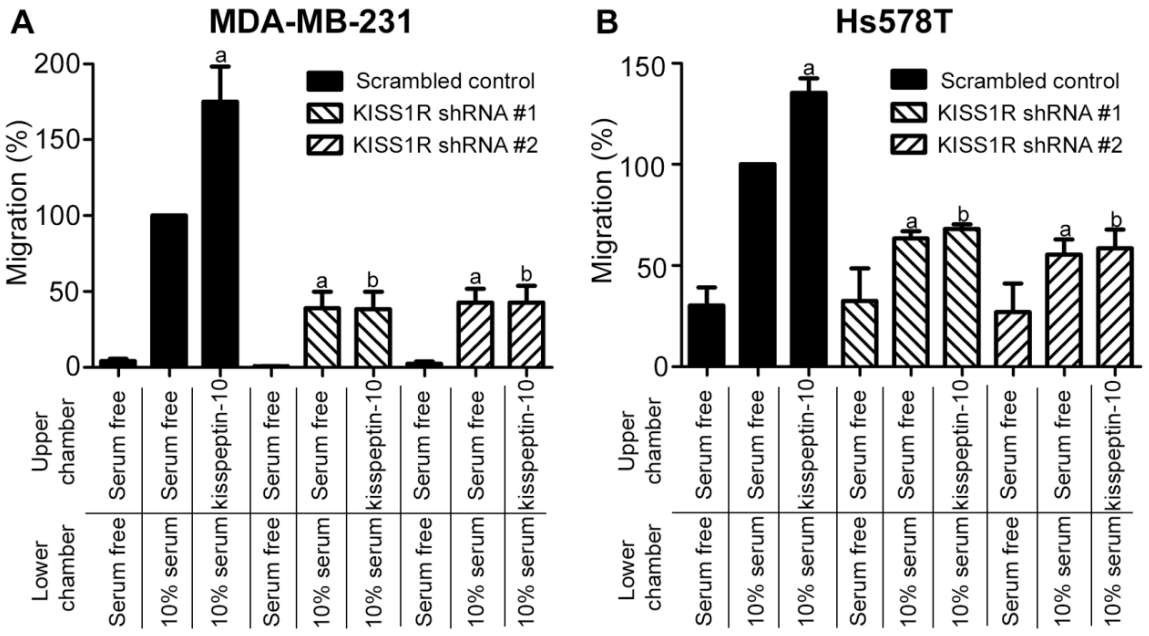

Figure 2.10. Depletion of KISS1R expression inhibits MDA-MB-231 and Hs578T cell transwell chamber migration. Depletion of KISS1R in (A) MDA-MB-231 and (B) Hs578T cells decreases the number of cells migrated towards 10% fetal bovine serum (FBS) under basal conditions and inhibits kisspeptin-10 induced cell migration (n=3-6). Two-way ANOVA followed by Bonferroni post hoc test: a, $P < 0.05$ for significant difference vs. scrambled control non-stimulated; b, $P < 0.05$ for significant difference vs. scrambled control 100 nM kisspeptin-10. Columns represent cells migrated to lower chamber ± SEM.

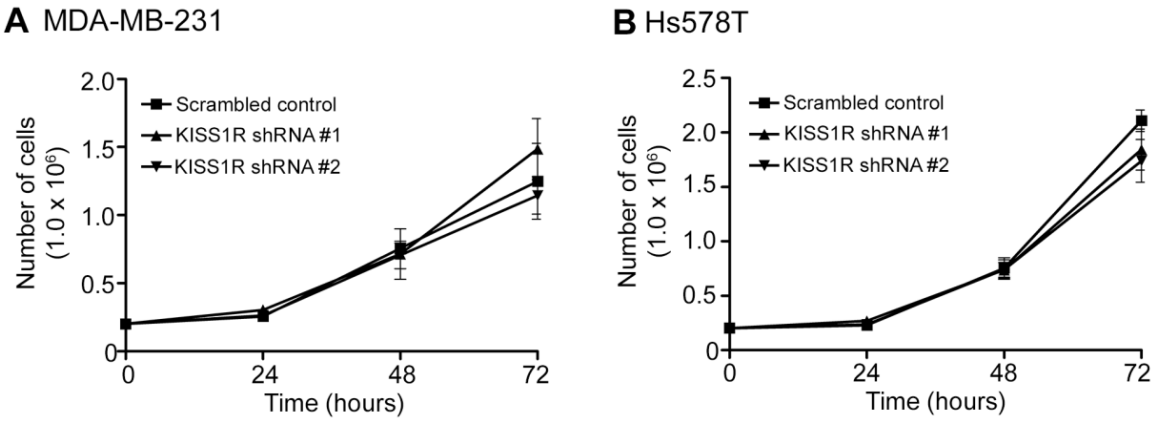

Figure 2.11. KISS1R depletion does not affect cell proliferation rate over a 72 hour period. (A) MDA-MB-231 or (B) Hs578T cells expressing KISS1R shRNA or scrambled control where cultured for 72 hours and counted at 24 hour intervals (n=3).

Decreased KISS1R expression diminishes breast cancer cell invasion.

Both malignant and non-malignant breast cell lines can be cultured in Matrigel, a reconstituted extracellular matrix that mimics the *in vivo* tumor micro-environment (2-4, 7). MDA-MB-231 and Hs578T breast cancer cells cultured in the three-dimension (3D) Matrigel assay formed less invasive (stellate) colonies when depleted of KISS1R in comparison to cells expressing scrambled control (Figure 2.12, 2.13). Furthermore, we observed that KISS1R depletion reduced basal and kisspeptin-10 induced breast cancer cell invasion using transwell membrane invasion assays (Figure 2.14). Overall, these findings suggest that KISS1R signaling promotes the invasiveness of breast cancer cells and that this may be a mechanism by which depletion of KISS1R inhibited metastasis *in vivo*.

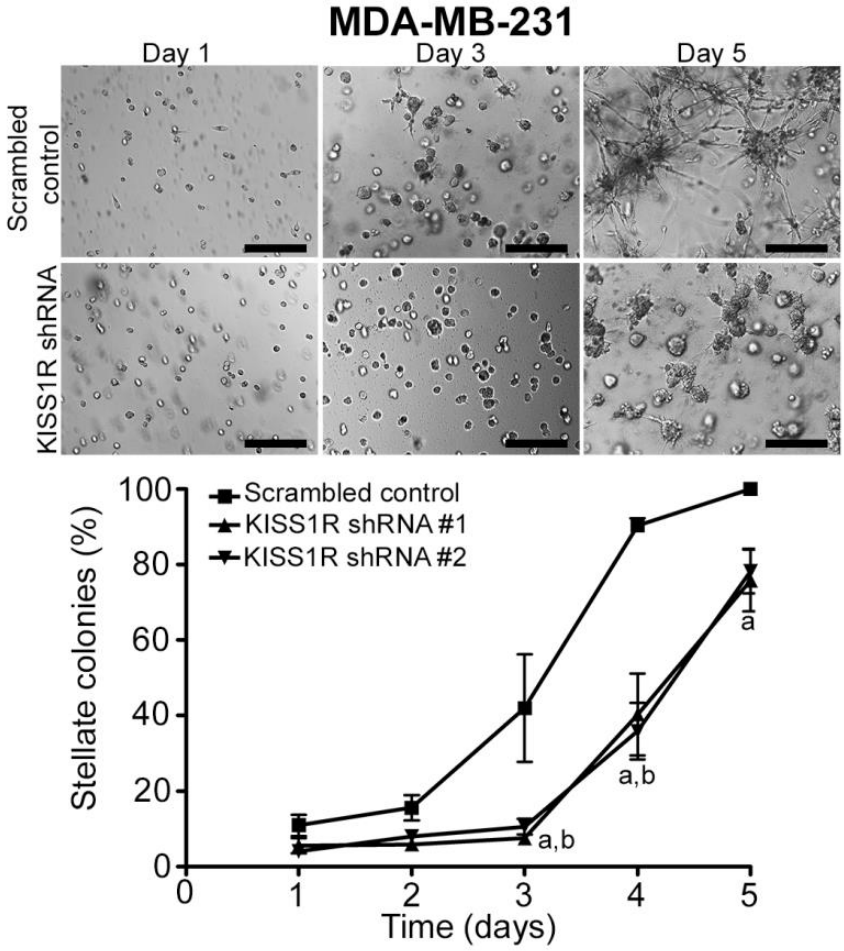

Figure 2.12. Decreased KISS1R expression inhibits MDA-MB-231 breast cancer cell invasion in three-dimensional Matrigel assay. MDA-MB-231 cells expressing KISS1R shRNA suspended in Matrigel for 5 days display reduced growth of invasive stellate structures relative to cells expressing scrambled control (n=3-5). Scale bar, 100 μm. Two-way ANOVA followed by Bonferroni post hoc test: a, $P < 0.05$ for KISS1R shRNA construct #1 vs. scrambled control; b, $P < 0.05$ KISS1R shRNA construct #2 vs. scrambled control.

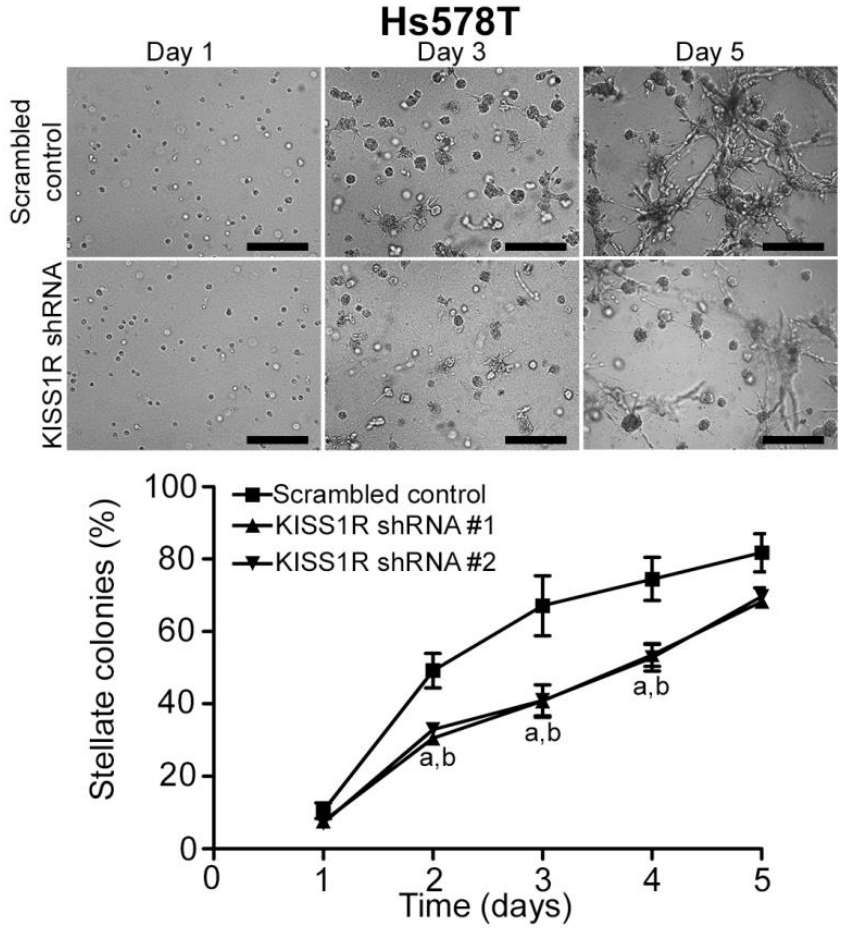

Figure 2.13. Decreased KISS1R expression inhibits Hs578T breast cancer cell invasion in three-dimensional Matrigel assay. Hs578T cells expressing KISS1R shRNA suspended in Matrigel for 5 days display reduced growth of invasive stellate structures relative to cells expressing scrambled control (n=3). Scale bar, 100 μm. Two-way ANOVA followed by Bonferroni post hoc test: a, $P < 0.05$ for KISS1R shRNA construct #1 vs. scrambled control; b, $P < 0.05$ KISS1R shRNA construct #2 vs. scrambled control.

