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# KISS1R Induces Human Mammary Epithelial Cell Invasiveness and Promotes Breast Cancer Cell Migration and Invasion via IQGAP1

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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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# KISS1R INDUCES HUMAN MAMMARY EPITHELIAL CELL INVASIVENESS AND PROMOTES BREAST CANCER CELL MIGRATION AND INVASION *VIA* IQGAP1

(Thesis format: Monograph)

By

Donna Cvetković

Graduate Program in Physiology

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

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**KISS1R Induces Human Mammary Epithelial Cell Invasiveness and Promotes Breast Cancer Cell Invasion *via* IQGAP1**

is accepted in partial fulfillment of the
requirements for the degree of
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**ABSTRACT**

Kisspeptins (KP), peptide products of the kisspeptin-1 (*KISS1*) gene are the endogenous ligands for a G protein-coupled receptor (KISS1R). *KISS1* acts as a metastasis suppressor in numerous human cancers. However, recent studies have demonstrated that an increase in *KISS1* and *KISS1R* expression in human breast tumors correlates with higher tumor grade and metastatic potential. We have previously shown that KP-10, the most potent KP, stimulates invasion of estrogen receptor (ER)-negative breast cancer cells *via* transactivation of the epidermal growth factor receptor (EGFR). Here, I report that KP-10 treatment of the ER-negative non-malignant mammary epithelial MCF10A cells, or stable expression of KISS1R in MCF10A and SKBR3 breast cancer cells stimulated cell invasiveness. KISS1R expression in these cells induced a partial epithelial-to-mesenchymal transition (EMT)-like phenotype. However, KP-10 had no effect on migration and invasion of the ER-positive T47D and MCF7 breast cancer cells. Furthermore, KP-10 stimulated EGFR transactivation in the ER-negative, but not in the ER-positive cells. KP-10-stimulated cell migration, invasion and EGFR transactivation were ablated upon stable expression of ERα in the ER-negative MDA-MB-231 cells. Lastly, I found that KISS1R was localized at the leading edge of motile cells, where it co-localized with the actin scaffolding protein, IQGAP1. Furthermore, I identified IQGAP1 as a novel binding partner of KISS1R and have demonstrated that KISS1R regulates breast cancer cell migration and invasion in an IQGAP1-dependent manner. Overall, these data reveal for the first time that the ER status of mammary cells may dictate whether KISS1R signaling pathway may be a novel target for breast cancer metastasis.

**Keywords:** Breast cancer, metastasis, cell migration, cell invasion, G protein-coupled receptor (GPCR), kisspeptin (KP), KP receptor (KISS1R), epidermal growth factor receptor (EGFR), epithelial-to-mesenchymal transition (EMT), three dimensional (3D) cultures, IQGAP1.

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Lastly, this work would not have been possible without the funds from the Translational Breast Cancer Research Unit (TBCRU) and the CIHR Strategic Training Program (STP) in Cancer Research and Technology Transfer (CaRTT) from the London Regional Cancer Research Institute.


*“Success is not the result of spontaneous combustion. You must set yourself on fire.”*

*-Reggie Leach*

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*Unless otherwise noted, all figures are original works by Donna Cvetković.*

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Angiotensin II | (Ang II) |
| AP-2α | Activator protein 2 alpha |
| BRCA1 | Breast cancer type 1 susceptibility protein |
| cDNA | Complementary deoxyribonucleic acid |
| CHO | Chinese hamster ovary |
| CSC | Cancer stem cells |
| CXCR4 | Chemokine receptor 4 |
| DAG | Diacylglycerol |
| DCIS | Ductal carcinoma *in situ* |
| $E_2$ | Estradiol |
| 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 |
| FAK | Focal adhesion kinase |
| FBS | Fetal bovine serum |
| FOX | Forkhead box |
| FSH | Follicle stimulating hormone |
| GATA | Trans-acting T-cell-specific transcriptional factor |
| G protein | Guanine-nucleotide binding protein |
| GnRH | Gonadotropin-releasing hormone |
| GPCR | G protein-coupled receptor |
| GRK | G protein-coupled receptor kinase |
| HEK | Human embryonic kidney |
| hMEC | Human mammary epithelial cell |
| HPG | Hypothalamic-pituitary-gonadal |
| HUVEC | Human umbilical vein endoethelial cell |
| IP(s) | Inositol phosphate(s) |

| | |
|---|---|
| $IP_3$ | Inositol-(1,4,5)-trisphosphate |
| IQGAP1 | IQ motif containing GTPase activating protein 1 |
| KP(s) | Kisspeptin(s) |
| KISS1R | Kisspeptin receptor |
| LCIS | Lobular carcinoma *in situ* |
| LH | Luteinizing hormone |
| LPA | Lysophosphatidic acid |
| LPA-R | Lysophosphatidic acid receptor |
| MAPK | Mitogen-activated protein kinase |
| MET | Mesenchymal-to-epithelial transition |
| MMP | Matrix metalloproteinase |
| MMTV | Mouse mammary tumor virus |
| mRNA | Messenger ribonucleic acid |
| P-234 | Peptide-234 |
| PAR-1 | Protease-activated receptor-1 |
| PI3K | Phosphatidylinositol-3-kinase |
| $PIP_2$ | Phosphatidylinositol bisphosphate |
| $PIP_3$ | Phosphatidylinositol (3,4,5)-trisphosphate |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PyMT | Polyoma virus middle T antigen |
| PR | Progesterone receptor |
| q-PCR | Quantitative-polymerase chain reaction |
| RNAPII | Ribonucleic acid polymerase II |
| RT-PCR | Real time-polymerase chain reaction |
| RTK(s) | Receptor tyrosine kinases (s) |
| SH2 | Src homology 2 |
| shRNA | Short-hairpin RNA |
| siRNA | Short interfering RNA |
| Sp1 | Specificity 1 protein |
| TGF | Transforming growth factor |

| | |
|---|---|
| TIMP | Tissue inhibitor of metalloproteinase |
| TNBC | Triple negative breast cancers |
| VEGF | Vascular endothelial growth factor |

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# CHAPTER 1: INTRODUCTION

**1.1. Breast Cancer**

Breast cancer is the second leading cause of cancer-related deaths in Canadian women, with the majority of these deaths resulting from metastasis of cancer to other tissues (Canadian Cancer Society, 2012). In 2012, an estimated 22,700 Canadian women will be diagnosed with breast cancer and 5,100 will suffer from cancer-related deaths. On average, 62 Canadian women are diagnosed with breast cancer each day and 14 women die of it every day (Canadian Cancer Society, 2012). When breast carcinomas are confined to breast tissue, cure rates exceed 90%; however as cancer cells colonize surrounding or distant tissues, long-term survival shows a pronounced decline (Martin *et al.*, 2005; Gupta and Massague, 2006; Geiger and Peeper, 2009). The dissemination of cancer cells to secondary sites, resulting in disruption of normal tissue function is the principal cause of fatality and the main impediment to improving prognosis in breast cancer patients (Chambers *et al.*, 2002; Martin *et al.*, 2005).

Although many of the exact mechanisms and etiologies underlying the development of human breast cancer are not fully understood, the most commonly proposed model posits that invasive breast cancer initiates from the sequential and compounded malignant transformation of epithelial cells that comprise either the mammary ducts (ductal carcinoma *in situ*, DCIS) or the lobules of the mammary glands (lobular carcinoma *in situ*, LCIS) (Sakorafas and Tsiotou, 2000; Cichon *et al.*, 2010). The early stages of the abnormal growth are typically classified as benign breast disease (Sakorafas and Tsiotou, 2000; Cichon *et al.*, 2010). Abnormal proliferation progresses through stages during which the epithelium becomes increasingly proliferative, without acquiring atypical characteristics (proliferative disease without atypia) (Cichon *et al.*,

2010). Atypical hyperplasia can manifest as either ductal or lobular forms (atypical ductal hyperplasia or atypical lobular hyperplasia, respectively) (Cichon *et al.*, 2010), before it develops into DCIS or LCIS (Cichon *et al.*, 2010). These abnormal epithelial cells remain confined to the primary site of origin until they acquire additional genetic alterations that render these cells capable of evading anti-growth and anti-apoptotic cues and thus continue to proliferate in perpetuity (Sakorafas and Tsiotou, 2000). Progressive growth and dedifferentiation produce cells that have acquired the ability to invade into neighboring tissues or to more distant organs (Sakorafas and Tsiotou, 2000) **(Figure 1.1)**.

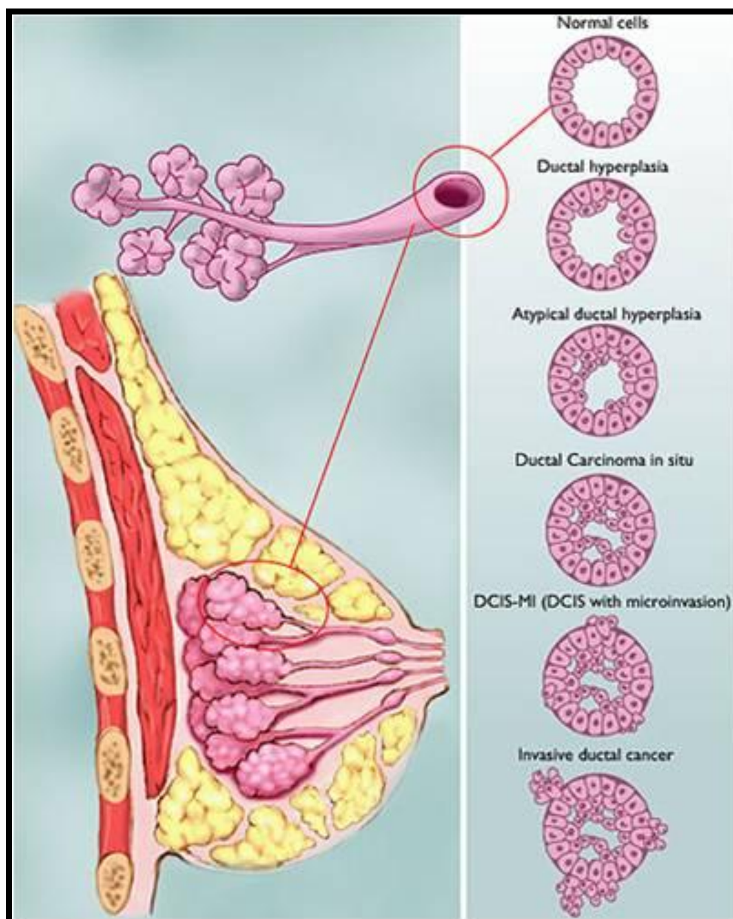


**Figure 1.1. Breast cancer progression.** Normal luminal epithelial cell growth becomes unrestricted, resulting in ductal hyperplasia. Cells begin to lose their normal morphology, progressing to an appearance indicative of ductal carcinoma *in situ* (DCIS). Progressive growth and dedifferentiation may result in cells with invasive capabilities and metastasis to distant sites. Schematic is adapted from the National Institutes of Health (NIH) website (http://health.nih.gov/topic/BreastCancer/).

### *1.1.1. Structure of the Mammary Gland*

The human mammary breast epithelium is composed of a series of branched parenchymal ductal networks that, during lactation, drain milk-producing alveoli into the nipple (Cichon *et al.*, 2010). The milk-producing structures of the mammary gland are collections of multiple small acini at the distal ends of the ducts and are known as terminal duct lobular units, and the entire epithelium is embedded within a collagenous surrounding stroma (Cichon *et al.*, 2010; Russo and Russo, 2011).

There are two main lineages of epithelial cells within the mammary epithelium: the luminal (or apical) cells that line the central lumen and the underlying myoepithelial cells that are adjacent to the basement membrane (Perou *et al.*, 2000; Russo and Russo, 2011; Valastyan, 2012). The basal layer of the mammary ductal epithelium is composed of myoepithelial cells (Perou *et al.*, 2000; Russo and Russo, 2011; Valastyan, 2012). The two cell types may be distinguished by immunohistochemical analysis; luminal epithelial cells stain with antibodies against simple cytokeratins 8/18/19, whereas basal epithelial cells stain with antibodies against cytokeratin 5/6/14 (Petersen and Polyak, 2010). In culture, luminal cells display standard epithelial cobblestone morphology, with junctional complexes and apicobasal polarity, and express luminal/epithelial markers such as E-cadherin (Perou *et al.*, 2000; Russo and Russo, 2011; Valastyan, 2012), whereas basal cells exhibit expression of mesenchymal markers, such as N-cadherin, vimentin, fibronectin and possess a spindle-like morphology (Perou *et al.*, 2000; Russo and Russo, 2011; Valastyan, 2012) **(Figure 1.2)**.

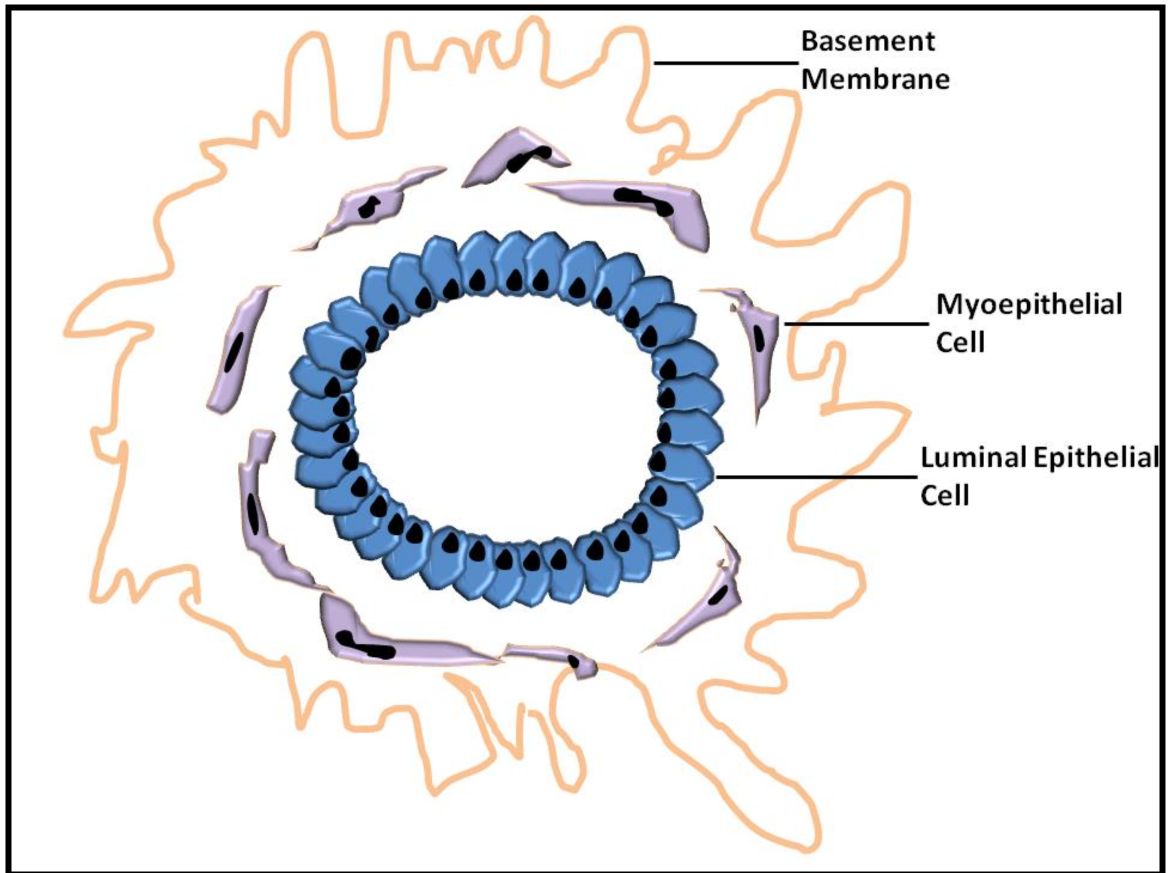


**Figure 1.2. Structure of a normal mammary acinus.** The mammary epithelium possesses an apico-basal polarized architecture surrounding a hollow lumen, surrounded by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells.

### *1.1.2. Breast Carcinoma Subtypes*

Breast cancer presents as a molecularly and phenotypically heterogeneous disease, displaying a variety of histopathological features, genetic markers and diverse prognostic outcomes (Perou *et al.*, 2000). Advances in microarray technology and transcriptional profiling techniques have led to improvements in tumor classification (Perou *et al.*, 2000). Gene expression profiling has enabled a subdivision of tumors into five individual subclasses (known as the Sorlie–Perou subtypes) found to convey a distinct prognostic and biological message in breast cancer above and beyond established clinical markers (Perou *et al.*, 2000; Sorlie *et al.*, 2001). The five groups are: luminal A, luminal B, basal-like, ErbB2-positive (HER2-positive) and normal breast-like subtypes (Perou *et al.*, 2000; Sorlie *et al.*, 2001). The majority of breast cancers arise from the luminal epithelium of the small mammary ducts (Perou *et al.*, 2000). Most newly diagnosed breast cancers are classified as the luminal A subtype, which are typically estrogen receptor (ER)-positive, low-grade, weakly proliferative and invasive and have a favorable prognosis (Perou *et al.*, 2000). Although the cellular origin of luminal A tumors remains unresolved, these tumors are termed luminal because they display epithelial phenotypic markers, such as E-cadherin and retain some degree of epithelial organization (Perou *et al.*, 2000). Luminal B tumors are also mostly ER-positive, but may express low levels of hormone receptors and usually are of high-grade and have a higher proliferation rate (Perou *et al.*, 2000). The basal-like subtype, on the other hand, is often characterized by triple-negative tumors (ER-, progesterone receptor (PR)-, and HER2-negative). The ErbB2-positive subtype shows amplification and high expression of the *ErbB2* gene (also known as *HER2*, which is often constitutively active) (Perou *et al.*,

2000). Lastly, there is the normal breast-like subtype, which displays expression of genes generally present in the non-epithelial cell types, such as adipose tissue, shows strong expression of basal epithelial genes, and minimal to non-existent expression of luminal epithelial genes (Perou *et al.*, 2000). However, it is unclear whether the latter subtype is a unique group or represents poorly sampled tissue (Perou *et al.*, 2000; Sorlie *et al.*, 2001). Given the heterogeneity and diversity of breast cancers, with clinical behavior that is difficult to predict, prescribing an adequate treatment must take into account numerous factors such as patient's age, previous treatments, and co-morbidities. Moreover, the molecular profile of the tumor usually takes the priority when making the ultimate decision. Therefore, ER, PR and HER2 expressions help determine which treatment will be most effective in combating the disease (Perou *et al.*, 2000; Sorlie *et al.*, 2001).

### *1.1.3. Estrogen Receptor Status*

The steroid hormone estrogen plays a critical role in the development of the mammary epithelium during puberty (Perou *et al.*, 2000; Yan *et al.*, 2010; Stingl, 2011; Guttilla *et al.*, 2012). Considering the role estrogen has in promoting mammary gland development, it is not surprising that there is a strong positive correlation between lifetime exposure to estrogen and breast cancer risk (Perou *et al.*, 2000; Yan *et al.*, 2010; Stingl, 2011; Guttilla *et al.*, 2012). The hormone estradiol ($E_2$), acting through ERα, is required for the normal growth and development of the mammary ductal network (Stingl, 2011; Rosen, 2012). ERα is an important prognostic indicator in breast cancer (Parl *et al.*, 1984), given that breast tumors are typically categorized as being ER-positive or ER-negative (Stingl, 2011). A significant fraction of cells within a luminal A tumor express

ERα, and $E_2$/ERα signaling promotes and sustains proliferation in these cells (Parl *et al.*, 1984; Perou *et al.*, 2000; Guttilla *et al.*, 2012). The expression of and dependence on ERα in luminal A cancers form the rationale for hormonal therapies involving anti-estrogens or aromatase inhibitors (Parl *et al.*, 1984; Perou *et al.*, 2000; Guttilla *et al.*, 2012). The $E_2$/ERα signaling pathway promotes differentiation of mammary epithelia along a luminal/epithelial lineage, in part through transcriptional stimulation of transcription factors such as the trans-acting T-cell-specific transcriptional factor (GATA3) and Forkhead box (FOX) (Eeckhoute *et al.*, 2007; Yan *et al.*, 2010; Guttilla *et al.*, 2012; Rosen, 2012). GATA3 is required for luminal differentiation in normal breast epithelia and, furthermore, studies have shown that ERα and GATA3 stimulate each other (Eeckhoute *et al.*, 2007; Yan *et al.*, 2010). FOXA1 is another ERα-interacting transcription factor that is required to establish the luminal lineage in mammary epithelia and specifically promotes ductal growth in mice (Eeckhoute *et al.*, 2007; Yan *et al.*, 2010). FOXA1 promotes accessibility of estrogen-response elements for ERα binding and stimulates ERα gene expression (Eeckhoute *et al.*, 2007; Yan *et al.*, 2010). In turn, $E_2$ appears to stimulate FOXA1 expression in breast cancer cells (Eeckhoute *et al.*, 2007; Yan *et al.*, 2010). Hence, ERα, FOXA1 and GATA3 are all favorable prognostic indicators in breast cancer (Eeckhoute *et al.*, 2007; Yan *et al.*, 2010). Given the dependence of mammary development on ERα signaling, any perturbations within this signaling pathway are likely to contribute to abnormalities in the homeostatic maintenance of mammary tissue, ultimately leading to breast cancer.

### *1.1.4. Metastasis*

Metastasis is the spread of a disease from one organ or part to another non-adjacent organ or part (Fidler, 2003; Eccles and Welch, 2007; Geiger and Peeper, 2009). Metastasis consists of a sequential, multistep cascade that must be completed to generate a metastatic tumor (Fidler, 2003; Eccles and Welch, 2007; Geiger and Peeper, 2009). Continual growth and survival of the tumor requires adequate blood supply to support its metabolic requirements, which is achieved through the process of angiogenesis (Chambers *et al.*, 2002). The poorly organized architecture and increased permeability of the new vasculature allows for cancerous cells to leave the primary site and enter the systemic circulation and/or the lymphatic system through the process of intravasation (Chambers *et al.*, 2002).

At the cellular level, several processes facilitate motility and invasion of cancer cells, including cytoskeletal reorganization of the filamentous actin, focal adhesion formation and a transition from an epithelial to spindle-like morphology (Jiang *et al.*, 2009). The initial stages of cell motility are characterized by the formation of broad cell membrane protrusions (lamellipodia) in the direction of the extracellular stimuli and the attachment of thin actin-containing membrane projections (filopodia) to the extracellular matrix (ECM) at sites of focal adhesions (Jiang *et al.*, 2009). These events are followed by contraction of the intracellular filamentous actin with subsequent disassembly of the focal adhesion at the rear of the cell to ultimately allow the cell to be dragged forward in a directional manner (Jiang *et al.*, 2009). Cytoskeletal reorganization and cell movement are regulated by several intracellular signaling pathways that are yet to be fully elucidated. In addition to the capacity to migrate, tumor cells must acquire the ability to

invade through surrounding tissues and vessels in order for metastasis to take place (Geiger and Peeper, 2009). Proteases such as matrix metalloproteinases (MMPs) are recruited to the leading edge of the cell, where they degrade and remodel the ECM (Geiger and Peeper, 2009). The cells that survive in the circulation might extravasate and settle in the surrounding tissue, where they must initiate and maintain growth for a macroscopic tumor to form (Chambers *et al.*, 2002). Although considerable progress has been made in early detection of breast cancer and, consequently, lowering mortality, metastatic breast cancer is a terminal disease and treatment goals focus on prolonging survival and providing palliative care (Chambers *et al.*, 2002; Gupta and Massague, 2006; Eccles and Welch, 2007; Geiger and Peeper, 2009; Tkaczuk, 2009).

### *1.1.5. Epithelial-to-Mesenchymal Transition*

An understanding of the molecular and cellular underpinnings of metastasis is required to develop targeted treatments against metastatic cells. At present, considerable attention is being directed towards epithelial-to-mesenchymal transition (EMT) as the probable first step in the complex process of metastasis (Hugo *et al.*, 2007; Sarrio *et al.*, 2008; Micalizzi *et al.*, 2010; Yan *et al.*, 2010; Creighton *et al.*, 2012; Guttilla *et al.*, 2012). EMT is defined as a multistep process, resulting in culmination of protein modifications and transcriptional events in response to a defined set of extracellular stimuli leading to a long term and sometimes reversible cellular changes (Hugo *et al.*, 2007). EMT has been described over the past decade as a process that is required for the remodeling of cells and tissues during embryogenesis, wound healing, and during the acquisition of malignant traits by carcinoma cells (Reya *et al.*, 2001; Hugo *et al.*, 2007;

Mani *et al.*, 2008; Polyak and Weinberg, 2009). One of the essential features differentiating embryonic and tumorigenic or oncogenic EMT events is that the tumorigenic processes involve genetically abnormal cells that progressively lose their responsiveness to normal growth-regulatory cues and acquire the characteristics associated with the hallmarks of cancer (Reya *et al.*, 2001; Mani *et al.*, 2008; Polyak and Weinberg, 2009). EMT is mediated by specific molecular signals that promote the loss of cell-cell junctions, cell-ECM adhesion and reorganization of the actin cytoskeleton (Gupta and Massague, 2006; Hugo *et al.*, 2007; Sarrio *et al.*, 2008; Stingl, 2011; Guttilla *et al.*, 2012). This results in the loss of the apical polarity associated with epithelial cells and gain of mesenchymal characteristics such as spindle-shaped morphology and a high degree of motility (Gupta and Massague, 2006; Hugo *et al.*, 2007; Sarrio *et al.*, 2008; Stingl, 2011; Guttilla *et al.*, 2012) **(Figure 1.3)**. The genetic and epigenetic changes resulting in EMT are only one source of morphologic heterogeneity observed within tumors (Reya *et al.*, 2001; Mani *et al.*, 2008; Polyak and Weinberg, 2009).

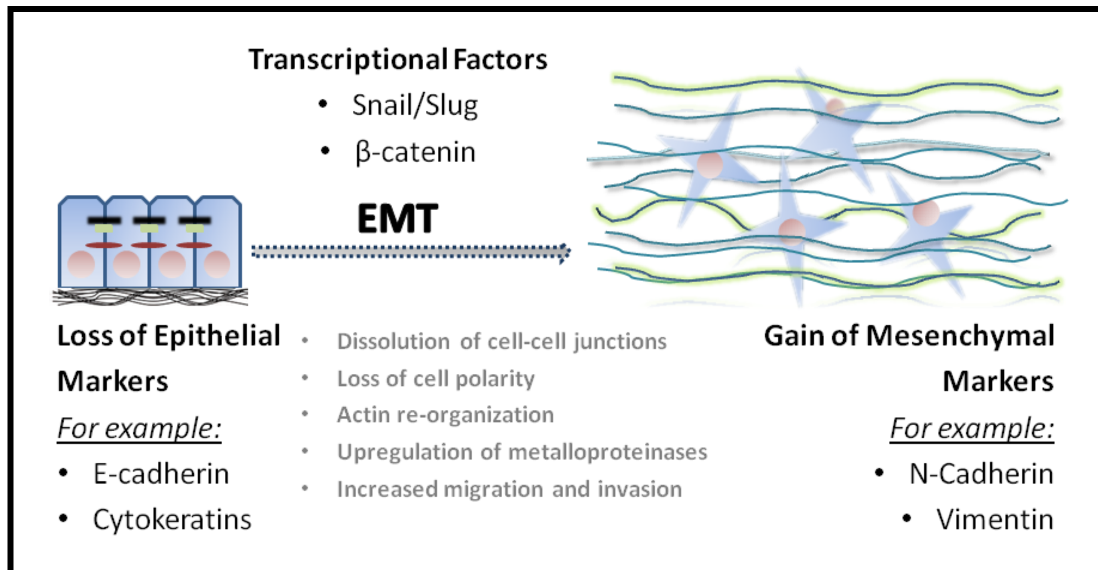


**Figure 1.3. Epithelial-to-mesenchymal transition (EMT).** EMT occurs when epithelial cells lose their epithelial cell characteristics, including dissolution of cell-cell junctions, loss of apico-basal polarity, and acquire a mesenchymal phenotype, characterized by actin cytoskeleton re-organization and stress fiber formation, increased migration and invasion.

Thus far, a large body of research has described stem cells in normal tissues, capable of self-renewal, whilst concurrently generating committed progenitor cells, whose descendants may eventually differentiate and carry out tissue-specific functions (Reya *et al.*, 2001). Moreover, recent studies have provided evidence of self-renewing, stem-like cells within tumors, which have been called cancer stem cells (CSCs) (Reya *et al.*, 2001). Due to their ability to generate new tumors, these cells have been termed tumor-initiating cells (Reya *et al.*, 2001). During the process of metastasis, which is often enabled by EMT, disseminated cancer cells would seem to require self-renewal capability, similar to that exhibited by stem cells (Reya *et al.*, 2001; Mani *et al.*, 2008; Polyak and Weinberg, 2009).