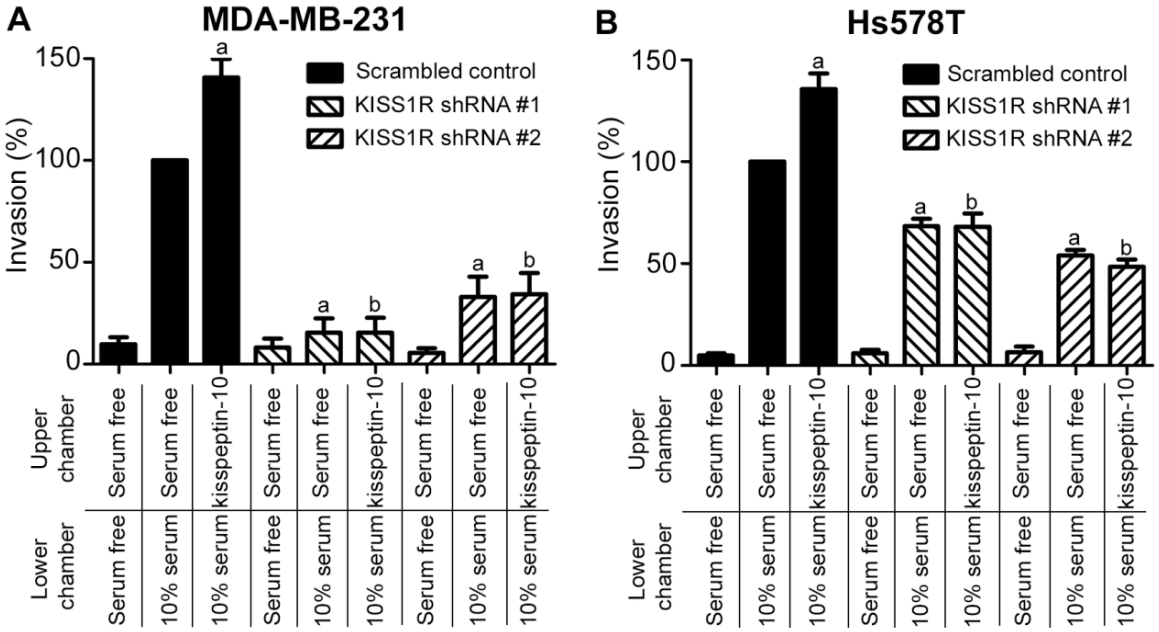

Figure 2.14. Depletion of KISS1R expression inhibits breast cancer cell invasion in Matrigel transwell chamber invasion assay. Decreased KISS1R expression reduces (A) MDA-MB-231 and (B) Hs578T cell invasion towards 10% fetal bovine serum (FBS) at basal conditions and inhibits kisspeptin-10 induced invasion (n=3-5). Two-way ANOVA followed by Bonferroni post hoc test: a, $P < 0.05$ for significant difference vs. scrambled control non-stimulated; b, $P < 0.05$ for significant difference vs. scrambled control 100 nM kisspetin-10. Columns represent cells invaded to lower chamber ± SEM

KISS1R depletion decreases colony formation by breast cancer cells

To establish a role for KISS1R in breast cancer tumorigenicity, we conducted the anchorage independent assays. MDA-MB-231 cells depleted of KISS1R or expressing the scrambled control were cultured in agar and then the numbers of colonies that formed were counted. Compared to the scrambled control, cells depleted of KISS1R showed a dramatic decrease in colony formation (Figure 2.15). Thus, our data suggest that KISS1R expression regulates the ability of metastatic breast cancer cells to grow in an anchorage-independent environment suggesting a mechanism by which KISS1R signaling may influence primary tumor growth.

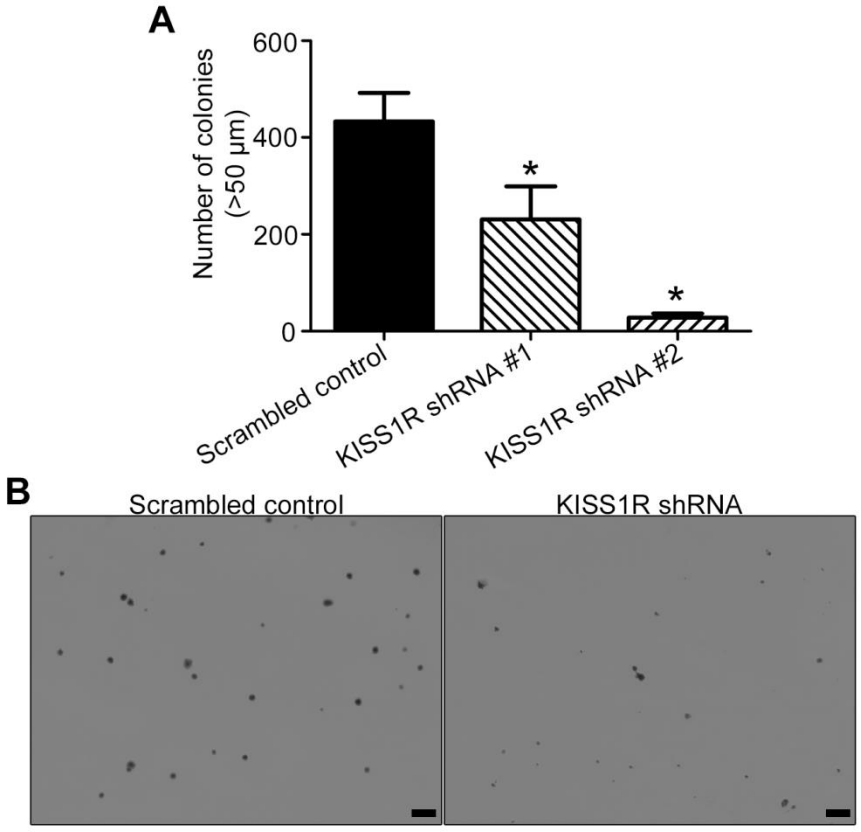

Figure 2.15. KISS1R depletion in MDA-MB-231 cells reduces anchorage independent growth. (A) MDA-MB-231 expressing scrambled control shRNA or KISS1R shRNA was grown in agarose for 6 weeks (n=4-5). Reduction in KISS1R decreased the number of colonies greater than 50 µm in diameter as compared to cells expressing scrambled control. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent colonies greater than 50 µm in diameter, ± SEM. (B) Representative pictures of MDA-MB-231 cellular colonies at 6 weeks. Scale bar, 200 µm.

Kisspeptin Signaling Stimulates Invadopodia Formation

Our data thus far indicates that KISS1R signaling plays a role in promoting breast cancer cell invasion. To evaluate the mechanism by which KISS1R promotes cell invasion, we used a gelatin degradation assay to assess the ability of kisspeptin-10 to stimulate invadopodia. Invadopodia formed by cells degrade the gelatin leaving darkened areas or “holes” in the areas depleted of gelatin (22, 23). The ability for invadopodia to form and degrade the gelatin is dependent on cortactin and F-actin; these two proteins display a punctate pattern and co-localize at points of degradation (23, 24). Hence, invadopodia formation was determined by measuring the number of cells forming F-actin-cortactin co-localization punctate pattern with associated underlying gelatin degradation (Figure 2.16A). MDA-MB-231 or Hs578T cells were plated on gelatin for 24 hours and then stimulated with 100 nM kisspeptin-10 for 30 minutes. Upon kisspeptin-10 stimulation, the number of cells with invadopodia formation increased significantly compared to basal levels (Figure 2.16B). Furthermore, we observed that pre-treatment with the KISS1R antagonist, P-234 (1 μM), inhibited kisspeptin-10 induced invadopodia formation (Figure 2.16B). Overall, our data reveals for the first time that KISS1R stimulates invadopodia formation in breast cancer cells.