Multiple extracellular cues can initiate EMT events and there is a significant crosstalk among the downstream intracellular signaling pathways and transcription factors that choreograph this complex process (Reya *et al.*, 2001; Hugo *et al.*, 2007; Polyak and Weinberg, 2009). The transcription factors such as Slug and Snail (mesenchymal markers) induce EMT by repressing the transcription of E-cadherin (epithelial marker) in numerous cancers, including breast cancer (Hugo *et al.*, 2007). Inhibition of E-cadherin transcription is often the first step triggering EMT as this releases β-catenin, which is subsequently lost from the cell membrane and translocates to the nucleus to participate in EMT signaling events (Hugo *et al.*, 2007). Furthermore, Snail family proteins repress E-cadherin transcription by binding the regulatory segments on the E-cadherin promoter (Hugo *et al.*, 2007). Reduction or absence of E-cadherin expression is often accompanied by reciprocally increased expression of N-cadherin (a mesenchymal marker) (Hugo *et al.*, 2007). N-cadherin has been shown to promote breast

cancer cell invasion (Hugo *et al.*, 2007). The relevance of the mesenchymal phenotype and the utility of multiple mesenchymal markers such as N-cadherin and vimentin to aid in identifying EMT events, were reinforced by Zajchowski and colleagues, who found that vimentin and other mesenchymal gene products were part of a 24 gene signature predicting breast carcinoma cell invasiveness (Zajchowski *et al.*, 2001).

It is well established that ERα activation is mitogenic in that it promotes growth of the primary lesion, but is nevertheless able to keep EMT process in check (and therefore is anti-metastatic) up to a point (Guttilla *et al.*, 2012; Rosen, 2012). $E_2$/ERα antagonizes pathways that lead to EMT (Guttilla *et al.*, 2012; Rosen, 2012). For instance, transforming growth factor beta (TGF-β) has been shown to induce EMT in human mammary epithelial cells and overexpression of the EMT-inducing factor Snail in MCF7 cells increased TGF-β signaling and cell invasiveness, and decreased adhesion and ERα expression (Hajra *et al.*, 2002; Eeckhoute *et al.*, 2007; Creighton *et al.*, 2012; Guttilla *et al.*, 2012). More recently, Ye and colleagues examined the effects of either overexpression of ERα in ERα-negative breast cancer cell lines (MDA-MB-468, MDA-MB-231) or ERα knockdown in ERα-positive cell lines (MCF7, T47D) on the expression of Slug and Snail and the resulting phenotypes (Ye *et al.*, 2008; Ye *et al.*, 2010). Overexpression of ERα repressed Slug (but had no effect on Snail), increased protein expression levels of E-cadherin and induced cells to grow as adherent colonies with reduced invasiveness (Ye *et al.*, 2008; Ye *et al.*, 2010). In contrast, knockdown of ERα resulted in elevation of Slug expression, and subsequent loss of E-cadherin expression (Ye *et al.*, 2008; Ye *et al.*, 2010). Thus, ERα modulates EMT in breast cancer cells. Furthermore, in a recent study Prasad and colleagues provided clinical evidence in

support of Wnt/β-catenin to formation of invasive ductal carcinomas (Prasad *et al.*, 2009).

Growing evidence suggests that EMT is an essential regulator of cellular plasticity in carcinomas and has important roles in therapeutic resistance, tumor recurrence and metastatic progression (Reya *et al.*, 2001; Hugo *et al.*, 2007; Mani *et al.*, 2008; Polyak and Weinberg, 2009). Owing to its clinical importance of the EMT-induced processes, inhibition of EMT is an attractive therapeutic approach that could potentially have significant effects on the disease outcome.

## 1.2. Kisspeptins and Kisspeptin Receptor

### *1.2.1. Discovery and Distribution*

The metastasis of cancer cells hinges upon a series of choreographed cascade of events; hence, interruption of any step should effectively halt the process. Metastasis suppressors, defined by their abilities to inhibit metastasis without blocking orthotopic tumor growth are an attractive collection of contenders to treat metastasis (Beck and Welch, 2005). Over a decade ago, a new metastasis suppressor gene was identified and named *KISS1* gene in reference to its place of discovery - Hershey, Pennsylvania, the home of the famous Hershey Kisses (Lee and Welch, 1997a). The *KISS1* gene encodes a 145-amino acid protein, which is subsequently cleaved into a 54-amino acid fragment, which in turn may be cleaved by furin or prohormone convertases (deduced by the presence of pairs of basic residues flanking this sequence) into even shorter, biologically

active secreted peptides (10, 13, 14 amino acids long), collectively referred to as kisspeptins (KPs) (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001; Mead *et al.*, 2007) **(Figure 1.4)**. Currently, it is unclear whether the shorter forms are breakdown products (Kotani *et al.*, 2001). KPs are categorized as members of the Arg-Phe (RF)-amide family due to the C-terminal amidation site that leads to strong binding affinity with their receptor (Lee *et al.*, 1999; Clements *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001; Stafford *et al.*, 2002; Kutzleb *et al.*, 2005). In humans, reverse transcriptase polymerase chain reaction (RT-PCR) revealed *KISS1* mRNA to be present with high levels in the brain, breast, pancreas, placenta, testis, liver, heart and small intestine (Lee *et al.*, 1996; Muir *et al.*, 2001; Ohtaki *et al.*, 2001; Kirby *et al.*, 2010).

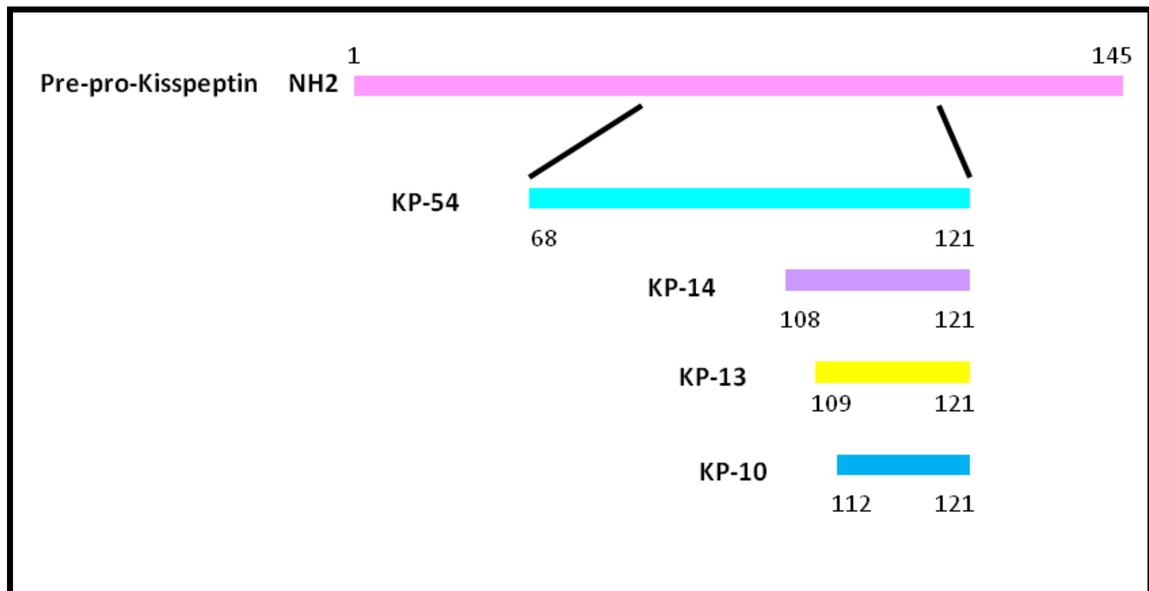


**Figure 1.4. The kisspeptins (KPs).** Cleavage of KP-145 results in the production of smaller peptides, designated KP-54 (metastin), KP-14, KP-13, and KP-10.

KP-10, the most potent KP, is the smallest active peptide comprised of the last ten amino acids of the full 145-amino acid peptide (Gutierrez-Pascual *et al.*, 2009). The structure of KP-10 is highly conserved across a range of species, differing from the human and primate sequence only by a single amino acid in rat, mouse, platypus, sheep, and cow (Kirby *et al.*, 2010). It was revealed through saturation binding experiments that KP-10 exhibited a $K_D$ of 1.0 ± 0.1nM (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001; Kirby *et al.*, 2010). Furthermore, KP-10 exhibited greater potency than KP-54 (5.47 ± 0.03nM), KP-14 (7.22 ±0.07 nM), or KP-13 (4.62 ± 0.02nM), with an $EC_{50}$ of 4.13 ± 0.02nM (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001; Kirby *et al.*, 2010).

Although the sequence for *KISS1* gene has been known since its initial discovery, it was not until 2001 that the peptide products of *KISS1* were identified as the endogenous ligands for the KP receptor (KISS1R; formerly known as AXOR12, GPR54 or hOT7T175) by three independent groups (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). The *KISS1R* mRNA displays similar tissue distribution as its ligand, with high levels expressed in the placenta, pituitary gland, pancreas, breast and spinal cord (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). *KISS1R* mRNA is also abundant in the heart, skeletal muscle, kidney, liver, and placenta, and also in regions of the central nervous system (Clements *et al.*, 2001). Radioligand binding of $^{125}$I-KP-14 was detected in aorta, coronary artery and umbilical vein (Mead *et al.*, 2007), suggesting expression of the KISS1R in the vasculature.

In a recent study, Roseweir and colleagues reported on the derivation of a KISS1R antagonist, termed Peptide-234 (P-234) (Roseweir *et al.*, 2009; Kirby *et al.*, 2010). P-234 was discovered by systematically substituting amino acid residues in the

KP-10 sequence and the resulting compounds were evaluated for their ability to block KP-10-induced inositol phosphate (IP) release in Chinese hamster ovary (CHO) cells stably expressing KISS1R (Roseweir *et al.*, 2009; Kirby *et al.*, 2010). P-234 contains seven residues conserved from KP-10, it showed an $IC_{50}$ of 7.0nM, and competed for the binding of $^{125}$I-KP-10 with an affinity of 2.7nM (Roseweir *et al.*, 2009; Kirby *et al.*, 2010). P-234 was demonstrated to inhibit the firing of gonadotropin-releasing hormone (GnRH) neurons in the brain of the mouse and to reduce pulsatile GnRH secretion in female pubertal monkeys (Pineda *et al.*, 2009; Roseweir *et al.*, 2009). Furthermore, P-234 inhibited the KP-10-induced release of luteinizing hormone (LH) in rats and mice and blocked the post-castration rise in LH in sheep, rats, and mice (Roseweir *et al.*, 2009). Therefore, the development of KISS1R antagonists provides a valuable tool to investigate the roles of the KP-10/KISS1R signaling pathway in physiological and pathophysiological states (Pineda *et al.*, 2009; Roseweir *et al.*, 2009; Kirby *et al.*, 2010).

### *1.2.2. KP/KISS1R Signaling*

KISS1R is a canonical G protein-coupled receptor (GPCR), which couples to the $G\alpha_{q/11}$ signaling pathway, activating phospholipase C (PLC), which results in phosphatidylinositol 4,5-bisphosphate ($PIP_2$) hydrolysis, followed by accumulation of inositol-(1,4,5)-trisphosphate ($IP_3$) and diacylglycerol (DAG) to cause subsequent calcium mobilization (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). Consequently, an increase in intracellular calcium concentration could induce hormone release as observed in the reproductive system, or mediate inhibition of cell proliferation (Stafford *et al.*, 2002), as it has been shown in some cancer cells (Lamprecht and Lipkin,

2001). Other signaling pathways activated by KP/KISS1R seem to be cell type-dependent, and proposed downstream mediators include protein kinase C (PKC), mitogen-activated protein kinases (MAPKs; such as extracellular signal-regulated kinase (ERK) 1/2 and p38), and phosphatidylinositol-3-kinase/Akt (Kotani *et al.*, 2001; Muir *et al.*, 2001). Thus, a unique pattern of activation of transduction signals selectively regulates biological functions by the KP/KISS1R system in a cell type-dependent manner **(Figure 1.5)**. The diversity of the pathways activated by the KP/KISS1R system to exert its distinct functions are yet to be fully understood and the mechanisms by which KP/KISS1R signals are yet to be resolved.

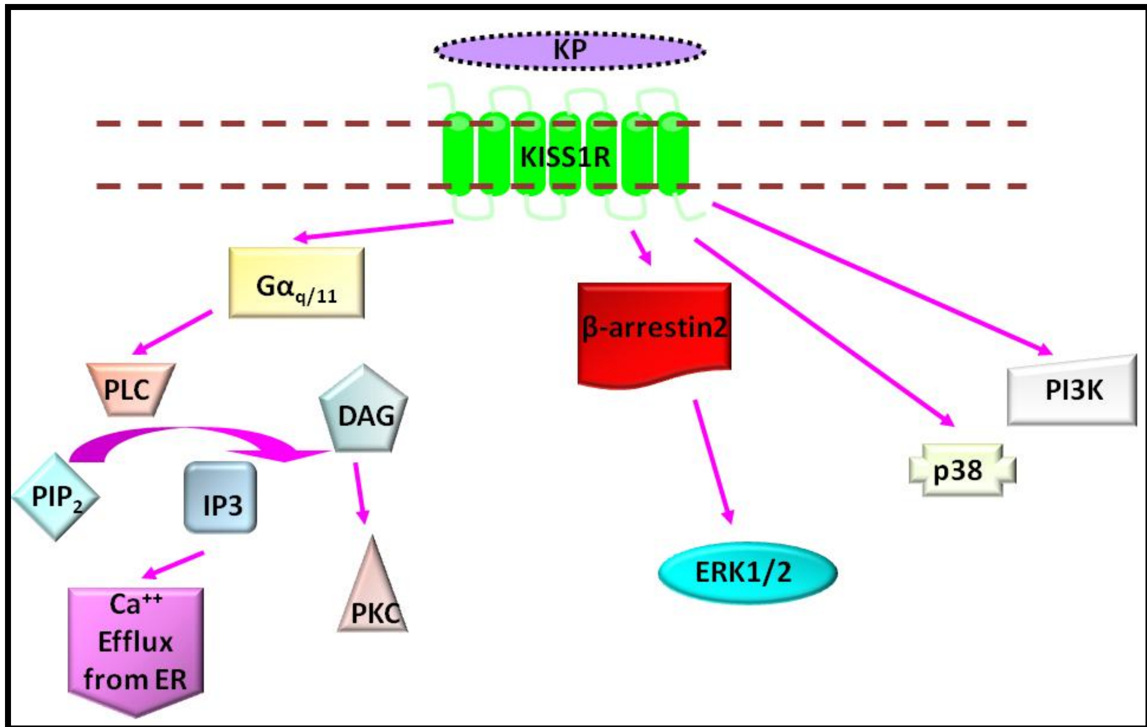


**Figure 1.5. Molecular signaling of the KPs *via* KISS1R.** KISS1R is a $G_{q/11}$ protein-coupled receptor (GPCR), resulting in the activation of phospholipase C (PLC), protein kinase C (PKC), and members of the mitogen-activated protein kinase (MAPK) pathway, including extracellular signal-regulated kinase (ERK)1/2, p38, and phosphatidylinositol-3-kinase (PI3K).