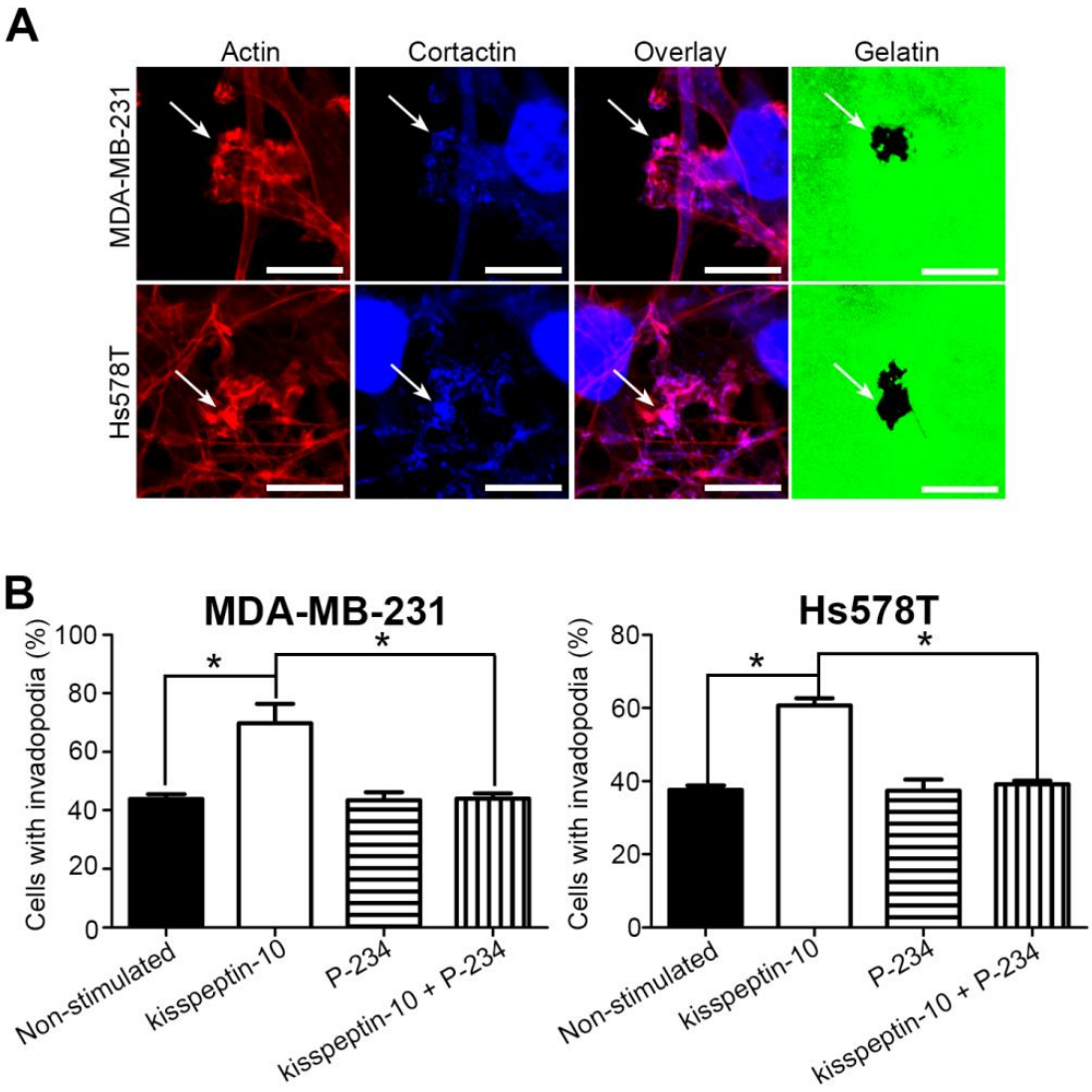

Figure 2.16. Activation of KISS1R promotes invadopodia formation. (A) Representative pictures of invadopodia formation via actin (phalloidin, red) and cortactin (blue) organization promoting gelatin degradation. Cortactin immunostaining detected using mouse monoclonal anti-cortactin antibody, followed by DyLight 350 secondary antibody. Images are representative of three independent experiments. Scale bar, 10 μm. (B) Treatment of MDA-MB-231 or Hs578T breast cancer cells with 100 nM kisspeptin-10 causes an increase in the number if cells with invadopodia formation. Pre-treatment with the KISS1R antagonist, P-234, inhibits kisspeptin-10 induced invadopodia formation (n=3). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent percent of cells with invadopodia ± SEM.

Kisspeptin stimulation increases cortactin and cofilin activity

Invadopodia formation is dependent on the activity of two proteins, cortactin and cofilin (23, 25). The activities of both of these proteins are dependent on their phosphorylation status, where dephosphorylation can be associated with increased activity and maturation of invadopodia (23, 26-29). To evaluate if KISS1R regulates the activity of cofilin and cortactin we stimulated MDA-MB-231 breast cancer cells with kisspeptin-10 (100 nM) for 5 and 15 minutes and evaluated changes in phosphorylation of either cortactin (Y421) or cofilin (S3). Upon stimulation of the MDA-MB-231 cells with kisspeptin-10, we saw a decrease in phosphorylation of both proteins, with maximal dephosphorylation occurring for cortactin at 5 minutes and maximal dephosphorylation for cofilin at 15 minutes (Figure 2.17). These results demonstrate that kisspeptin-10 treatment of breast cancer cells increases the activity of the proteins involved in invadopodia formation. Furthermore, we found that upon depletion of KISS1R, kisspeptin-10 mediated dephosphorylation of cortactin and cofilin was inhibited at both time points (Figure 2.18). Our results thus indicate that kisspeptin-10 signaling via KISS1R stimulates cortactin and cofilin activity necessary to promote invadopodia formation in breast cancer cells. Furthermore, we observed that KISS1R is co-localized in invadopodia with cortactin, IQGAP1, EGFR, and MT1-MMP, proteins essential for the formation and invasive capacity of invadopodia (22, 30) (Figure 2.23).

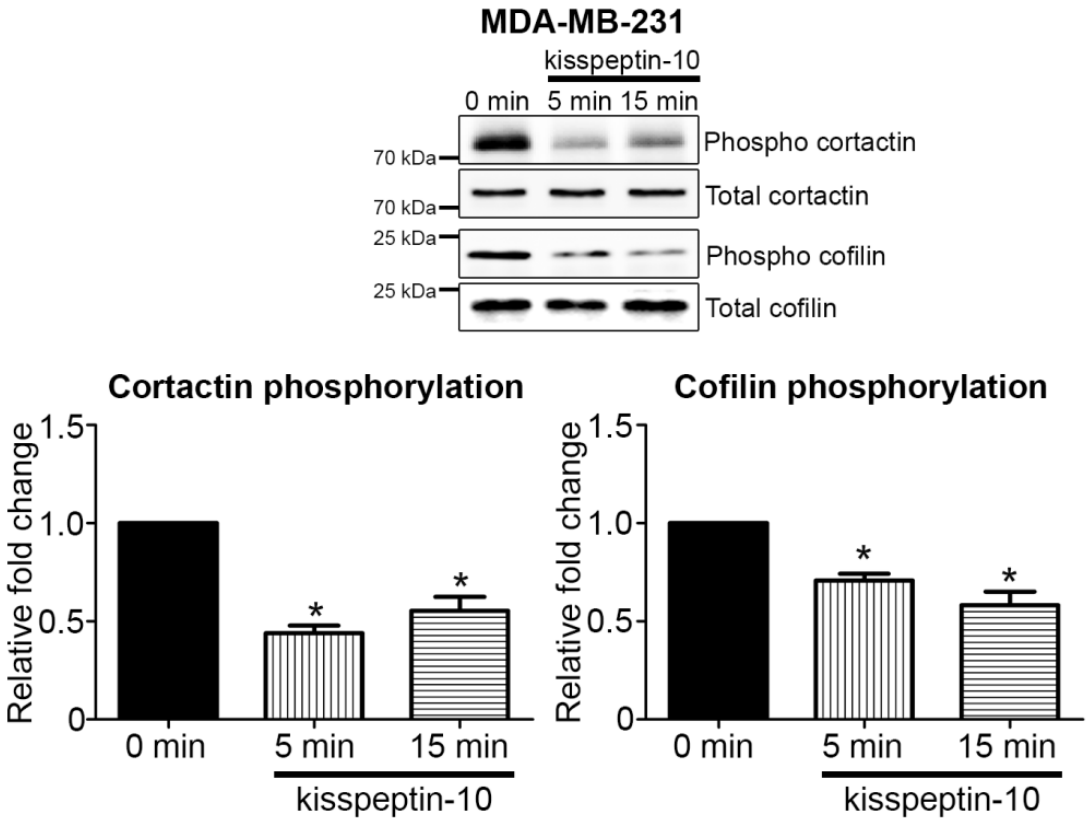

Figure 2.17. KISS1R signaling promotes cortactin and cofilin activity. Treatment of MDA-MB-231 breast cancer cells with 100 nM kisspeptin-10 caused a decrease in the phosphorylation of cofilin and cortactin. Western blot analysis with a rabbit polyclonal anti-phospho cortactin (Y421) antibody, rabbit polyclonal anti-phospho cofilin (S3), mouse monoclonal anti-cortactin, and mouse monoclonal anti-cofilin. Phosphorylation levels are normalized to total cofilin or total cortactin (n=5). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$. Columns represent protein phosphorylation ± SEM.

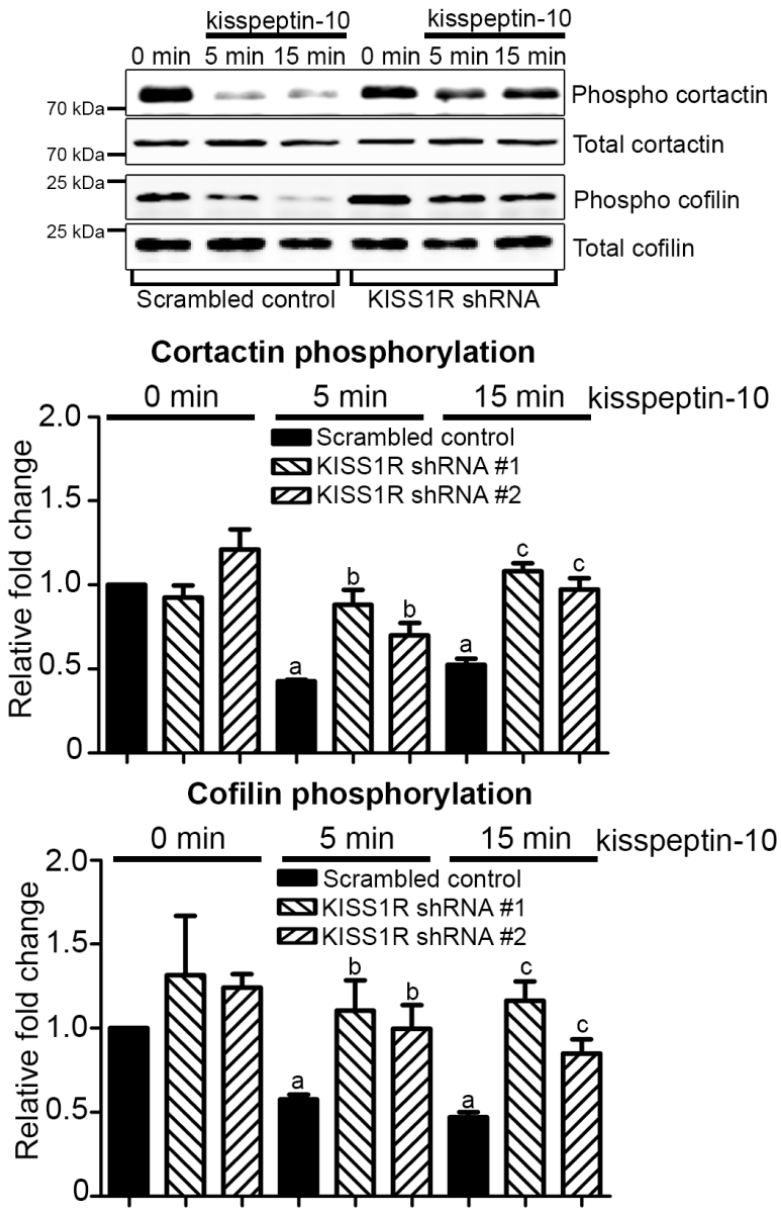

Figure 2.18. Depletion of KISS1R expression inhibits kisspeptin-10 induced decreases in cortactin and cofilin activity. Treatment with kisspeptin-10 100 nM was unable to decrease cofilin and cortactin phosphorylation of MDA-MB-231 breast cancer cells depleted of KISS1R. Phosphorylation levels are normalized to total cofilin or total cortactin (n=3-5). One-way ANOVA followed by Bonferroni post hoc test: a, $P < 0.05$ for significant difference vs. scrambled control kisspeptin-10 0 minutes; b, $P < 0.05$ for significant difference vs. scrambled control kisspeptin-10 5 minutes; c, $P < 0.05$ significant difference vs. scrambled control kisspeptin-10 15 minutes. Columns represent protein phosphorylation ± SEM.

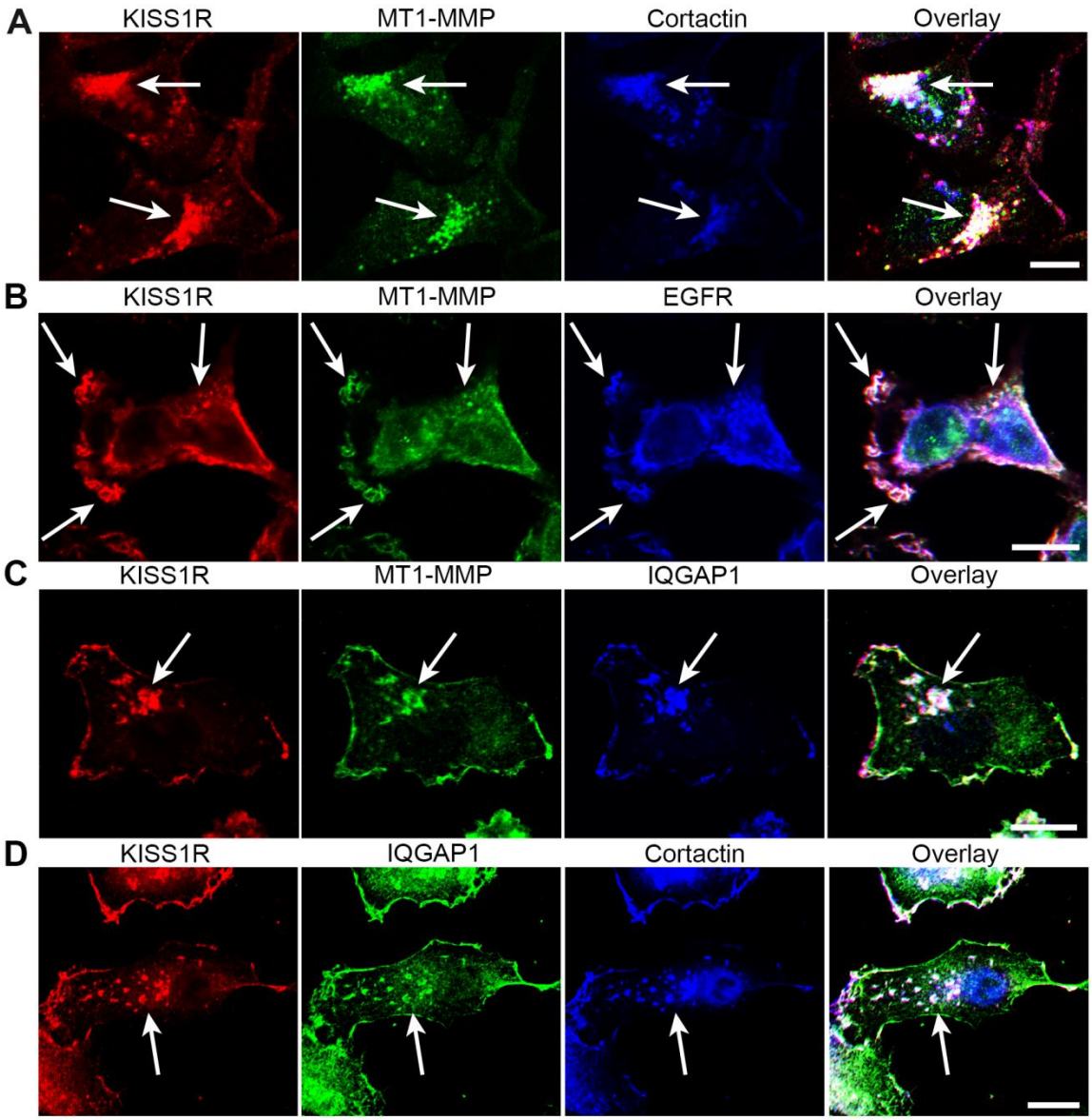

Figure 2.19. KISS1R co-localizes with endogenous cortactin, EGFR, MT1-MMP, and IQGAP1 in invadopodia. Co-localization of endogenous KISS1R with (A) MT1-MMP and cortactin or (B) MT1-MMP and EGFR or (C) MT1-MMP and IQGAP1or (D) IQGAP1 and cortactin. Images are representative of three independent experiments. Scale bar, 10 μm. KISS1R immunofluorescence detected using goat polyclonal KISS1R (N-20) antibody followed by an anti-goat Alexa Fluor 555 (red); EGFR detected using mouse monoclonal anti-EGFR antibody followed by anti-mouse DyLight 350 (blue); MT1-MMP detected using rabbit monoclonal anti-MT1-MMP antibody followed by anti-rabbit Alexa Fluor 488 (green); Cortactin detected using mouse monoclonal anti-cortactin antibody followed by anti-mouse DyLight 350 (blue); IQGAP1 detected using either mouse or rabbit monoclonal anti-IQGAP1 antibody followed by anti-mouse DyLight 350 (blue) or anti-rabbit Alexa Fluor 488 (green).

Kisspeptin-10 induces MT1-MMP Tyrosine Phosphorylation.

The activation of MT1-MMP by tyrosine phosphorylation of Tyr 573 by Src kinase has been recognized to increase cellular migration and matrix-degradation in 3D matrices (31, 32). Furthermore, an *in vivo* study using a subcutaneous tumor injection into the flanks of mice observed that Tyrosine 573 mutation inhibiting tyrosine phosphorylation resulted in decreased tumor progression, increased tumor necrosis, and increased survival suggesting a direct role for MT1-MMP tyrosine phosphorylation in cancer progression (33). Thus, to evaluate if KISS1R signaling could activate MT1-MMP, we stimulated MDA-MB-231 cells with kisspeptin-10 100 nM for 5 and 15 minutes and observed for changes in MT1-MMP tyrosine phosphorylation. Using immunoprecipitation of MT1-MMP and an anti-tyrosine-phosphorylation antibody, we observed increase in tyrosine phosphorylation at both 5 and 15 minutes of kisspeptin-10 stimulation, with a significant increase at 5 minutes (Figure 2.20). Thus, this result suggests KISS1R stimulation may cause an increased MT1-MMP activity which is important in invadopodia invasive function.

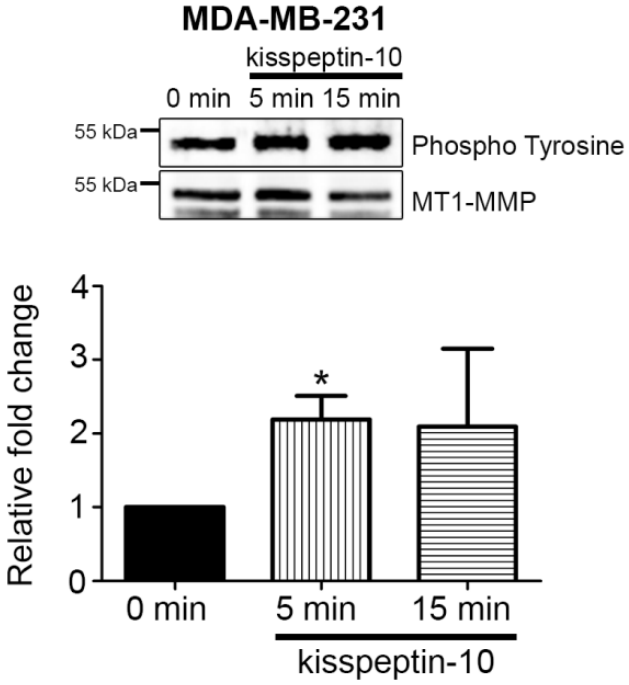

Figure 2.20. Kisspeptin stimulation increases MT1-MMP tyrosine phosphorylation. Treatment of MDA-MB-231 breast cancer cells with 100 nM kisspeptin-10 causes an increase in MT1-MMP tyrosine phosphorylation. Western blot analysis with a mouse monoclonal anti-phosphotyrosine antibody and rabbit monoclonal anti-MT1-MMP. Phosphorylation levels are normalized to total MT1-MMP (n=3). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$. Columns represent protein phosphorylation ± SEM.

β-arrestin2 regulates kisspeptin mediated invadopodia formation

KISS1R promotes invasion by stimulating the secretion and activity of MMP-9 *via* a β-arrestin2 dependent mechanism (3) Likewise, β-arrestin2 has shown to be a direct binding partner for the invadopodia precursors cofilin, cortactin, and IQGAP1 (6, 34, 35). Hence, we determined whether β-arrestin2 is also localized to invadopodia. Indeed, we observed that β-arrestin2 is localized to invadopodia and co-localizes with MT1-MMP, cortactin and KISS1R suggesting a possible role for β-arrestin2 in regulating *KISS1R mediated* invadopodia formation (Figure 2.21). Therefore, to investigate a role of β-arrestin2 in invadopodia formation, we depleted β-arrestin2 in MDA-MB-231 cells as verified through western blot (Figure 2.22A). Using the gelatin degradation assay, we observed a reduction of invadopodia formation in cells depleted of β-arrestin2 (Figure 2.22B). Furthermore, β-arrestin2 depletion inhibited the ability of kisspeptin-10 100 nM to increase invadopodia formation (Figure 2.22B). Finally, we investigated if β-arrestin2 regulated kisspeptin-10 mediated decrease in phosphorylation of both cortactin and cofilin. We found that upon depletion of β-arrestin2, kisspeptin-10 mediated dephosphorylation of cortactin and cofilin was inhibited (Figure 2.23).

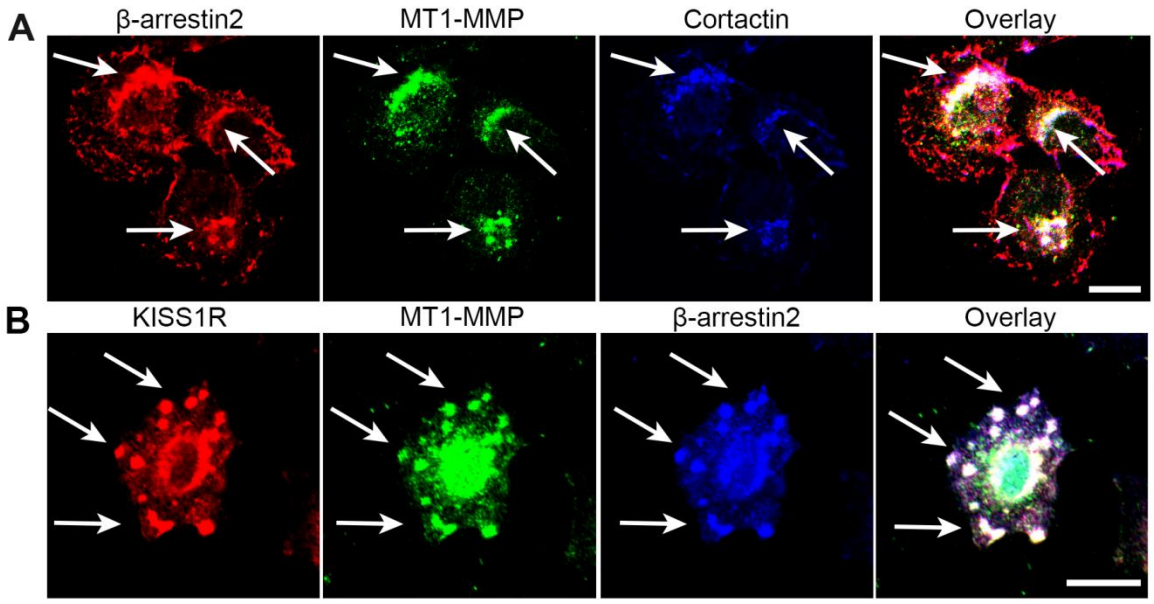

Figure 2.21. β-arrestin2 co-localizes with cortactin, MT1-MMP, and KISS1R in invadopodia. Co-localization of endogenous β-arrestin2 with (A) MT1-MMP and cortactin or (B) KISS1R and MT1-MMP. Images are representative of three independent experiments. Scale bar, 10 μm. β-arrestin2 detected using goat polyclonal anti-β-arrestin2 followed by anti-goat Alexa Fluor 555 (red); MT1-MMP detected using rabbit monoclonal anti-MT1-MMP antibody followed by anti-rabbit Alexa Fluor 488 (green); cortactin detected using a mouse monoclonal anti-cortactin antibody followed by anti-mouse DyLight 350 (blue); KISS1R immunofluorescence detected using goat polyclonal KISS1R (N-20) antibody followed by an anti-goat Alexa Fluor 555 (red).