One of the principal mechanisms for switching off many GPCRs is homologous desensitization, a process that involves the co-ordinated actions of two families of proteins, the GPCR kinases (GRKs) and β -arrestins (Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998). GRK-mediated GPCR phosphorylation specifically prepares the activated receptor for arrestin binding (Luttrell and Lefkowitz, 2002). Arrestin binding to the receptor blocks further G protein-mediated signaling, targets receptor for internalization to the endosomes *via* clathrin-coated pits (for recycling) or lysosomes (for degradation), and redirects signaling to alternative G protein-independent pathways (Luttrell and Lefkowitz, 2002). Pampillo and colleagues were the first to demonstrate that KISS1R is constitutively associated with GRK2 and β-arrestins-1 and -2, and that these interactions are mediated through residues in the second intracellular loop and cytoplasmic tail of KISS1R (Pampillo *et al.*, 2009). Additionally, they showed that KISS1R undergoes GRK-dependent desensitization in human embryonic kidney (HEK) 293 cells and that β-arrestin-2 mediates KISS1R activation of ERK1/2 in MDA-MB-231 breast cancer cells (Pampillo *et al.*, 2009). Furthermore, recent studies by this group have also shown that β-arrestin-1 inhibits, whereas β-arrestin-2 and $G_{q/11}$ activate ERK1/2 in a co-dependent manner following KISS1R activation (Szereszewski *et al.*, 2010). Since KISS1R is emerging as a GPCR of immense clinical importance, understanding the molecular mechanisms that regulate KISS1R signaling upon receptor activation is necessary to evaluate its potential as a therapeutic target.

### *1.2.3. Physiological Roles of KP/KISS1R Signaling*

Since the discovery of the KPs and their receptor, numerous reports about KP/KISS1R signaling have appeared, ranging from reproductive endocrinology, cardiovascular physiology, in addition to cancer biology. The crucial role that KPs and their receptor play in the regulation of the reproductive axis was first indicated by observations of loss-of-function mutations in the KISS1R in some patients with idopathic hypogonadotropic hypogonadism (de Roux *et al.*, 2003; Seminara *et al.*, 2003) and confirmed in transgenic mouse models (Funes *et al.*, 2003; Seminara *et al.*, 2003; Kauffman *et al.*, 2007). KPs have since been identified as major regulators of the hypothalamic-pituitary-gonadal (HPG) axis, governing pubertal onset in an increasing number of species.

#### *1.2.3.1. Reproductive Endocrinology*

There is substantial evidence that the KISS1R signaling is required for the onset of puberty. KP activation of KISS1R exerts a role in the neuroendocrine control of reproduction by regulating GnRH (de Roux *et al.*, 2003; Funes *et al.*, 2003; Seminara *et al.*, 2003; d'Anglemont de Tassigny *et al.*, 2007; Kauffman *et al.*, 2007; Lapatto *et al.*, 2007). *Kiss1r*-null mice experience a delayed onset of puberty or are unable to proceed through puberty altogether, with both sexes appearing to have immature sexual organs (de Roux *et al.*, 2003; Funes *et al.*, 2003; Seminara *et al.*, 2003; d'Anglemont de Tassigny *et al.*, 2007). Furthermore, *Kiss1* and/or *Kiss1r* gene deletion significantly reduced testicle size in male mice (Lapatto *et al.*, 2007). LH and follicle-stimulating hormone (FSH) serum levels were also decreased in male *Kiss1r*-null mice, whereas this was not observed in female mice (Lapatto *et al.*, 2007). Subcutaneous injection of KP-54

resulted in increased levels of LH and FSH (with the exception of the *Kiss1r*-null mice), indicating the crucial roles of *Kiss* and *Kiss1r* in GnRH release from the hypothalamus (Lapatto *et al.*, 2007). In addition to mice, humans with mutations of the *KISS1R* or *KISS1* gene fail to go through puberty (de Roux *et al.*, 2003; Funes *et al.*, 2003; Seminara *et al.*, 2003; d'Anglemont de Tassigny *et al.*, 2007; Kauffman *et al.*, 2007; Lapatto *et al.*, 2007). Contrary to the effects of decreased KP levels, KP administration can induce precocious puberty (Navarro *et al.*, 2005), whereas central injection of the KISS1R antagonist (P-234) counteracts the effects of KPs in that it delays puberty (Pineda *et al.*, 2009) in pre-pubertal rats.

#### *1.2.3.2. Pregnancy and Placentation*

In addition to the well established role of KP/KISS1R system in the regulation of the HPG axis, this signaling pathway has been proposed to have significant effects on other physiological systems, such as pregnancy and placentation. Initial studies of *KISS1* gene expression reported high levels of this protein in the human placenta (Lee *et al.*, 1996; Muir *et al.*, 2001; Ohtaki *et al.*, 2001; Kirby *et al.*, 2010). More specifically, KP and KISS1R expression has been demonstrated to be higher in the first trimester than in the third trimester placentas, correlating with reduced invasiveness of placental tissue (Janneau *et al.*, 2002; Horikoshi *et al.*, 2003). A radioimmunoassay revealed that in male and non-pregnant female humans, plasma KP circulates at very low concentrations (Dhillo *et al.*, 2006). However, during pregnancy, plasma KP concentrations display a 1000-fold increase in the first trimester, rising to a 10,000-fold increase in the third trimester (Horikoshi *et al.*, 2003).

In addition, the KP/KISS1R signaling pathway plays a major role in regulation of trophoblast invasion to allow remodelling of the maternal arteries, to provide sufficient blood flow to the developing fetus (Bilban *et al.*, 2004). KP-10, the form found in placenta (Bilban *et al.*, 2004) has been shown to inhibit migration of primary trophoblast explants and this is associated with a decrease in MMP expression (Bilban *et al.*, 2004).

***1.2.3.3. Cardiovascular System***

Recently it has been suggested that KPs may play a role in regulating the cardiovascular system, given that KP and KISS1R expression has been detected in human, rat and mouse myocardium and vasculature (Nijher *et al.*, 1111; Mead *et al.*, 2007; Kirby *et al.*, 2010; Ramaesh *et al.*, 2010; Maguire *et al.*, 2011; Sawyer *et al.*, 2011). Additionally, Mead and colleagues have identified presence of both *KISS1* and *KISS1R* mRNA in human aorta, umbilical vein and coronary artery (Mead *et al.*, 2007). Moreover, KPs have been shown to act as positive inotropes (modulators of force of muscular contraction) in the atria of these three species (Maguire *et al.*, 2011). It was further suggested that KPs act as vasoconstrictors *in vivo* as KPs stimulated contraction of human vessels with comparable potency to angiotensin II (Ang II), a potent vasoactive peptide (Mead *et al.*, 2007). In a different study, Ramaesh and colleagues have shown that KP-10 induced concentration-dependent inhibition of proliferation and migration of the human umbilical vein endoethelial cells (HUVEC), however KP-10 had no effect on the viability or apoptosis of these cells (Ramaesh *et al.*, 2010). Furthermore, KP-10 has been shown to inhibit angiogenesis by interfering with vascular endothelial growth factor (VEGF) signaling (Ramaesh *et al.*, 2010). Nevertheless, Nijher and colleagues have shown that elevation of plasma KP does not alter blood pressure in humans (Nijher *et*

*al.*, 1111). Therefore, a growing body of evidence suggests that the KP/KISS1R system has the ability to regulate cardiovascular system by modulating vasoconstriction and angiogenesis.

### *1.2.4. KP/KISS1R Signaling in Cancer*

To date, the metastasis suppressor activity of the KP/KISS1R system has been identified in numerous cancers, including thyroid (Ringel *et al.*, 2002; Stathatos *et al.*, 2005), ovarian (Gao *et al.*, 2007; Hata *et al.*, 2007; Zhang *et al.*, 2008), bladder (Sanchez-Carbayo *et al.*, 2003a), gastric (Dhar *et al.*, 2004; Guan-Zhen *et al.*, 2007), esophageal (Ikeguchi *et al.*, 2004), pancreatic (Masui *et al.*, 2004) and lung (Zohrabian *et al.*, 2007) cancers. KISS1R activity was shown to repress MMP-9 activity, inhibit migration and invasion, increase tissue inhibitor of metalloprotease (TIMP)-1 production and activate focal adhesion kinase (FAK), leading to the formation of excessive focal adhesions and stress fibre formation (Kotani *et al.*, 2001).

Numerous studies have confirmed that a reduction in *KISS1* expression correlates with poor prognosis in cancer patients. One of the first clinical studies evaluating the role of KPs in human cancer was performed in melanomas, where *KISS1* mRNA expression was examined at various stages of melanoma progression and found to be reduced in large primary melanomas and in metastases (Shirasaki *et al.*, 2001). Additionally, in gastric cancers, *KISS1* mRNA expression in patients with distant metastases (lymph node, liver metastases) was significantly reduced (Dhar *et al.*, 2004). In another gastric cancer study, analysis of KP protein expression was reduced in lymph node and liver metastases compared to primary gastric tumors (Guan-Zhen *et al.*, 2007). In another

study, comparing plasma KP-54 levels, pancreatic cancer patients were found to have higher plasma KP-54 levels compare to healthy individuals (Katagiri *et al.*, 2009). Collectively, these findings are intriguing in that they establish KPs as an important prognostic indicator in gastric and pancreatic cancers and suggest the potential significance of plasma KP levels within patients (Guan-Zhen *et al.*, 2007; Katagiri *et al.*, 2009).

In another clinical study, *KISS1* and *KISS1R* mRNA expression have been shown to be independent markers of favorable prognosis in patients with clear-cell subtype ovarian carcinoma (Prentice *et al.*, 2007). A loss of *KISS1* and *KISS1R* has been found to be a strong prognostic factor for lymph node metastasis of esophageal squamous cell carcinomas, given that the loss of *KISS1* and/or *KISS1R* gene expression was detected in 86-100% of primary tumors in cases with lymph node metastasis (Ikeguchi *et al.*, 2004). Finally, in bladder tissue, 80% of invasive tumors showed little to no expression of *KISS1*, compared to normal urothelium that displayed high expression of this gene (Sanchez-Carbayo *et al.*, 2003b). Thus, as indicated by these studies, *KISS1* and/or *KISS1R* expression could potentially prove useful prognostic markers in clinical settings.

Thus far, numerous types of cancers, including melanoma, gastric carcinoma, esophageal squamous cell carcinoma, pancreatic cancer and bladder cancer have shown that signaling of the KPs and KISS1R may have anti-metastatic and tumor-suppressant effects. Nevertheless, studies are emerging that indicate that KP/KISS1R may act in a pro-metastatic fashion. For example, in hepatocellular cancers, KP acts in a pro-metastatic fashion (Ikeguchi *et al.*, 2003; Hou *et al.*, 2007; Schmid *et al.*, 2007). Additionally, a recent study found that plasma KP-54 levels were elevated in patients

with colorectal cancer and the authors speculated that the measurement of plasma KP-54 levels could be a useful diagnostic and prognostic parameter for patients with colorectal cancer (Canbay *et al.*, 2012). Additionally, we have reported that KP/KISS1R system may positively regulate breast cancer cell invasion (Zajac *et al.*, 2011).

#### ***1.2.4.1. KP/KISS1R Signaling in Breast Cancer***

The role of KPs in breast cancer has been difficult to discern. In a 1997 study by Lee and colleagues, the human 'breast' cancer cell line MDA-MB-435 which is *KISS1*-negative was transfected with the KISS1 construct, and subsequently these cells were injected in athymic nude mice, resulting in a decrease in the number of macroscopic lung metastases compared to the non-transfected parental cells (Lee and Welch, 1997b). Therefore, in addition to anti-metastatic capacity of KPs in melanoma, the authors suggested that *KISS1* could also function as a metastasis suppressor of the MDA-MB-435 cells (Lee and Welch, 1997b). The authors went as far as to suggest that *KISS1* may inversely correlate with the progression of breast tumors, and thus may in fact be used as a prognostic marker in breast cancer patients (Lee and Welch, 1997a). The findings of this study had not been questioned until the controversial debate regarding the origins of MDA-MB-435 cells. Characterization of this cell line in 2000 has shown that the pattern of gene expression for MDA-MB-435 more closely resembled that of melanoma cell lines, rather than that of other breast tumor lines (Ross *et al.*, 2000). Therefore, in the breast cancer field, it is now generally accepted that the studies performed using these cells have to be conducted with caution (Chambers, 2009).

This controversy provided a gateway for a 2005 landmark study performed by Martin and colleagues, the first study of its kind to investigate the expression of *KISS1*

and its receptor in human breast cancer tissues. *KISS1* mRNA expression was elevated in tumor tissue compared to normal healthy mammary tissue as measured by quantitative-PCR (q-PCR)analysis, which was supported by immunohistochemistry (Martin *et al.*, 2005). Additionally, node positive tumors showed significantly increased *KISS1* mRNA levels compared to node negative tumors, yet no differences were observed in *KISS1R* mRNA levels (Martin *et al.*, 2005). This study has also shown that the introduction of the KISS1 construct into human breast cancer MDA-MB-231 cells increased their invasiveness and decreased their adhesive property using *in vitro* assays (Martin *et al.*, 2005). Therefore, overexpression of *KISS1* was correlated with poor prognosis in breast cancer patients and was proposed to act as a possible promoter of invasion in human breast cancer cells (Martin *et al.*, 2005).

Differential regulation of *KISS1* and *KISS1R* mRNA levels by steroid hormones has been reported to occur due to the direct effect of $E_2$, as ERα is expressed within KP-immunoreactive cells present in the preoptic area and arcuate nucleus of the ovine hypothalamus (Franceschini *et al.*, 2006). ERα-mediated pathways play a vital role in breast carcinogenesis, and thus, ERα level is consensually used as a prognostic marker of breast tumors and of the response to endocrine therapy (Clarke *et al.*, 2004). In a 2007 study, Marot and colleagues reported a significant $E_2$-induced decrease in *KISS1* mRNA level in adenovirus ERα- and adenovirus ERβ-infected ER negative MDA-MB-231 breast cancer cells, when compared with adenovirus control-infected cells (Marot *et al.*, 2007). Conversely, tamoxifen administration upregulated *KISS1* and *KISS1R* expression in ERα-positive breast tumor cells (Marot *et al.*, 2007). To provide further support to this conclusion, among ERα-positive tumor samples from patients treated with tamoxifen,

patients with shorter disease-free survival had elevated expression of KISS1 and KISS1R (Marot *et al.*, 2007). Recently, activator protein 2 alpha (AP-2α) has been described as a possible positive transcriptional regulator of *KISS1* in breast cancer cell lines *via* interaction with specificity 1 protein (Sp1) (Mitchell *et al.*, 2007). It has also been shown that $E_2$ rapidly down-regulates endogenous *KISS1* mRNA in a stable ERα-expressing MDA-MB-231 cell line (Huijbregts and de Roux, 2010). $E_2$-induced down-regulation of *KISS1* mRNA is mediated by a pathway combining ribonucleic acid polymerase II (RNAPII) loss at the proximal promoter and modulation of active RNAPII along the gene body, which is a novel mechanism in the complex process of $E_2$-induced repression of *KISS1* gene expression (Huijbregts and de Roux, 2010).