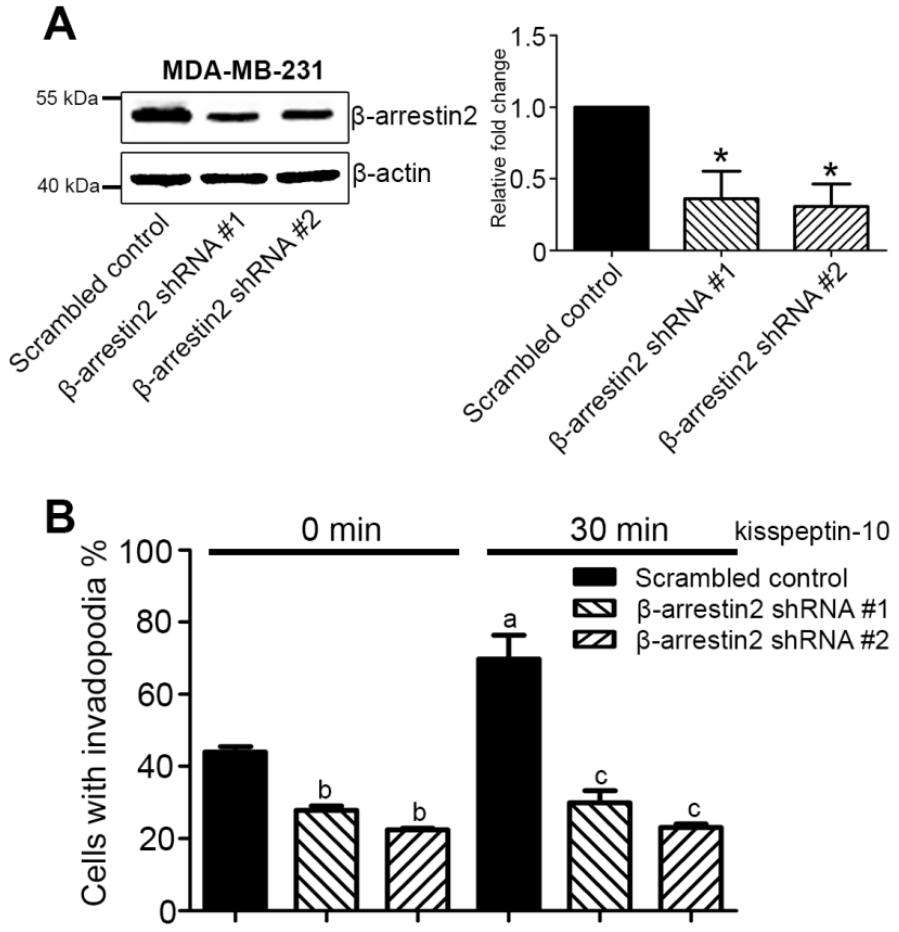

Figure 2.22. β-arrestin2 depletion inhibits invadopodia formation. (A) Representative Western blots showing expression levels of β-arrestin2 in MDA-MB-231 breast cancer cells expressing β-arrestin2 shRNA or scrambled control. Western blot analysis with mouse monoclonal anti-β-arrestin2 antibody. Quantification of Western blot expression normalized to β-actin (n=3). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent protein expression ± SEM. (B) Depletion of β-arrestin2 decreases invadopodia formation at basal conditions and inhibits kisspeptin mediated invasion. Two-way ANOVA followed by Bonferroni post hoc test: a, $P < 0.05$ for significant difference vs. scrambled control kisspeptin-10 0 minutes; b, $P < 0.05$ for significant difference vs. scrambled control kisspeptin-10 5 minutes; c, $P < 0.05$ significant difference vs. scrambled control kisspeptin-10 15 minutes. Columns represent protein phosphorylation ± SEM.

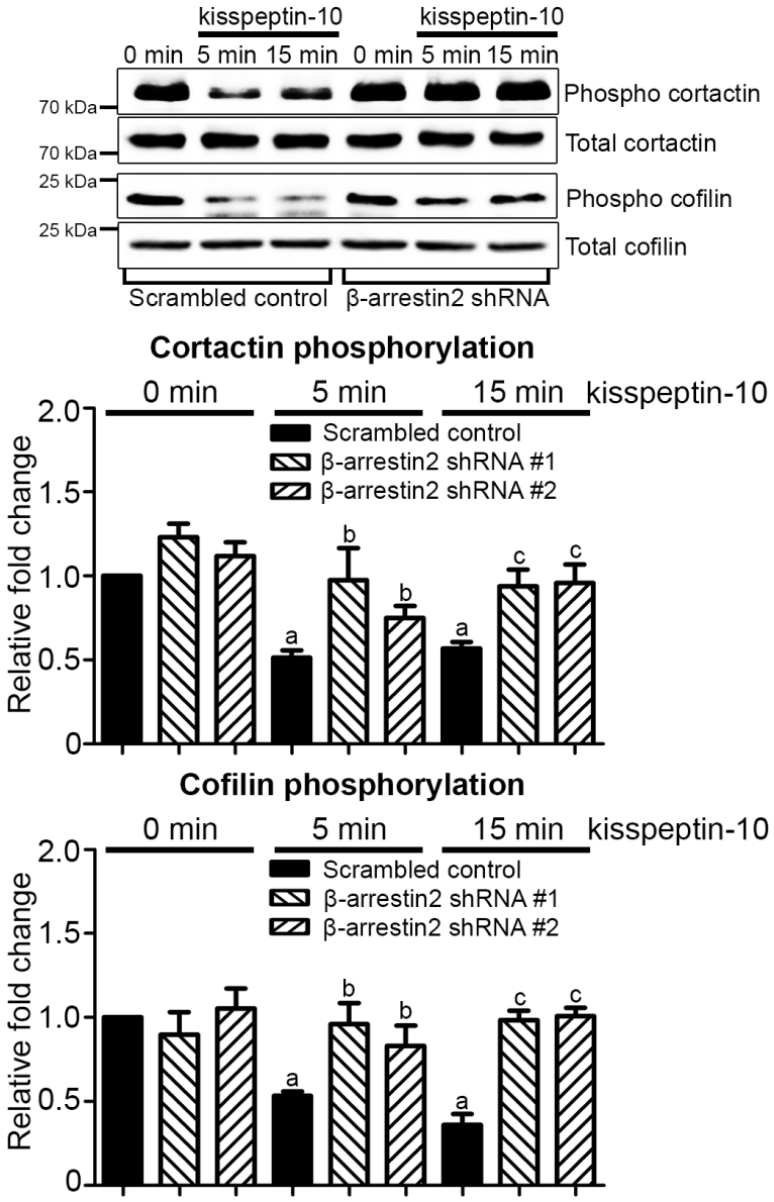

Figure 2.23. β-arrestin2 regulates kisspeptin-10 induced cortactin and cofilin activity. β-arrestin2 depletion in MDA-MB-231 breast cancer cells inhibits kisspeptin-10 100 nM induced cofilin and cortactin dephosphorylation. Phosphorylation levels are normalized to total cofilin or total cortactin (n=3-5). Two-way ANOVA followed by Bonferroni post hoc test: a, $P < 0.05$ for significant difference vs. scrambled control kisspeptin-10 0 minutes; b, $P < 0.05$ for significant difference vs. scrambled control kisspeptin-10 5 minutes; c, $P < 0.05$ significant difference vs. scrambled control kisspeptin-10 15 minutes. Columns represent protein phosphorylation ± SEM.

KISS1R signaling promotes breast cancer extravasation in vivo.

The ability of cells to form invadopodia has been linked to the capability of cancer cells to intravasate and extravasate during metastasis (18, 36). Extravasation is the event of metastasis where cancer cells leave the blood vasculature and enter the surrounding tissue. The chick chorioallantoic membrane (CAM) assay is a well-established model to measure cell extravasation by cancer cells *in vivo* (18, 36). MDA-MB-231 cells were injected into the chorioallantoic membrane of 12 day old chick embryos. A significant increase in cell extravasation was observed with 10 nM or 100 nM kisspeptin-10 treatments (Figure 2.24). Pre-treatment of the cells with 1 μM P-234 KISS1R antagonist or 1 μM AG-1478 EGFR antagonist or combination of both inhibitors significantly blocked extravasation (Figure 2.24). Also, KISS1R depletion significantly reduced MDA-MB-231 cell extravasation in comparison scrambled controls treated with vehicle or kisspeptin-10 10 nM (Figure 2.29). Overall, these results suggest that activation of KISS1R stimulates breast cancer cell extravasation, which can be blocked by inhibiting KISSR and/or EGFR activity.

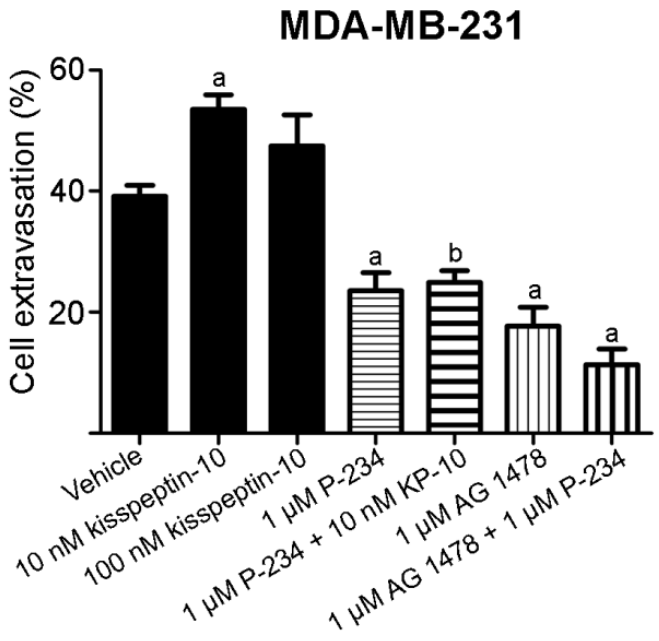

Figure 2.24. KISS1R antagonism attenuates MDA-MB-231 breast cancer cell extravasation in the chick chorioallantoic membrane (CAM) assay. Kisspeptin-10 (10 nM) significantly increased the number of MDA-MB-231 cells extravasating compared to vehicle control (n=14-18). Pre-treatment of cells with KISS1R antagonist P-234, and/or EGFR antagonist AG 1478 significantly reduced the percentage of cells extravasated compared to vehicle control or 10 nM kisspeptin-10 treatment (n=3-6). One-way ANOVA followed by Bonferroni post hoc test: a, $P<0.05$ compared to vehicle treated scrambled control; b, $P<0.05$ compared to 10 nM kisspetin-10 treated scrambled control. Columns represent % cell extravasation ± SEM.

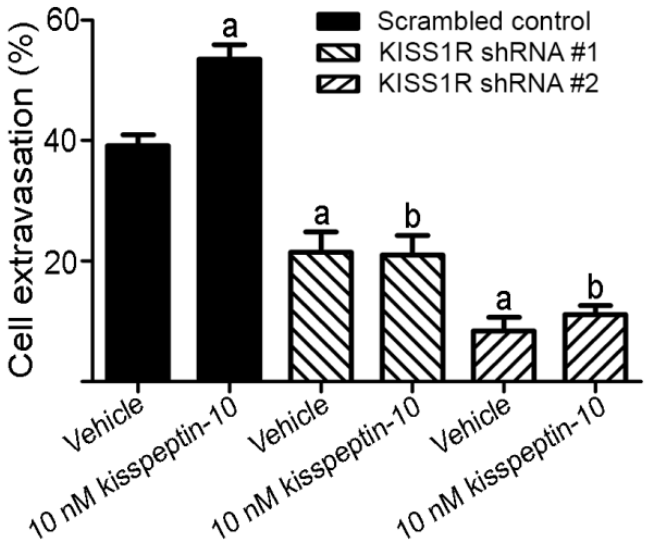

Figure 2.25. Depletion of KISS1R decreases breast cancer cell extravasation. Decreased KISS1R expression in MDA-MB-231 cells inhibits cell extravasation compared to scrambled control vehicle or 10 nM kisspeptin-10 treatments (control groups: n=14-18; KISS1R shRNA groups: n=5-6). Two-way ANOVA followed by Bonferroni post hoc test: a, $P<0.05$ compared to vehicle treated scrambled control; b, $P<0.05$ compared to 10 nM kisspeptin-10 treated scrambled control. Columns represent % cell extravasation ± SEM.

β-arrestin2 regulates KISS1R induced extravasation of breast cancer cells.

Thus far our data supports the premise that β-arrestin2 regulates kisspeptin-10 induced invadopodia formation. Hence, we sought to investigate whether KISS1R stimulates breast cancer extravasation via β-arrestin2. MDA-MB-231 cells depleted of β-arrestin2 were used in the CAM assay. Depletion of β-arrestin2 decreased basal and kisspeptin-10 induced extravasation as compared to scrambled controls (Figure 2.26). These results demonstrate that β-arrestin2 not only regulates invadopodia formation by also breast cell extravasation *in vivo*.

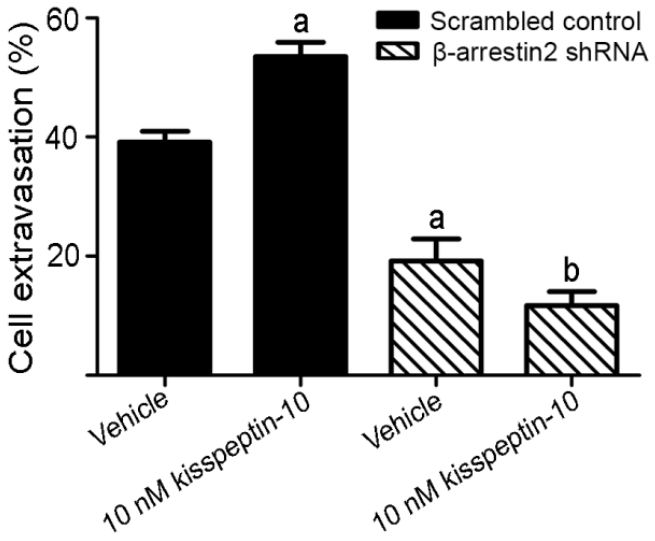

Figure 2.26. β-arrestin depletion attenuates breast cancer cell extravasation. Depletion of β-arrestin2 in MDA-MB-231 cells significantly reduced percent cell extravasation in comparison scrambled control vehicle and 10 nM kisspeptin-10 treatments (control groups: n=14-18, β-arrestin shRNA groups: n=5-6). Two-way ANOVA followed by Bonferroni post hoc test: a, $P<0.05$ compared to vehicle treated scrambled control; b, $P<0.05$ compared to 10nM kisspetin-10 treated scrambled control. Columns represent % cell extravasation ± SEM.

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Chapter 3 - Discussion and Conclusions

3.1 Summary of Novel Findings and Conclusions

Objective #1: To investigate if KISS1R depletion inhibits human breast cancer metastasis using an orthotopic xenograft mouse model.

Orthotopic xenograft tumors established with MDA-MB-231 human breast cancer cells depleted of KISS1R in NOD/SCID/IL2Rγ null mice resulted in decreased size and number of lung metastases. Hence, our results indicate that KISS1R signaling promotes human breast cancer metastasis *in vivo*.

Objective #2: To determine the underlying mechanisms of KISS1R signaling in breast cancer metastasis and invadopodia formation.

Depletion of KISS1R expression resulted in decreased orthotopic tumor blood vessel density and decreased expression of breast cancer cell pro-angiogenic factors. Furthermore, KISS1R depletion resulted in a reduction in breast cancer cell mesenchymal phenotype and decreased breast cancer cell migration and invasion, two important cellular characteristics involved in cancer metastasis. Additionally, we found that KISS1R signals *via* β-arrestin2 to regulate invadopodia formation and extravasation. Thus, our results reveal a mechanism by which KISS1R signaling promotes breast cancer metastases by means of angiogenesis, migration and invasion, invadopodia formation, and extravasation.

3.2 Contributions of Research to Current State of Knowledge

The importance of GPCR signaling in physiological and pathological events is well established (1, 2). GPCR signaling involves a complex cascade of G protein dependent and G protein-independent signaling pathways. The transactivation of EGFR by GPCRs has been demonstrated in breast, lung, ovarian, colon and prostate cancers (3). Similarly, EGFR overexpression in several cancers leads to increased cell survival, proliferation, and invasion (4, 5). Tyrosine kinase inhibitors targeting EGFR activity have been developed. However, clinical results show EGFR inhibition has limited anti-metastatic affects due to the acquisition of drug resistance (6). Studies from our laboratory have revealed that KISS1R transactivates EGFR, through a β-arrestin2 and IQGAP1 mechanism, leading to breast cancer cell invasion (7, 8). Thus, targeting both KISS1R and EGFR together may provide an effective therapeutic strategy in the prevention of breast cancer progression and metastasis. Likewise in triple-negative breast cancer, an effective therapeutic target remains elusive resulting in poor patient survival (9, 10). The identification of KISS1R as novel therapeutic target may provide a solution for those suffering from triple-negative cancer.

Role of KISS1R Signaling in Breast Tumor Formation and Metastasis

In agreement with our findings, Cho and colleagues (2011) showed that the loss of KISS1R in heterozygotic PyMT-*KISS1R*$^{+/-}$ mice resulted in attenuated breast tumor initiation, growth, latency, multiplicity and lung metastasis as compared to the homozygotic PyMT-*KISS1R*$^{+/+}$ mice (11). This study used a

MMTV-PyMT virus mediated initiation of breast cancer in mice mammary cells. Hence, whether KISS1R signaling in *human* breast cancer promotes metastasis remains unknown. For our study, we used a pre-clinical orthotopic xenograft mouse model to determine the role of KISS1R signaling in *human* breast cancer metastasis. The orthotopic xenograft mouse model has been shown to accurately recapitulate breast cancer gene expression profiles as seen in patient primary breast tumors providing an accurate model to assess the role of KISS1R in human breast cancer metastasis (12). Thus, our results support and augment the findings from the Cho et al. (2011) study by demonstrating for the first time that depletion in KISS1R expression in the human breast cancer cells decreases the number and area of lung metastases *in vivo*.

In our orthotopic xenograft mouse model we observed that KISS1R depletion significantly reduced primary tumor volume, thus possibly contributing to the amount of lung metastases observed. The capacity of tumors to stimulate angiogenesis has been shown to control tumor growth and volume (13). We saw that KISS1R depletion decreased blood vessel formation in the primary tumors established *in vivo*. Furthermore, depletion of KISS1R expression in MDA-MB-231 cells decreased the pro-angiogenic factors VEGF and HIF-1α. *Cho et al.* (2011) also reported that mouse breast cancer cells with reduction in KISS1R expression correlated with significant reduction in *VEGF* mRNA expression (11). A second possible mechanism by which the *in vivo* tumor volumes differed is through KISS1R regulation of cancer cellular proliferation. Cho et al. (2011) demonstrated that breast cancer cells cultured from *KISS1R* heterozygotic

PyMT-*KISS1R*$^{+/-}$ mice had a significant reduction in cellular proliferation compared to cancer cells cultured from *KISS1R* homozygotic PyMT-*KISS1R*$^{+/+}$ mice (11). Our study did not find a difference in cell proliferation in breast cancer cells depleted of KISS1R over a relatively short 72 hour period. However, using an anchorage-independent growth assay, we observed for the first time that depletion of KISS1R expression reduced colony formation over a 6 week period. The anchorage-independent growth assay is a well-established model to measure cell proliferation independent of external signals and cell anchorage, trademarks of malignant transformation (14). Therefore, the xenograft primary tumors depleted of KISS1R expression may have had reduced tumor volume due to reduced cell proliferation. Thus, a mechanism by which KISS1R may reduce breast cancer metastasis is by limiting primary tumor growth through angiogenesis and cell proliferation, thereby reducing the number of cancer cells available to metastasize.

KISS1R Signaling in Breast Cancer Invasion and Migration

For our study, we used two human breast cancer cell lines, MDA-MB-231 and Hs578T. These cell lines endogenously express KISS1R and EGFR, and kisspeptin-10 stimulation of these cells promotes migration and invasion (7, 8, 15, 16). Our study extended these findings by demonstrating that depletion of KISS1R expression reduces breast cancer cells capacity to form stress fiber formation. Similarly, our findings showed that KISS1R depletion reduces cellular migration as assessed through scratch and transwell migration assays supporting a role for KISS1R signaling in breast cancer cell migration. However,

kisspeptin-10 treatment was only able to significantly increase migration as assessed in the transwell chamber migration assay but not the scratch assay, possibly due to the scratch assay's lack of a chemical gradient as established in the transwell chamber assay (Supplementary Figure 1)(17).

The ability of cancer cells to acquire a migratory phenotype is thought to occur through EMT. Previous findings in our lab have demonstrated that exogenous expression of KISS1R in MCF10A breast epithelial cells or SKBR3 breast cancer cells initiated an EMT process, leading to increased expression of mesenchymal markers Snail/Slug, N-cadherin, and vimentin (7). In this study, we observed that depletion of KISS1R in MDA-MB-231 and Hs578T cells decreased expression of mesenchymal markers Snail/Slug, β-catenin, and smooth muscle actin. Lastly, our findings supported a role for KISS1R in breast cancer invasion by demonstrating that depletion of KISS1R expression resulted in decreased invasion of MDA-MB-231 and Hs578T cells in the transwell Matrigel invasion and the 3D-Matrigel invasion assays. High expression of mesenchymal markers in cancer cells, leading to cell invasion and migration, has been linked to increased cancer progression and metastasis (18). Our results support a role for KISS1R signaling in the promotion EMT, migration and invasion of breast cancer cells, thus conceivably contributing to the amount of lung metastases we observed in our xenograft mouse model.