To date, one study has investigated whether KP/KISS1R signaling regulates breast cancer metastasis *in vivo*. A mouse model that is widely used to investigate the relationship between human and mouse breast cancer development and metastasis is the polyoma virus middle T antigen (PyMT) under the control of the mouse mammary tumor virus (MMTV) promoter (MMTV-PyMT) transgenic model (Lin *et al.*, 2003). MMTV-PyMT mice show widespread transformation of the mammary epithelium and development of multifocal mammary adenocarcinomas and metastatic lesions in the lymph nodes and in the lungs (Lin *et al.*, 2003). The close similarity of this model to human breast cancer is also exemplified by the fact that in these mice there is a gradual loss of steroid hormone receptors (estrogen and progesterone) is observed (Lin *et al.*, 2003). In a recently published study, Cho and colleagues (2011) have shown that *Kiss1r* heterozygosity (*Kiss1r*$^{+/-}$) delayed PyMT-induced breast cancer development and metastasis in mice. More specifically, *Kiss1r* heterozygosity attenuated breast tumor

initiation, growth, latency, multiplicity and metastasis induced in MMTV-PyMT/Kiss1r mouse models (Cho *et al.*, 2011). Orthotopic injection into NOD.SCID/NCr mice of isolated mouse primary breast cancer MMTV-PyMT/Kiss1r$^{+/-}$ cells showed attenuated breast tumor growth compared to MMTV-PyMT/Kiss1r$^{+/+}$ cells (Cho *et al.*, 2011). To confirm the roles of human KISS1R for tumorigenicity in human breast epithelium, non-malignant human mammary epithelial MCF10A cells were transformed by overexpressing the constitutively active H-Ras (H-RasV12) to induce tumorigenesis (Cho *et al.*, 2011). Overexpression of the active H-RasV12 transformed the MCF10A breast epithelial cells and induced anchorage-independent colony growth on soft agar (Cho *et al.*, 2011). Knockdown of KISS1R using specific shRNA for human KISS1R reduced Ras-induced anchorage-independent colony formation, suggesting that human KISS1R plays a vital role in Ras-induced MCF10A cell tumorigenesis (Cho *et al.*, 2011). To date, the mechanisms by which KP/KISS1R regulates breast cancer cell migration and invasion, two processes required for metastasis remain largely unknown. Recent studies from our laboratory have demonstrated that KP-10, the most potent KP, stimulates breast cancer cell migration and invasion of ER-negative MDA-MB-231 and Hs578T that endogenously express KISS1R, *via* transactivation of epidermal growth factor receptor (EGFR) (Zajac *et al.*, 2011), a known pharmacological target, that is upregulated in numerous cancers, including breast cancer (Eccles, 2011).

**1.3. The Epidermal Growth Factor Receptor**

The human epidermal growth factor receptor (EGFR)/HER/ErbB family comprises of four closely related receptor tyrosine kinases (RTKs): ErbB1/EGFR/HER1 (hereafter termed EGFR), ErbB2/EGFR2/HER2 (hereafter termed HER2), HER3, and HER4 (Lurje and Lenz, 2009). Each member of the ErbB family has a similar structure consisting of a large extracellular domain, a single transmembrane-spanning domain, an intracellular juxtamembrane region, a tyrosine kinase domain and a C-terminal regulatory region (Ferguson, 2008; Liebmann, 2011).

Numerous ligands can bind and activate EGFR, including epidermal growth factor (EGF), TGF-α, amphiregulin, heparin-binding EGF, and betacellulin (Herbst, 2004). Binding of the ligand to the receptor leads to homodimerization or heterodimerization of the receptor, followed by internalization of the dimerized receptor (Herbst, 2004). Once the dimerized receptor becomes internalized, autophosphorylation of the cytoplasmic EGFR tyrosine kinase domains occurs (Franklin *et al.*, 2002). These phosphorylated tyrosine residues serve as binding sites for enzymes (e.g., phospholipase Cγ1 (PLCγ1)) or adaptor proteins (e.g. Grb2 or Shc) containing Src homology 2 (SH2) domains (Franklin *et al.*, 2002; Wetzker and Bohmer, 2003; Liebmann, 2011). The principal signaling cascades of RTKs that ultimately result in the modulation of various downstream targets include: MAPKs, PI3K family, members of the signal transducers and activator of transcription family, and the PLCγ1pathway (Liebmann, 2011). Given that these signaling systems are vital in development, it is not unexpected that their activation results in multiple co-ordinated cell responses in normal cells, but these are subverted by overexpression/misregulation in pathological processes such as cancer.

### *1.3.1. EGFR in Cancer*

The influence of growth factor-driven signaling in pathogenesis has been long recognized. ErbB receptors, their ligands and their signaling are essential in the control of cell proliferation, survival, differentiation and deregulated signaling of these receptors has been extensively studied as potential targets for inhibition of tumor growth and progression (Liebmann, 2011). Deregulation of ErbB signaling pathways has been described in many cancers, including breast, linked to a multiplicity of molecular mechanisms including epigenetic mechanisms, activating mutations of the receptors themselves or activation induced by autocrine/paracrine ligands (Eccles, 2011). EGFR is overexpressed in the majority of solid tumors, including breast cancer, head-and-neck cancer, non-small-cell lung cancer, renal cancer, ovarian cancer, and colon cancer (Herbst and Langer, 2002). Additionally, overexpression of the receptor occurs also in a smaller percentage of bladder cancers, pancreatic cancers, and gliomas (Salomon *et al.*, 1995; Herbst and Langer, 2002). Such overexpression produces excessive activation of downstream signaling pathways, resulting in cells displaying more aggressive growth and metastatic potential (Herbst and Langer, 2002; Herbst, 2004). In particular, overexpression of HER2, which occurs in 25% to 30% of breast cancers, is associated with poor prognosis and shorter survival (Slamon *et al.*, 1987). EGFR expression was found to be higher in patients with nodal or distant metastases than in those without (Sutton *et al.*, 2010). Mutations in EGFR are rare in breast cancer, but it is amplified in some cases (such as metaplastic subtype) (Burness *et al.*, 2010) and it is also highly expressed in basal breast cancers, a subset of triple negative breast cancers (TNBC) (Eccles, 2011). TNBC are characterized by a lack of expression (or minimal expression)

of ER and PR as well as an absence of HER2 overexpression (Eccles, 2011). TNBCs represent 10-17% of all breast cancers, are more common in certain non-Caucasian ethnic groups (e.g. those of African descent) and tend to occur at less than 50 years of age (Eccles, 2011). These cancers are also generally of high grade and show distinct patterns of metastasis; notably visceral, liver and brain involvement, leading to a particularly poor prognosis (Dawson *et al.*, 2009). TNBC is particularly prevalent in women carrying a breast cancer type 1 susceptibility protein (*BRCA1*) gene mutation and EGFR overexpression is found in 67% of *BRCA1* related cancers compared to 18% of sporadic cancers (Eccles, 2011). Using human mammary epithelial cell (hMEC) cultures it has been demonstrated that even partial suppression of BRCA1 function induced EGFR expression and an increase in EGFR-positive cancer stem-like cells, suggesting that this receptor could provide a growth advantage at early stages of transformation (Eccles, 2011).

EGFR and HER2 have been the main receptors considered as targets for immunotherapeutic approaches in breast cancer, mainly via antibody-based therapies (Ladjemi *et al.*, 2010; Eccles, 2011). Trastuzumab, a humanised anti-HER2 monoclonal antibody targeting the juxtamembrane region of the extracellular domain, has been successful in clinical trials, particularly in combination with standard chemotherapy and in adjuvant settings (Goel *et al.*, 2011). Trastuzumab is reportedly most active in tumors driven by HER2 homodimers and is also effective in combination with anti-endocrine therapies in ER-positive tumors (Goel *et al.*, 2011). Pertuzumab is another humanised antibody that inhibits HER2 heterodimerization with other family members by binding to the dimerization loop of the former (i.e a different site from trastuzumab). It has shown

some promise in HER2 breast and ovarian cancer patients and is also being evaluated in combination with trastuzumab or chemotherapy (CLEOPATRA trials) (Baselga and Swain, 2010). In general, HER2-targeted therapies are only effective in cancers with gene amplifications, and sensitive assays are needed to determine those who may benefit (e.g. HercepTest or Oncotype Dx).

Unfortunatley, some HER2-positive breast cancer patients treated with trastuzumab can become resistant. Hence, understanding the molecular mechanisms underlying HER2 signaling and trastuzumab resistance is vital to reduce breast cancer mortality (White *et al.*, 2011). IQ motif containing guanine triphosphatase activating protein 1 (IQGAP1) is a ubiquitously expressed scaffolding protein that contains multiple protein interaction domains (White *et al.*, 2011). By regulating its binding partners IQGAP1 integrates signaling pathways, several of which contribute to breast tumorigenesis. White and colleagues have shown that IQGAP1 is overexpressed in HER2-positive breast cancers and binds directly to HER2 (White *et al.*, 2011). Furthermore, IQGAP1 is overexpressed in trastuzumab-resistant breast epithelial cells, and reducing IQGAP1 both augments the inhibitory effects of trastuzumab and restores trastuzumab sensitivity to trastuzumab-resistant breast cancer cells (White *et al.*, 2011). These data suggest that inhibiting IQGAP1 function may represent a rational strategy for treating HER2-positive breast carcinoma.

### *1.3.2. IQGAP1 in Cancer*

IQGAP1 is a ubiquitously expressed scaffolding protein with multiple binding partners which allow for integration of diverse signaling pathways (Briggs and Sacks,

2003; Brown and Sacks, 2006). Proteins that are known to bind IQGAP1 include actin, calmodulin, E-cadherin, β-catenin, components of the MAPK pathway (White *et al.*, 2011) and EGFR (McNulty *et al.*, 2011). By interacting with these proteins, IQGAP1 regulates multiple cellular activities, such as cytoskeletal organization, cell-cell adhesion, cell migration, gene transcription and signal transduction (Briggs and Sacks, 2003; Brown and Sacks, 2006). For example, binding of IQGAP1 to β-catenin both disrupts the E-cadherin-catenin complex, inhibiting epithelial cell-cell adhesion (Kuroda *et al.*, 1996) and increases β-catenin-mediated transcriptional activation (Briggs and Sacks, 2003).

Accumulating evidence strongly supports a role for IQGAP1 in tumorigenesis (Johnson *et al.*, 2009; White *et al.*, 2011). More than 50% of the identified IQGAP1 binding partners have defined roles in neoplastic transformation and tumor progression, and many cellular functions regulated by IQGAP1 are important in cancer biology (Johnson *et al.*, 2009; White *et al.*, 2011). IQGAP1 is upregulated in numerous human cancers, including breast carcinoma (Jadeski *et al.*, 2008), oligodendroglioma (French *et al.*, 2005) and colorectal carcinoma (Bertucci *et al.*, 2004). In addition, overexpression of IQGAP1 stimulates tumorigenesis of human breast epithelial cells (Jadeski *et al.*, 2008). Modulating IQGAP1 expression levels in malignant human breast epithelial cells significantly alters their tumorigenicity (Mataraza *et al.*, 2003; Jadeski *et al.*, 2008). Overexpression of IQGAP1 enhances *in vitro* motility and invasion of both MCF7 and MDA-MB-231 cells (Mataraza *et al.*, 2003; Jadeski *et al.*, 2008). Conversely, siRNA-mediated knockdown of IQGAP1 reduces MCF7 anchorage-independent growth, motility and invasion *in vitro*, as well as growth and invasion *in vivo* (Jadeski *et al.*, 2008). Collectively, these data suggest that IQGAP1 overexpression contributes to

tumorigenesis of human breast epithelium. These findings support the concept that IQGAP1 functions as an oncogene (Johnson *et al.*, 2009; White *et al.*, 2011). To further implicate IQGAP1 relevance in breast cancer, IQGAP1 was found to be overexpressed in trastuzumab-resistant human breast epithelial cells and knockdown of IQGAP1 both enhances the inhibitory effects of trastuzumab *in vitro* and abrogates trastuzumab resistance (White *et al.*, 2011). These findings imply that IQGAP1 is a potential target for the development of additional therapeutic strategies for patients with HER2-positive breast cancers.

### *1.3.3. Signaling Cross-Talk between GPCR-EGFR*

Over the last decade, significant progress has been made in understanding the complexity of GPCR-RTK signaling. Once seen as isolated receptors connecting extracellular stimuli to the activation of G proteins, GPCRs are now regarded as complex receptors capable of initiating a vast array of G protein-dependent and -independent signaling cascades, including signaling and interacting both directly and indirectly with other receptor families through the involvement of scaffolding molecules (Freedman and Lefkowitz, 1996; Fischer *et al.*, 2003; Wetzker and Bohmer, 2003; Liebmann, 2011). RTKs such as the EGFR are overexpressed in numerous cancers where signaling through this receptor contributes to cell survival, proliferation, and invasion (Thomas *et al.*, 2006; Rodland *et al.*, 2008). Inhibition of EGFR alone using RTK inhibitors, although highly promising in preclinical models has resulted in limited anti-metastatic effects due to acquired resistance to these agents (Engelman and Janne, 2008).

An interesting example of non-classical information integration is the ability of GPCR-mediated RTK transactivation. It is well established that GPCR-mediated EGFR transactivation results in increased migration and invasion, hallmarks of the metastatic process (Thomas *et al.*, 2006). Numerous GPCRs have been shown to increase motility and invasion of cancer cells through mechanisms involving the transactivation of EGFR. These include the protease-activated receptor-1 (PAR-1) (Arora *et al.*, 2007), lysophosphasitic acid receptor 1 ($LPA_1$-R) (Shida *et al.*, 2008), bombesin (Chao *et al.*, 2009) and chemokine receptor 4 (CXCR4) (Kasina *et al.*, 2009). Additionally, GPCR-mediated EGFR transactivation may participate not only in cancer development and progression, but also has been found to influence clinical responses to EGFR-targeted therapies. For example, cell-specific crosstalk between GPCRs and EGFR has been reported for various cancer types such as lung, breast, prostate, ovarian, colon, and head and neck cancers (Fischer *et al.*, 2003; Kalyankrishna and Grandis, 2006).

Recently, we have demonstrated that KISS1R mediates EGFR transactivation in highly aggressive human breast cancer cell lines to promote their migration and invasion (Zajac *et al.*, 2011). Given the clinical importance of understanding the mechanism by which KISS1R may regulate cell transformation, invasion and metastasis, I set out to determine the influence of KISS1R signaling in non-malignant human mammary MCF10A epithelial cells and to determine the mechanism by which KISS1R signaling promotes breast cancer cell migration and invasion. MCF10A cells were chosen to investigate the effect of KP-10/KISS1R signaling on the acquisition of a malignant phenotype.

## 1.4. Rationale and Hypothesis

*Findings in humans:*

Martin and colleagues first reported that expression of *KISS1* and *KISS1R* mRNA and protein levels were elevated in human breast cancer tissues using real-time PCR and immunohistochemistry, respectively (Martin *et al.*, 2005). This study found a positive correlation between *KISS1* and *KISS1R* mRNA expression with increases in breast tumor grade (Martin *et al.*, 2005). Additionally, patients with high *KISS1* and *KISS1R* mRNA have shown the shortest relapse-free survival (Marot *et al.*, 2007). To provide further support to this conclusion, another study reported that among ERα-positive tumor samples from patients treated with tamoxifen, patients with shorter disease-free survival had elevated expression of *KISS1* and *KISS1R* mRNA (Marot *et al.*, 2007).

*Finding from animal models:*

Recently, Cho and colleagues (2011) have shown that loss of KISS1R decreased tumor growth *in vivo* in immunocompromised mice. They have also shown that Kiss1r heterozygosity ($Kiss1r^{+/-}$) attenuated breast tumor initiation, growth, latency and metastasis induced in MMTV-PyMT/Kiss1r mouse models (Cho *et al.*, 2011). The authors demonstrated that knockdown of KISS1R in the H-RasV12-transformed MCF10A cells reduced anchorage-independent colony formation, suggesting that human KISS1R plays a key role in Ras-induced MCF10A cell tumorigenesis (Cho *et al.*, 2011).

*Findings from the Bhattacharya laboratory:*

We have previously shown that KP-10, the most potent KP stimulates ER-negative breast cancer cell invasion concomitant with MMP-9 secretion and activity, and have implicated β-arrestin 2 in this process (Zajac *et al.*, 2011). We found that treatment of

ER-negative breast cancer cells with KP-10 results in the transactivation of EGFR, and this is required for KP-10-stimulated invasion (Zajac *et al.*, 2011). Furthermore, we discovered that KISS1R directly complexes with EGFR, and that stimulation of breast cancer cells with either KP-10 or EGF regulates the endocytosis of KISS1R and EGFR (Zajac *et al.*, 2011). *However, the role of endogenous KP/KISS1R signaling in non-malignant mammary epithelial cells and the mechanism by which KP/KISS1R stimulates migration and invasion is still unclear and was examined in this study.*

**Hypothesis:** KP-10 signaling *via* KISSIR will not stimulate invasion and motility of non-malignant mammary epithelial MCF10A cells.

## 1.5. Objectives

To determine:

**Aim 1)** Whether or not KP-10/KISS1R signaling promotes migration and invasion of non-malignant mammary epithelial MCF10A cells.

**Aim 2)** Whether or not KP-10 promotes invasion and migration of ER-positive T47D and MCF7 breast cancer cells.

**Aim 3)** The mechanism by which KISS1R regulates breast cancer cell migration and invasion.

## 1.6. Significance

Gaining a better understanding of the molecular pathways involved in cell migration and invasion is expected to be vital in the identification of novel targets for the design of new therapeutics for cancer patients. The proposed studies will reveal the underlying mechanisms by which KISS1R signaling regulates cell invasiveness, shedding light on whether or not the ER status of breast epithelia influences KISS1R signaling and targeting KISS1R in breast cancer is a potentially useful therapy.

# CHAPTER 2: METHODS AND RESULTS

## 2.1. Statement of Co-Authorship

The contents of this chapter will be submitted to Molecular Biology of the Cell.

Donna Cvetković [1], Magdalena Dragan [1], Cynthia Pape [1], Macarena Pampillo [3,4], Andy V. Babwah [1,2,3,4], Moshmi Bhattacharya [1].

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Magdalena Dragan generated the following stable cell lines (MDA-MB-231 pcDNA3.1, MDA-MB-231 ERα, SKBR3 pFLAG-A1 and SKBR3 FLAG-KISS1R) and helped with the immunofluorescence microscopy for the EMT studies (**Figure 2.16**).

Cynthia Pape and Dr. Macarena Pampillo provided technical guidance for IP formation assays.

Dr. Macarena Pampillo performed FRET analysis experiments (data not included in the thesis).

Dr. Andy Babwah provided supervisory guidance for this project and provided reagents for some of the experiments, and supervised Dr. Macarena Pampillo for FRET experiments.

Dr. Moshmi Bhattacharya supervised all experiments performed in her laboratory, aided in confocal microscopy and assisted in writing the manuscript.

## 2.2. Materials and Methods

**Cell culture.** The cell lines used in this study are summarized in **Table 2.1**. Cell lines were purchased from ATCC (Manassas, VA), some of which were genetically modified as presented in Table 1. Cells were maintained at 37°C with 5% $CO_2$. Human breast cancer cells, MDA-MB-231, MDA-MB-231 pcDNA3.1, MDA-MB-231 ERα, MDA-MB-231 pSuperRetro Renilla B (Scrambled), MDA-MB-231 pSuperRetro siIQGAP1 #1 (siIQGAP1 #1), MDA-MB-231 pSuperRetro siIQGAP1 #2 (siIQGAP1 #2), T47D, SKBR3, SKBR3 pFLAG-A1 and SKBR3 FLAG-KISS1R were cultured in RPMI 1640 (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma). MCF7 breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS and 0.3% insulin. MCF10Ca1h cells were cultured in DMEM supplemented with 10% (v/v) FBS. MCF10A, MCF10A pFLAG-A1 (vector control), MCF10A FLAG-KISS1R (generated from a single clone), and MCF10A FLAG-KISS1R heterogenous pooled cell population (from here on referred to as MCF10A FLAG-KISS1R mixed) were grown in mammary epithelial basal medium (MEBM; Clonetics-Cambrex) supplemented with a MEGM Single Quots kit (bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, gentamicin/amphotericin) and cholera toxin at 100ng/mL.

**Table 2.1. Summary of the human cell culture models used in the study.**

| CELL LINES | CHARACTERISTICS OF THE PARENTAL CELL LINE |
|---|---|
| ***Human Mammary Epithelial Cells*** | |
| **MCF10A**<br>Derivatives:<br>• *MCF10A pFLAG-A1*<br>• *MCF10A FLAG-KISS1R*<br>• *MCF10A FLAG-KISS1R mixed* | • Non-malignant mammary epithelial cells isolated from a patient with fibrocystic desease (Soule *et al.*, 1990)<br>• Non-motile, non-invasive (Soule *et al.*, 1990)<br>• ERα-negative (Petersen and Polyak, 2010)<br>• Express KISS1R endogenously (Cho *et al.*, 2011) |
| ***Human Breast Cancer Cell Lines*** | |
| **MCF10Ca1h** | • Malignant mammary epithelial cell line derived from a pre-malignant mammary epithelial MCF10AT cells (Santner *et al.*, 2001)<br>• ERα-negative (Santner *et al.*, 2001) |
| **T47D** | • Infiltrating ductal carcinoma (Keydar *et al.*, 1979)<br>• ERα-positive (Keydar *et al.*, 1979)<br>• Express KISS1R endogenously (Marot *et al.*, 2007) |
| **MCF7** | • Invasive ductal carcinoma (Soule *et al.*, 1973)<br>• ERα-positive (Soule *et al.*, 1973)<br>• Express KISS1R endogenously (Marot *et al.*, 2007) |
| **SKBR3**<br>Derivatives:<br>• *SKBR3 pFLAG-A1*<br>• *SKBR3 FLAG-KISS1R* | • Moderately invasive, HER2-overexpressing breast carcinoma (Trempe, 1976)<br>• ERα-negative (Trempe, 1976) |

**Table 2.1. Summary of the human cell culture models used in the study (*continuation*).**

| CELL LINES | CHARACTERISTICS OF THE PARENTAL CELL LINE |
| --- | --- |
| *Human Breast Cancer Cell Lines* | |
| **MDA-MB-231**<br>Derivatives:<br>• *MDA-MB-231 pcDNA3.1*<br>• *MDA-MB-231 ERα*<br>• *MDA-MB-231 Scrambled*<br>• *MDA-MB-231 siIQGAP1#1*<br>• *MDA-MB-231 siIQGAP1 #2* | • Highly invasive breast adenocarcinoma (Cailleau *et al.*, 1974)<br>• ERα-negative (Cailleau *et al.*, 1974)<br>• Express KISS1R endogenously (Martin *et al.*, 2005) |

**Stable transfections and gene knockdowns.** FLAG-KISS1R and pFLAG-A1 (vector control) constructs were generated as described (Oved *et al.*, 2006; Pampillo *et al.*, 2009) and obtained from Dr. Andy Babwah. MCF10A cells (1 x $10^6$ cells) were transfected with 5µg cDNA constructs by microporation (1700V, 10 pulse width, 3#) using the Neon™ Transfection System (Invitrogen) according to the manufacturer's instructions. MDA-MB-231 cells were transfected with either pcDNA3.1 or ERα constructs (a generous gift from Dr. Bonnie Deroo; (Deroo *et al.*, 2004)) (5µg/1 x $10^6$ cells in 100µL) by microporation as described above. A heterogeneous population of stable transfectants was selected by using media containing 750µg/mL G418 (Invitrogen). To silence IQGAP1 expression, a Bio-Rad microporator was used to transfect 6.0 x $10^5$ cells with two individual siRNA constructs that targeted IQGAP1 or a scrambled control (generously provided by Dr. David Sacks (NIH, Bethesda, MD)) as described (Mataraza *et al.*, 2003). The siRNA against IQGAP1 targets the 4959-4977 nucleotide region of the IQGAP1 mRNA with +1 representing the first nucleotide of the first codon. Stable heterogenous population of cells was selected by culturing in puromycin (1mg/mL) and mixed population cells (siIQGAP#1 and #2) generated with two different siIQGAP1 constructs as described (Mataraza *et al.*, 2003), showing the best knockdown (as was verified by Western blot analysis) were chosen for the rest of the experiment.

**Cell migration and invasion assays.** Transwell chamber migration and invasion assays were performed as previously described (Li *et al.*, 2009; Zajac *et al.*, 2011). Briefly, transwell filters (8µm pores) were placed into a 24-well plate containing either serum-

free medium or medium supplemented with 10% FBS. Cells were serum-starved for 4 hours. T47D (1.5 x $10^5$), MCF7 (1.0 x $10^5$), MDA-MB-231 pcDNA3.1, MDA-MB-231 ERα, MDA-MB-231 scrambled, MDA-MB-231 siIQGAP1 #1 or MDA-MB-231 siIQGAP1 #2 (4.0 x $10^4$) cells were plated in the upper chamber in either serum-free medium or serum-free medium supplemented with 10nM or 100nM KP-10 (Phoenix Pharmaceuticals; Burlingame, CA) and incubated for 20 hours. For invasion assays, filters were coated with 1 in 10 dilution of Matrigel dissolved in serum-free RPMI 1640 (9.4mg/mL stock, BD Biosciences). The top of the filter was scraped to remove cells that did not migrate or invade. Cells were then fixed using a solution of 20% acetone and 80% methanol and nuclei were stained using 0.1% Hoechst 33258 (Invitrogen, Burlington, Ontario, Canada). Two replicates were conducted for each condition, and ten random fields of the filter were imaged using an Olympus IX-71 inverted microscope. The average number of cells (nuclei) that migrated or invaded were counted. Results are presented as a ratio of cells that migrated relative to cells that migrated in control conditions (cells seeded in serum-free media and migrating towards 10% (v/v) FBS-supplemented appropriate medium). Results are from at least three independent experiments.

**3D Matrigel invasion assays.** Typical mononolayer cultures of mammary epithelial cells do not closely mimic the features of cells and tissue architecture *in vivo* (Weaver *et al.*, 1995; Weaver and Bissell, 1999). Moreover, they do not recapitulate the genetic modifications acquired during growth and progression of breast tumors (Lelievre *et al.*, 1998). Both normal and malignant breast cells can be cultured in reconstituted

extracellular matrix as a three-dimensional (3D) model, resembling the *in vivo* micro-environment (Li *et al.*, 2009; Zajac *et al.*, 2011). To determine the oncogenic potential of KISS1R in breast cells, the ability of non-malignant human mammary epithelial MCF10A cells and moderately invasive SKBR3 stably expressing either FLAG-KISS1R or pFLAG-A1 (vector control), to form organotypic invasive structures in 3D invasion assay was examined. Cells were seeded in a 1:1 dilution of phenol red-free Matrigel and culture medium at $2.5 \times 10^4$ cells/mL on Matrigel-coated 35mm glass-bottomed culture dishes (Mattek, Ashland, MA). Cultures were overlaid with culture medium and maintained for up to three weeks in the presence of 10nM or 100nM KP-10 or left untreated. Cell colonies were scored blindly as being either stellate or spheroidal after growth in Matrigel. A colony was deemed to be stellate if one or more projections from the central sphere of cells were observed. To examine the effect of KP-10 on invasion of ER-positive breast cancer cells, 3D Matrigel invasion assays were also performed with T47D and MCF7 cells (with the same conditions kept as for the MCF10A and SKBR3 cells). Images were taken with an Olympus IX-81 microscope (Olympus, Center Valley, PA), using InVivo Analyzer Suite (Media Cybernetics).