KISS1R Signaling in Invadopodia Formation

Over the last decade, significant progress has been made in understanding the role of invadopodia in cell invasion and its contribution to cancer metastasis. First implicated in focal invasion of cells, invadopodia have been demonstrated to play a significant role in many metastatic events as revealed through *in vivo* studies. Invadopodia formation has been directly shown to be involved in cancer cell invasion into the basement membrane surrounding primary tumors as well as playing a direct role in cancer cell intravasation and extravasation (19, 20). Thus, understanding how invadopodia formation occurs and which proteins control invadopodia establishment and invasion is essential for understanding the events that regulate cancer metastasis. Previous studies have revealed that two proteins, cortactin and cofilin, are major invadopodia precursors that control invadopodia formation and maturity. Upon stimulation of growth factors, such as lysophosphatidic acid (LPA) or EGF, a cascade of signaling leads to increased activity of cofilin and cortactin through dephosphorylation of Serine 3 and Tyrosine 421, respectively (21-23). Growth factor stimulation has been shown to influence the phosphorylation status of cofilin and cortactin as quick as 30 seconds to as long as a hour, with peak times within the 3-15 minute range (20, 24, 25). Therefore, for our study we stimulated MDA-MB-231 cells with kisspeptin-10 for 5 and 15 minutes and observed for subsequent changes in phosphorylation of cortactin and cofilin. Upon kisspeptin-10 stimulation we saw a peak dephosphorylation of cofilin at 15 minutes and cortactin at 5 minutes after stimulation. However, to directly determine if

kisspeptin signaling is involved in invadopodia formation, we used gelatin degradation assays, the gold standard assay, to quantify invadopodia formation in numerous cells (19, 20, 24, 26-28). Changes to invadopodia formation due to growth factor stimulation has been demonstrated to take 15 minutes to 3 hours, depending largely on cell type (29, 30). Upon stimulation with kisspeptin-10 we observed peak invadopodia formation at 30 minutes, thus all subsequent experiments were performed with 30 minute kisspeptin-10 stimulation (Supplementary Figure 2). Overall, these results suggest that kisspeptin/KISS1R signaling can promote the formation of invadopodia in human breast cancer cells.

Invadopodia maturation occurs through several stages of protein localization and activity (31). Changes in cortactin and cofilin activity only account for the initial stage of invadopodia formation and protrusion. At maturation, MT1-MMP co-localizes at the invadopodia tip and mediates ECM remodeling (31, 32). Phosphorylation of MT1-MMP at tyrosine 573 regulates protein activity leading to matrix-degradation resulting in cell invasion and tumor progression (33-35). We observed that stimulation with kisspeptin-10 increased tyrosine phosphorylation of MT1-MMP. In combination with our findings that kisspeptin increases invadopodia formation and gelatin degradation, the ability of kisspeptin to increase MT1-MMP tyrosine phosphorylation supports the previous studies implicating MT1-MMP tyrosine phosphorylation increases MT1-MMP invasive activity (33-35). However, due to the use of a non-specific tyrosine antibody, it is unknown if the change in tyrosine phosphorylation is that of tyrosine 573 or another tyrosine residue specific to KISS1R signaling. We have previously

demonstrated that KISS1R transactivates EGFR leading to invasion through increased MMP-9 activity (8). MT1-MMP degradation of ECM occurs through MT1-MMP mediated activation of related MMPs including MMP-9 (36). Hence, kisspeptin-10 mediated increase in MT1-MMP supports the previous findings that kisspeptin signaling leads to increase MMP-9 activity (8). Furthermore, we observed that KISS1R co-localizes with EGFR and MT1-MMP in invadopodia. Our laboratory has previously shown that KISS1R transactivates EGFR to mediate breast cancer cell invasion and EGFR has been demonstrated to play an important role in invadopodia invasion (8, 24). Thus, these results support a possible role for KISS1R in the formation and function of invadopodia.

The scaffolding protein IQGAP1 is considered an oncogene and has been shown to promote breast cancer cell tumorigenesis (37, 38). A previous study investigating MT1-MMP delivery in invadopodia determined that the scaffolding protein IQGAP1 regulates exocyst complex and MT1-MMP delivery to the invadopodia tip (39). Our study supported this finding, by observing that IQGAP1 co-localized with MT1-MMP and cortactin in invadopodia. Our laboratory has previously shown that IQGAP1 co-localizes with KISS1R at the leading edge of migrating breast cancer cells (7). Furthermore, KISS1R transactivates EGFR in an IQGAP1 dependent manner (7). In this study, we observed that KISS1R co-localizes with IQGAP1 and MT1-MMP in invadopodia. Hence, KISS1R may regulate invadopodia invasion through regulation of IQGAP1 mediated MT1-MMP delivery as well as regulating MT1-MMP phosphorylation. Overall, our results suggest that KISS1R signaling regulates the initial stages of invadopodia

formation through the regulation of cofilin and cortactin activity, as well as invadopodia maturation through regulation of MT1-MMP activity and delivery. Due to the evidence for invadopodia in the promotion of metastasis, the ability of KISS1R to promote invadopodia provides an additional mechanism by which KISS1R expression may influence breast cancer metastasis as seen *in vivo* (40, 41).

KISS1R Signals via β-arrestin2 in Invadopodia formation and Extravasation

The scaffolding protein β-arrestin2 is a critical regulator of breast cancer cell migration and invasion (8, 42, 43). We have previously found β-arrestin2 regulates EGFR transactivation by regulating secretion and activity of MMP-9 (7, 8). Global proteomic analysis has demonstrated that β-arrestins interact directly with the invadopodia precursors, cofilin and cortactin (44). Additionally in MDA-MB-468 breast cancer cells, cofilin activity has shown to be regulated by β-arrestin downstream of protease-activated receptor-2 (45). However, whether β-arrestins play a role in invadopodia formation and activity is unknown. We observed that depletion of β-arrestin2 decreased basal levels of invadopodia formation in MDA-MB-231 breast cancer cells thus supporting a role for β-arrestin2 in invadopodia formation. Due to the ability of β-arrestin2 to bind both cortactin and cofilin and evidence that β-arrestin2 regulates KISS1R signaling, we investigated if β-arrestin2 regulated kisspeptin-10 mediated cofilin and cortactin dephosphorylation (44-46). Upon depletion of β-arrestin2, kisspeptin-10 was unable to decrease both cofilin and cortactin phosphorylation. Likewise, we observed co-localization of β-arrestin2 with MT1-MMP and cortactin during

invadopodia formation. Thus, for the first time, our results suggest a role for β-arrestin2 in kisspeptin-10 mediated invadopodia formation and invasion.

Conclusive evidence has demonstrated that invadopodia are necessary for cancer intravasation and extravasation, and metastasis *in vivo* (40, 41). The chick embryonic chorioallantoic membrane (CAM) is a suitable model for observing cancer cell extravasation as the CAM microvasculature exhibit a continuous endothelial layer with an underlying basement membrane (47-49). Using appropriate visualization equipment, this assay enables the observation of cancers cells within blood capillaries, cells undergoing extravasation through the endothelial layer, and cancer cells within stroma (47). Previous findings in our laboratory have demonstrated that exogenous expression of KISS1R in SKBR3 breast cancer cells alone could significantly increase cell extravasation over basal levels as observed using the CAM assay (7). Furthermore, treatment of SKBR3 cells expressing exogenous KISS1R with kisspeptin-10 greatly increased SKBR3 extravasation which in turn could be inhibited using the KISS1R antagonist, P-234 (7). Our findings in this study support these previous results whereas kisspeptin-10 treatment of MDA-MB-231 cells increased extravasation in the CAM assay, which was in turn inhibited with P-234. Additionally, we found that the EGFR antagonist, AG1478, was able to inhibit basal and kisspeptin-10 mediated extravasation of MDA-MB-231 cells, thus supporting a role for both EGFR and KISS1R in breast cancer cell extravasation. Moreover, we found MDA-MB-231 cells depleted of KISS1R expression had reduced extravasation compared to basal levels and inhibited kisspeptin-10 mediated extravasation.

Finally, we observed that β-arrestin2 depletion significantly reduced breast cancer cell extravasation below basal levels and inhibited kisspeptin-10 stimulated extravasation increase. These results demonstrate that KISS1R signaling contributes to breast cancer cell extravasation through a β-arrestin2 dependent mechanism. Thus, in combination with our findings that KISS1R signaling plays a role in invadopodia formation and activity, it is possible that cancer cells with decreased KISS1R or β-arrestin2 expression are unable to form invadopodia which in turn inhibits extravasation and metastasis.

3.3 Future Directions

Our research has revealed a possible mechanism by which KISS1R signaling *via* β-arrestin2 promotes invadopodia formation (Figure 3.1). However, further research must be performed to fully understand kisspeptin-10 mediated effects of invadopodia precursor activity and localization. Previous studies, have reported conflicting roles for the phosphorylation status of cortactin and subsequent invadopodia activity. Several studies have reported that increase in Src mediated cortactin phosphorylation at residue Y421 is required for invadopodia activity (24, 25, 27). We observed that kisspeptin-10 stimulation caused a decrease in cortactin Y421 phosphorylation leading to increased invadopodia formation. These findings suggest that decreased cortactin phosphorylation is associated with increased cortactin activity which has also been reported by two previous studies, Martinez-Quiles *et al.* (2004) and Meiler *et al.* (2012) (21, 22). Martinez-Quiles *et al.* (2004) demonstrated that extracellular-regulated kinase (ERK) induces cortactin serine/threonine

phosphorylation necessary for cortactin N-WASP binding and ensuing actin polymerization. Consequently, Src phosphorylation of cortactin residue Y421 inhibited the ability of cortactin to bind to N-WASP and promote actin polymerization (21).

It has been established that KISS1R signaling leads to β-arrestin2 dependent activation of ERK1/2 (15, 50). Our laboratory has previously demonstrated that kisspeptin-10 stimulation causes transactivation of EGFR, a well-known ERK activator (8, 51). Additionally, EGFR has been reported to bind to and increase activity of protein tyrosine phosphatase 1B (PTP1B), the phosphatase that causes dephosphorylation of cortactin Y421 (52, 53). Therefore, it is possible that kisspeptin-10 stimulation may lead to ERK activity though β-arrestin2 or EGFR transactivation, leading to subsequent cortactin mediated invadopodia formation. Hence, further research must be performed to evaluate such a mechanism, specifically whether kisspeptin-10 signaling leads to increased ERK and PTP1B activity in breast cancer cells.