**Scratch assays for cell motility.** T47D, MCF7, MCF10A, MCF10A pFLAG-A1 or MCF10A FLAG-KISS1R cells were seeded into a 12-well dish in appropriate culture medium and allowed to grow to confluency. Cells were then serum-deprived in serum-free medium for 4 hours. The scratch was made with a sterile pipette tip passed across the monolayer. Cells were then treated with 10nM KP-10 in appropriate media supplemented with FBS (5% for MCF10A and stable transfectants, 10% for MDA-MB-231 cells). For

the experiments with the KISS1R antagonist - Peptide-234 (P-234; Phoenix Pharmaceuticals (Burlingame, CA) was dissolved in 20% acetonitrile in sterile water (vehicle). Cells were pre-treated with P-234 in serum-free medium for 4 hours, and then stimulated in the presence or absence of P-234 and allowed to migrate for the duration of the experiment. Cells were allowed to migrate into the scratch for 24 hours and visualized every 60 minutes using an Olympus IX-81 microscope (Olympus). Distance travelled (in μm) was then measured over the course of the 24 hours, using duplicates for each condition and seven fields per duplicate. Data were analyzed using ImagePro software (Media Cybernetics) and graphed as a function of time.

**Cell growth assays.** To measure cell growth in monolayer culture, 4.0 x $10^5$ MCF10A pFLAG-A1 or MCF10A FLAG-KISS1R cells were initially seeded into 60mm dishes. At 24 hour intervals (24, 48, 72), duplicate samples (2 dishes per day) were trypsinized and the number of cells was enumerated using a hemocytometer.

**MTT cell viability assays.** MTT cell viability assays (Cell Signaling) were conducted as described (Li *et al.*, 2009), according to the protocol of the manufacturer. Briefly, 5.0 x $10^4$ cells (MCF10A pFLAG-A1, MCF10A FLAG-KISS1R or MDA-MB-231) were plated in 96-well plates and stimulated with 10nM KP-10 dissolved in FBS (5% or 10% for MCF10A pFLAG-A1 and FLAG-KISS1R or MDA-MB-231 cells, respectively), in the presence or absence of P-234. Cells were then incubated with 0.5mg/mL of MTT labeling agent (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) for 4 h and subsequently solubilized for 24h. Absorbance was measured at 570nm using a

SpectraMax M5 MultiMode Microplate Reader (Molecular Devices) with a background subtraction at 750nm.

**KP-10-stimulated EGFR transactivation.** These experiments were conducted as previously described (Zajac *et al.*, 2011). T47D, MCF7, SKBR3, MDA-MB-231 pcDNA3.1, MDA-MB-231 ERα, MCF10A, MCF10A pFLAG-A1, MCF10A FLAG-KISS1R, MCF10A FLAG-KISS1R mixed or MCF10A-CA1h were cultured to 80% confluency, serum-starved for 24 hours to attenuate basal EGFR activity and then stimulated with various ligands for the indicated times. After stimulation, cultures were washed with ice-cold phosphate-buffered saline (PBS) and were solubilised in lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM $Na_3VO_4$, 1 mM NaF, 10% glycerol, 1% Triton X-100) containing protease inhibitors (1 µL/mL aprotinin, 1 µL/mL leupeptin and 10 µL/mL AEBSF). Lysates (850 µg of total protein) were used for immunoprecipitation studies. EGFR was immunoprecipitated using a rabbit polyclonal anti-EGFR antibody (1:100, Upstate Millipore) and protein G-sepharose beads (Sigma) over-night at 4°C. Immunoprecipitated proteins were resolved by 7.5% SDS-PAGE, and then semi-dry transferred onto a 0.45µm nitrocellulose membrane. Phosphorylation of EGFR was examined probing the membrane with a mouse monoclonal anti-phosphotyrosine antibody (PY-20, Santa Cruz), diluted in Tris Buffered Saline-Tween 20 (TBS-T) containing 1% bovine serum albumin (BSA) at 1:1000. Membranes were rinsed with TBS-T and then incubated with enhanced chemiluminescence (ECL) anti-mouse horseradish peroxidase (HRP)-conjugated IgG used at 1:3000 dilution and visualized by ECL Western blotting detection reagents

(Fisher, Nepean, Ontario, Canada). Western blots were then re-probed with rabbit polyclonal anti-EGFR antibody (1:4000, Upstate Millipore) to assess total EGFR. ECL anti-rabbit HRP-conjugated IgG was used at 1:3000 dilution in 1% bovine serum albumin (BSA) dissolved in TBS-T and subsequently proteins were visualized using ECL detection reagents (Fisher, Nepean, Ontario, Canada). Densitometric analysis was done using VersaDoc Imaging System (Bio-Rad).

**Inositol phosphate production assays.** These experiments were conducted as we have previously described (Aziziyeh *et al.*, 2009; Pampillo *et al.*, 2009). MCF10A, MCF10A pFLAG-A1, MCF10A FLAG-KISS1R or MCF10A FLAG-KISS1R mixed cells were incubated overnight with 1 μCi/mL [$^3$H]myo-inositol in DMEM to radiolabel inositol lipids. Unincorporated [$^3$H]myo-inositol was removed by washing the cells with HBSS. Cells were incubated for one hour in HBSS at 37°C and then incubated in 500μL of the same buffer containing 10mM LiCl for an additional 15 minutes at 37°C. Next, the cells were incubated either in the absence or presence of 100nM KP-10 for one hour at 37°C. The reaction was stopped on ice by adding 500μL of 0.8M perchloric acid and then neutralized with 400μL of a solution containing 0.72M KOH and 0.6M $KHCO_3$. Total [$^3$H]inositol incorporated into the cells was determined by counting the radioactivity present in 50μL of the cell lysate. Total IP was purified from the cell extracts by anion exchange chromatography using AG® 1-X8 (analytical grade, 200-400 mesh, formate form) resin (Bio-Rad). [$^3$H]IP formation was determined by LS 6500 Scintillation Counter (Beckman). The means ± SEM are shown for the number of independent experiments indicated in the figure legends.

**Co-immunoprecipitations.** Co-immunoprecipitation assays were conducted as previously described (Aziziyeh *et al.*, 2009; Li *et al.*, 2009; Zajac *et al.*, 2011). MDA-MB-231 pFLAG-A1 or MDA-MB-231 FLAG-KISS1R cells were serum-starved for 4 hours, and subsequently they were left untreated or stimulated for the indicated times with 100nM KP-10. Cells were solubilized in lysis buffer (20mM HEPES, 150mM NaCl, 1.5mM $MgCl_2$, 1% Triton X-100, protease inhibitors). Cell lysates (850 µg of total protein) were used for co-immunoprecipitation studies. FLAG-tagged receptor was immunoprecipitated using a rabbit monoclonal anti-FLAG antibody and protein G-Sepharose beads (Sigma) over-night at 4°C. Immunoprecipitated proteins were separated by SDS-PAGE and IQGAP1 expression was examined using a rabbit monoclonal anti-IQGAP1 antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, California) and visualized by ECL, following the manufacturer's protocol.

**Immunofluorescence microscopy.** MCF10A, MCF10A pFLAG-A1, or MCF10A FLAG-KISS1R cells were serum starved for 4 hours. Cells were washed three times, fixed and permeabilized with 4% paraformaldehyde (PFA) along with 0.2% Triton-X at room temperature for 20 minutes. Cells were incubated with the following antibodies: goat anti-GPR54 (N-20) sc-48220, (1:50, Santa Cruz Biotechnology), rabbit anti-E-cadherin (1:350, BD), rabbit anti-Snail/Slug (1:500, Santa Cruz Biotechnology), mouse anti-N-cadherin (1:500, BD) or phalloidin (1:100, Molecular Probes) conjugated to Alexa Fluor (AF) 555 for 1 hour and then washed 3 times. The following AF secondary antibodies were used: AF 555 anti-goat (1:250, Invitrogen), AF 568 anti-rabbit (1:250, Invitrogen) or AF 488 anti-mouse (1:250, Invitrogen). Images were acquired using an

LSM-510 META laser scanning microscope (Zeisss, Oberkochen, Germany) using a Zeiss 63X objective, oil immersion lens.

**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 were performed using GraphPad Prism 5 (GraphPad Software, Inc.). Differences were considered statistically significant at $P < 0.05$.

## 2.3. Results

**KISS1R stimulates invasion of non-malignant mammary epithelial cells.** We have previously reported that KP-10 increases migration and invasion of the highly invasive ER-negative MDA-MB-231 breast cancer cells in a dose-dependent manner, starting at 10nM and with a maximal response obtained using 100nM KP-10 (Zajac *et al.*, 2011). Therefore, all of the subsequent studies were performed using either 10 or 100nM KP-10, which is in accordance with published literature (Navenot *et al.*, 2005; Pampillo *et al.*, 2009). To test my hypothesis that KP-10/KISS1R signaling will not stimulate motility and invasion of non-malignant mammary epithelial MCF10A cells, I first sought to determine the effect of KP-10 treatment on MCF10A cells, using 3D Matrigel invasion assays. Treatment with either 10nM or 100nM KP-10 significantly stimulated MCF10A cells, which endogenously express KISS1R (Cho *et al.*, 2011) to form invasive structures compared to untreated cells (**Figure 2.1**).

In order to assess the role for KISS1R signaling in mediating the invasive phenotype in MCF10A cells, a stable cell line expressing KISS1R was generated from a single clone, named MCF10A FLAG-KISS1R cells. The exogenous expression of FLAG-KISS1R was verified by immunoprecipitation experiments **(Figure 2.2A)** and by immunofluorescence microscopy. Endogenous KISS1R in MCF10A cells cultured in 3D was detected by immunofluorescence microscopy using a commercially available KISS1R (N-20) antibody **(Figure 2.2B)**. Antibody binding to antigen was blocked by pre-absorption with the blocking peptide **(Figure 2.2B)**. Expression of FLAG-KISS1R was detected using an anti-FLAG antibody **(Figure 2.2C)**; confocal Z-stacks illustrate the staining pattern of the receptor. The endogenous expression of KISS1R in MCF10A cells and the exogenous expression of KISS1R in MCF10A FLAG-KISS1R cells exhibited a similar localization to the cell surface membranes and intracellularly **(Figure 2.2B, Figure 2.2C)**.

To examine the effects of stable exogenous expression of KISS1R in MCF10A cells on cell invasion, MCF10A FLAG-KISS1R cells were grown in 3D Matrigel cultures. These cells exhibited invasive structures, even in the absence of KP-10 **(Figure 2.3A)**. On the contrary, MCF10A pFLAG-A1 (vector control) cells showed an increase in invasive stellate structure formation only when treated with KP-10 (both 10nM and 100nM) **(Figure 2.3A)**, as was observed with the MCF10A parental cells **(Figure 2.1)**. To rule out any effects due to clonal selection, a pooled population of MCF10A FLAG-KISS1R cells (named MCF10A FLAG-KISS1R mixed) was generated. MCF10A FLAG-KISS1R mixed cells also invaded both in the presence and absence of KP-10 **(Figure 2.3B)**, thus corroborating observation with the MCF10A FLAG-KISS1R cells. Therefore,

these results reveal that treatment of non-malignant and non-invasive MCF10A cells with KP-10, or expression of KISS1R in these cells induces an invasive phenotype.

**KISS1R expression stimulates invasiveness of the ER-negative SKBR3 breast cancer cells.** To corroborate the observations that KISS1R expression stimulates invasiveness, I expressed KISS1R in another ER-negative, weakly invasive cell line - the SKBR3 breast cancer cell line. KP-10 did not induce invasive structure formation in SKBR3 pFLAG-A1 (vector control) cells **(Figure 2.4A)**. However, stable expression of KISS1R in SKBR3 cells induced the formation of invasive structures both in presence and absence of either 10nM or 100nM KP-10 **(Figure 2.4B)**. Thus, KISS1R expression induces an invasive phenotype in the ER-negative MCF10A **(Figure, 2.1, Figure 2.3A, Figure 2.3B)** and SKBR3 **(Figure 2.4B)**, in addition to ER-negative MDA-MB-231 and Hs578T breast cancer cells (Zajac *et al.*, 2011).

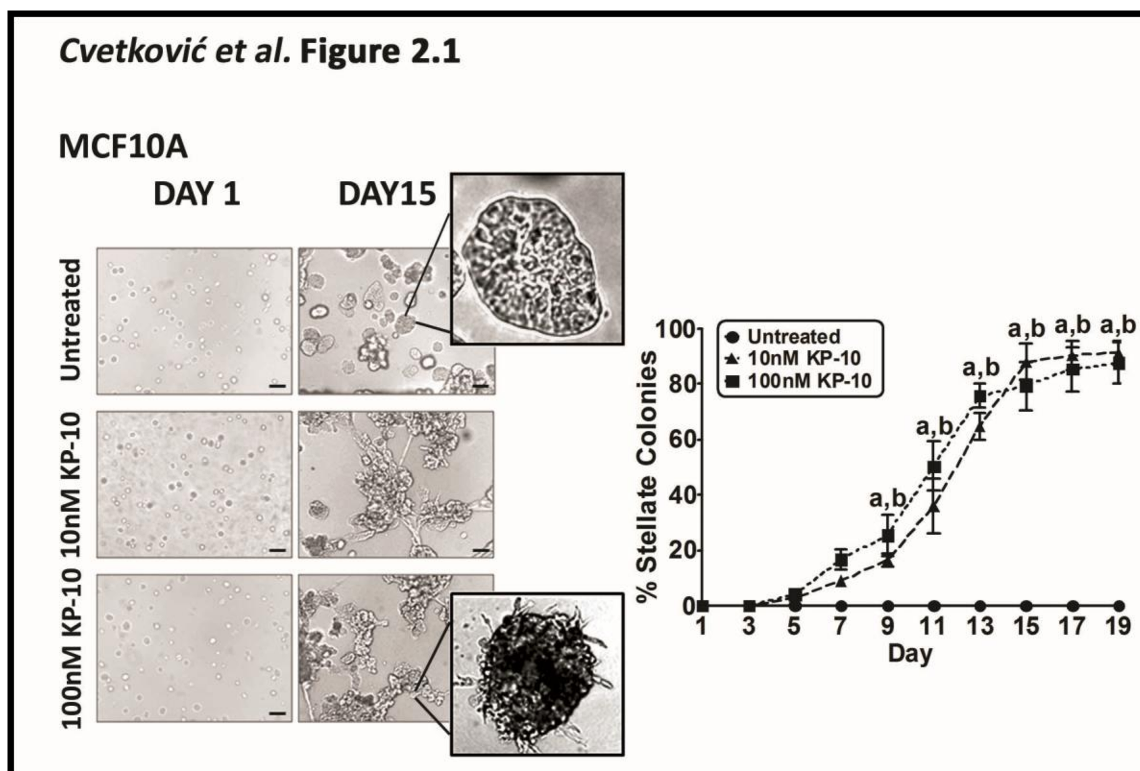


**Figure 2.1. KP-10 stimulates invasion of non-malignant mammary epithelial MCF10A cells.** Treatment of MCF10A cells with either 10 or 100nM KP-10 significantly increases invasive stellate structure formation in 3D cultures, when compared to untreated cells (n=5). Two-way ANOVA followed by Bonferroni post-hoc test: *a*, *P<0.05* for 10nM KP-10 when compared to untreated cells; *b*, *P<0.05* for 100nM KP-10 when compared to untreated cells. *Scale bar*, 100 µm.