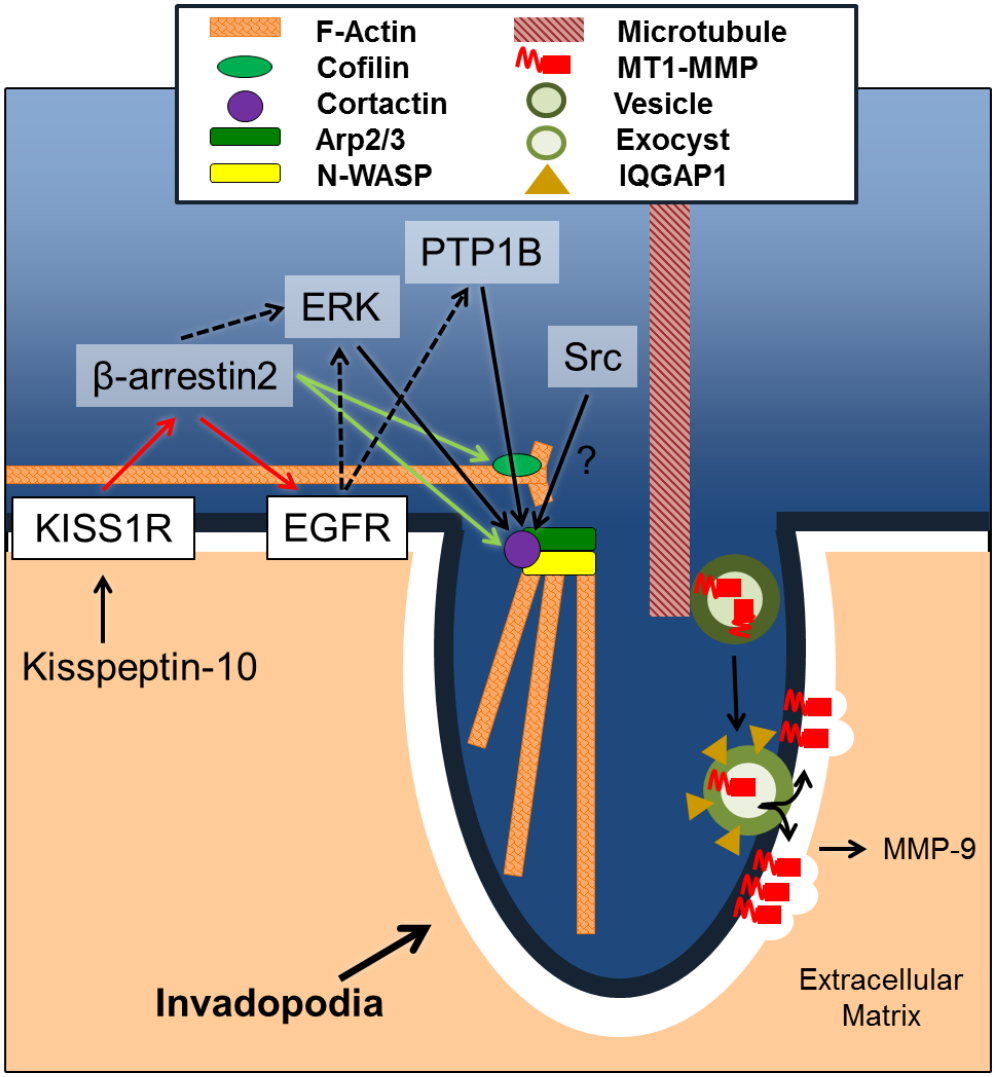

Figure 3.1 Proposed mechanism of KISS1R mediated invadopodia formation. This study (green lines) revealed that kisspeptin-10 stimulation of KISS1R increases cortactin and cofilin activity via β-arrestin2**.** Previous work in the Bhattacharya laboratory (red lines) has demonstrated that KISS1R transactivates EGFR via a β-arrestin2 dependent mechanism. Consequently, kisspeptin-10 signaling may lead to ERK and PTP1B activity, subsequent cortactin mediated actin polymerization and invadopodia formation.

3.4 Conclusion

Our results demonstrate for the first time that KISS1R signaling regulates human breast cancer metastasis. *In vitro* and *in vivo* experimentation has demonstrated that KISS1R expression plays a role in events that both regulate breast primary tumor development, such as angiogenesis, as well as events that that dictate metastasis, such as invadopodia formation and extravasation. Identifying KISS1R as a regulator of these events poses an intriguing therapeutic possibility; developing a therapeutic to inhibit KISS1R signaling or expression could both decrease breast primary tumor progression as well as inhibit breast cancer metastasis. Thus, this study has identified KISS1R as a novel therapeutic target for prevention of human breast cancer metastasis.

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Appendices

Animal Approval:

Western

AUP Number: 2012-015
PI Name: Bhattacharya, Moshmi
AUP Title: Beta-arrestins and Ral Signalling in Breast Cancer Metastasis

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2012-015 has been approved.

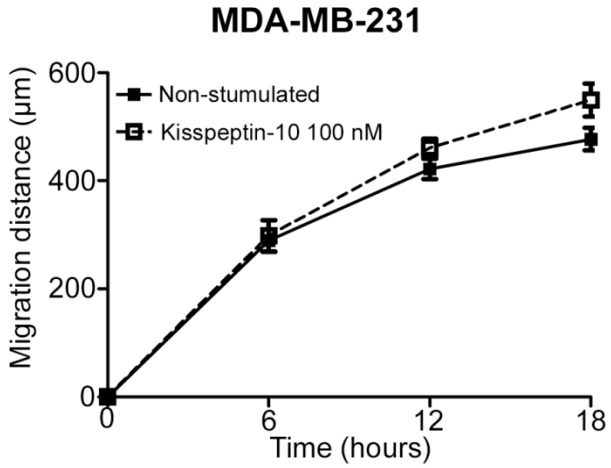
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: [redacted]
on behalf of the Animal Use Subcommittee

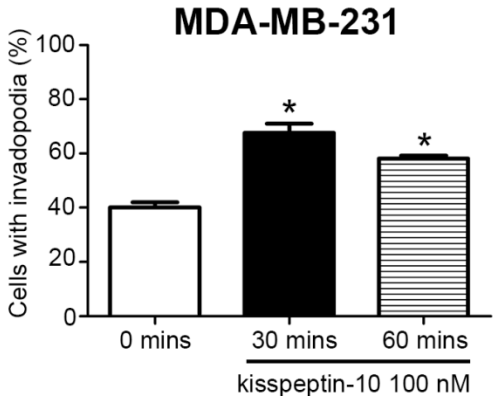
Signature removed for thesis submission.

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, [redacted]

Supplementary Figures



Supplementary Figure 1. Kisspeptin-10 stimulation did not affect scratch wound closure. Treatment with kisspeptin-10 100 nM did not significantly increase migration of MDA-MB-231 breast cancer cells as assessed using the scratch assay (n=4).



Supplementary Figure 2. Kisspeptin-10 stimulation causes peak invadopodia formation at 30 minutes. MDA-MB-231 breast cancer cells treated with kisspeptin-10 100 nM caused increased invadopodia formation at 30 and 60 minutes (n=3-6). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Columns represent percent of cells with invadopodia ± SEM.

Cameron Goertzen
Curriculum Vitae

Education:

University of Western Ontario, MSc. 2014, Department of Physiology & Pharmacology, Supervisor: Dr. Moshmi Bhattacharya. (Entrance Average 89%)

University of Western Ontario, BMSc. 2012 (Western Scholar), Honors Specialization in Pathology & Toxicology with distinction.

Publications:

Goertzen C G-F, Dragan M, Hess D A, Tuck A B, Babwah A V, Bhattacharya M. KISS1R Signaling Promotes Human Breast Cancer Invadopodia Formation and Metastasis. *Cancer Research (In Submission)*

Goertzen C G-F, Cvetkovic D, Bhattacharya M. Quantification of Breast Cancer Cell Invasiveness Using a Three-dimensional (3D) Model. *Journal of Visualized Experiments, 2014*

Research Experience:

June 2014 Oncology Research Day: **Goertzen C**, Dragan M, Hess D, Tuck A, Bhattacharya M. Role of KISS1R in Breast Cancer Metastasis. -Platform Presentation

March 2014 London Health Research Day: **Goertzen C**, Dragan M, Hess D, Tuck A, Bhattacharya M. Role of KISS1R in Breast Cancer Metastasis.-Platform Presentation (1st Place Platform Presentation)

November 2013 Physiology & Pharmacology Research Day: **Goertzen C**, Dragan M, David Hess, Alan Tuck, Bhattacharya M. Molecular Regulation of KISS1R in Breast Cancer Cell Invasion and Metastasis.-Poster Presentation

June 2013 Oncology Research & Education Day: **Goertzen C**, Cvetkovic D, Dragan M, Babwah A, Bhattacharya M. Molecular Regulation of KISS1R Induced Breast Cancer Invasion.-Poster Presentation

April 2013 American Association of Cancer Research Annual Meeting 2013: **Goertzen C**, Cvetkovic D, Dragan M, Babwah A, Bhattacharya M. Molecular Regulation of KISS1R Induced Breast Cancer Invasion.-Poster Presentation

March 2013 London Health Research Day: **Goertzen C**, Cvetkovic D, Dragan M, Bhattacharya M. Molecular Regulation of KISS1R Induced Breast Cancer Invasion.-Poster Presentation

March 2012 Western Undergraduate Research Journal Forum: **Goertzen C**, Alturkustani M, Derry K, Hammond R. Digital Quantitative Pathology of Carotid Atheromas: 3D Correlative Studies with Ultrasound, PET/CT and MRI. - Poster & Podium presentation (Top Podium Presentation)

February 2012 Annual Pathology & Toxicology Research day: **Goertzen C**, Alturkustani M, Derry K, Hammond R. Digital Quantitative Pathology of Carotid Atheromas: 3D Correlative Studies with Ultrasound, PET/CT and MRI. - Poster presentation (Second Prize for Poster Presentation)

Awards & Scholarships:

Graduate:

- 2014 1st Place Platform Presentation: London Health Research Day
- 2013-14 CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT) Trainee Studentship
- 2013-14 Translational Breast Cancer Research Studentship
- 2012-14 Western Graduate Research Scholarship
- 2012-13 Translational Breast Cancer Research Studentship

Undergraduate:

- 2012 Top Podium Presentation: Western Undergraduate Research Journal Forum
- 2012 Second Prize for Outstanding Poster Presentation: Annual Pathology & Toxicology Research day
- 2009, 2010, 2011, 2012 Dean's Honor List
- 2010, 2011 Excellence in Leadership Award Bronze
- 2010 Western Foot Patrol Male Patroller of the Year
- 2010 Accomplishment in Individual Leadership from the UWO Leadership and Mentorship Program
- 2008 J. Curtis Collard Memorial Trophy and Scholarship
- 2008 St. Davids & District Lions Club Award
- 2008 Meridian Credit Union Award
- 2008 Niagara District Secondary School Subject Award in Calculus & Vectors, Physics, Data Management, and Chemistry
- 2008 Ontario Scholar

Employment:
2012-14 Graduate Teaching Assistantship, University of Western Ontario
2009-11 HomeSense: Sales Associate/ Warehouse Receiver
2007-08 Leon's Home Furniture: Warehouse Associate
2006 Minor Brothers Farm and Country: Warehouse Associate

Volunteer Experience:
2013-2014 Society of Graduate Students (SOGS), Department of Physiology and Pharmacology Representative
2009-2014 Western Foot Patrol
2011-2012 Department of Pathology, UWO, Prospective Student Information Assistance
2010, 2012 Leave the Pack Behind
2011 Off to Western
2009-10 UWO Event Staff
2009-10 UWO Pre-Med Society: London Food Bank

Certifications:
- 2013 Basic Animal Care and Handling
- 2012 Biosafety Training
- 2012 Comprehensive WHMIS Training
- 2012 Laboratory Safety – Hazardous Waste
- 2012 Radiation Safety Awareness
- 2011 Health & Safety Orientation
- 2011 Safe Campus Community
- 2011 Accessibility at Western
- 2010 Canadian Red Cross: Standard First Aid with CPR/AED Level C