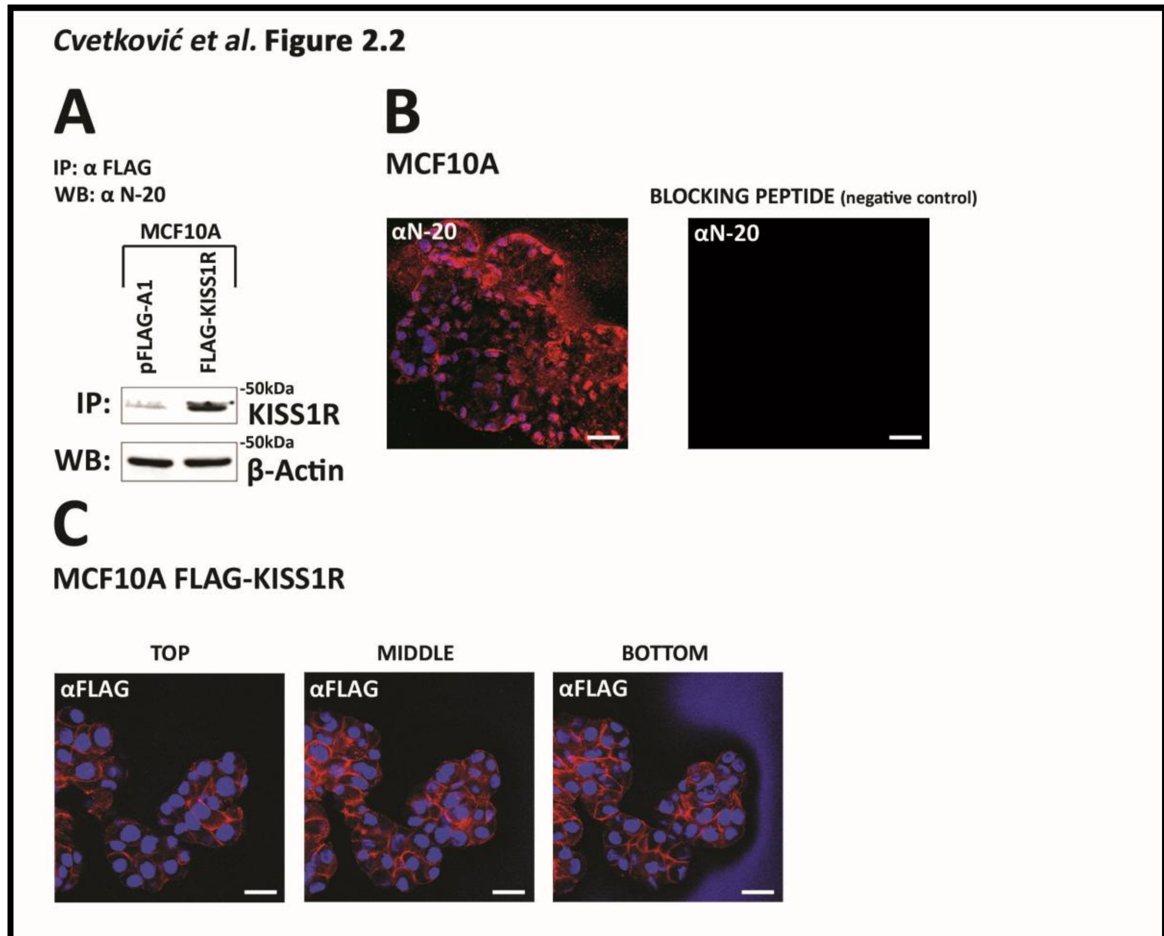


**Figure 2.2. KISS1R expression in MCF10A and stable transfectants.** **(A)** KISS1R expression in MCF10A FLAG-KISS1R stables. Immunoprecipitation with a rabbit polyclonal anti-FLAG antibody, followed by Western blot analysis with a goat polyclonal KISS1R (N-20) antibody reveals expression of FLAG-KISS1R in MCF10A FLAG-KISS1R stables. β-Actin, *loading control* (n=3). **(B)** Endogenous expression of KISS1R in MCF10A parental cells grown in 3D Matrigel cultures. KISS1R immunofluorescence was detected using goat polyclonal KISS1R (N-20) followed by anti-goat Alexa Fluor 555 (red). Presence of the blocking peptide against N-20 ablated immunofluorescence observed with N-20 (n=3). **(C)** Localization of KISS1R in MCF10A FLAG-KISS1R cells grown in 3D Matrigel cultures. Confocal microscopy

(displaying three sequential Z-stacks) of FLAG-KISS1R immunofluorescence was detected using a rabbit polyclonal FLAG antibody followed by anti-rabbit Alexa Fluor 568 (red). Nuclei stained using Hoechst (blue) (n=3). *Scale bar*, 20 μm.

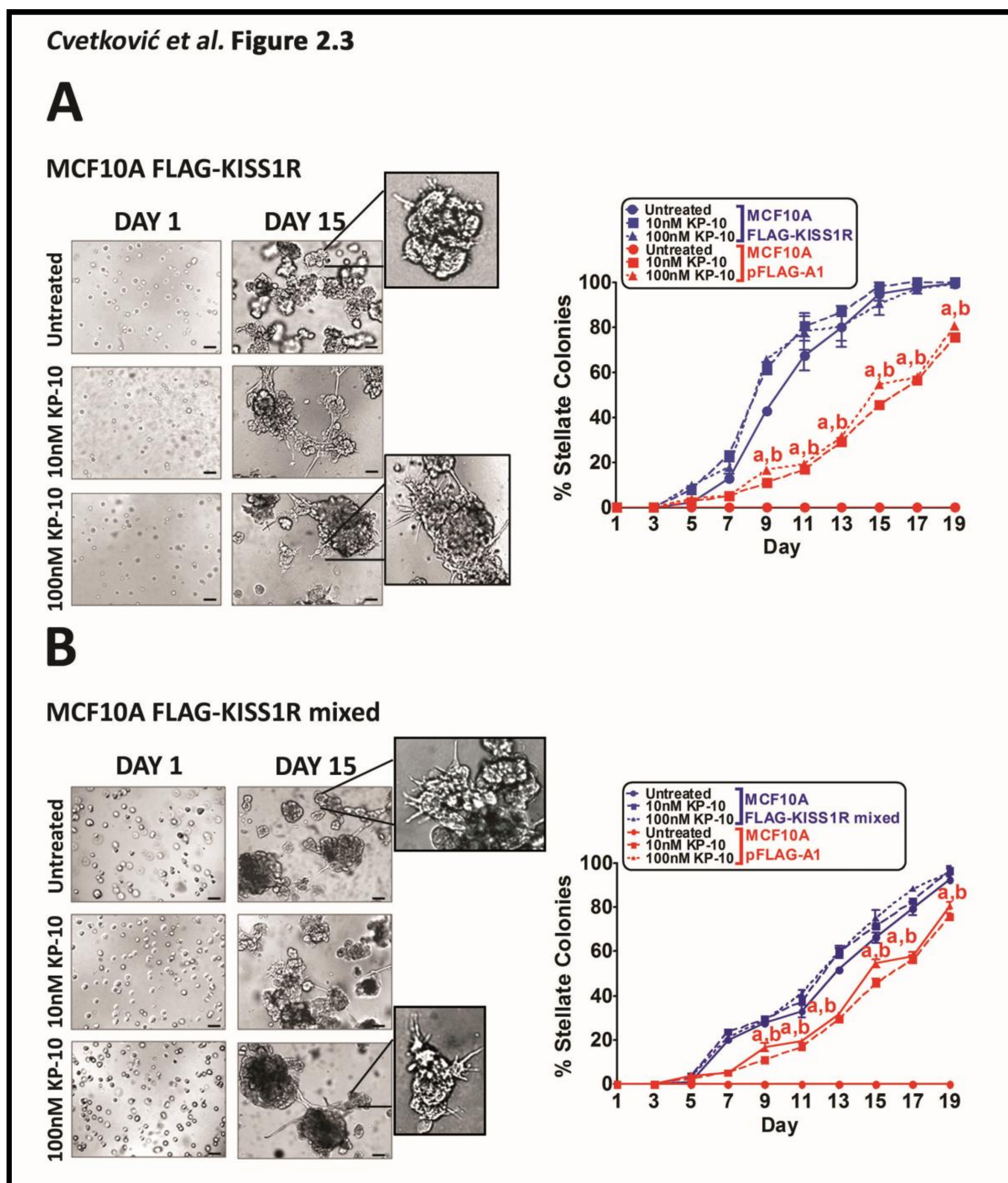


**Figure 2.3. KISS1R expression stimulates invasion of non-malignant mammary epithelial MCF10A cells.** Stable expression of FLAG-KISS1R in MCF10A cells **(A)** monoclonal population and **(B)** mixed pooled population induces stellate colony formation in 3D cultures, both in the presence and in the absence of KP-10. MCF10A

pFLAG-A1 (vector control) cells only invade in the presence of KP-10 (n=3). Two-way ANOVA followed by Bonferroni post-hoc test: *a*, $P<0.05$ for 10nM KP-10 MCF10A pFLAG-A1 (vector control) cells when compared to untreated MCF10A pFLAG-A1 (vector control) cells; *b*, $P<0.05$ for 100nM KP-10 MCF10A pFLAG-A1 (vector control) cells when compared to untreated MCF10A pFLAG-A1 (vector control) cells. *Scale bar*, 100 μm.

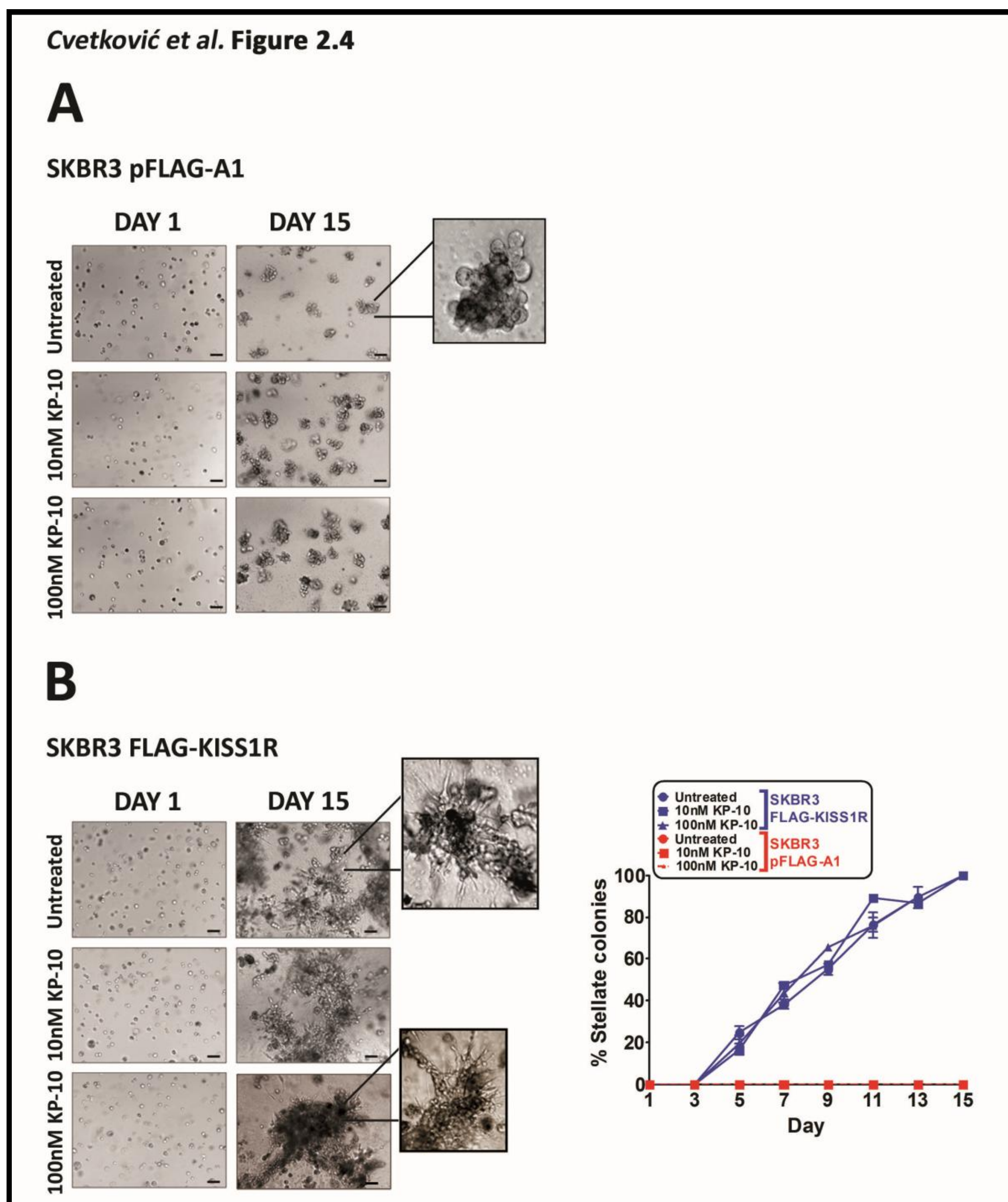


**Figure 2.4. Stable KISS1R expression stimulates invasion of SKBR3 breast cancer cells. (A)** KP-10 does not induce stellate colony formation in SKBR3 pFLAG-A1 (vector control) cells in 3D cultures (n=3). **(B)** Stable expression of FLAG-KISS1R in SKBR3

cells induces stellate colony formation, both in the presence and in the absence of KP-10 in 3D cultures (n=3). *Scale bar*, 100 µm.

**KISS1R stimulates motility of mammary epithelial cells**. Cell motility is required for many important physiological processes and unregulated cell motility can be the cause for progression of cancer. In order to visualize whether or not activation of KISS1R by KP-10 stimulates MCF10A cell motility, scratch assays were performed, as we have previously described (Zajac *et al.*, 2011). Addition of KP-10 (10nM) significantly enhanced the distance travelled by MCF10A cells, compared to cells stimulated with medium supplemented with 5% FBS **(Figure 2.5A)**. When KP-10 was dissolved in medium lacking FBS (i.e., serum-free medium), no migration was observed (data not shown), as we have previously reported in the MDA-MB-231 cells (Zajac *et al.*, 2011). Furthermore, 10nM KP-10 significantly stimulated cell motility in MCF10A pFLAG-A1 (vector control) and MCF10A FLAG-KISS1R cells, compared to cells seeded only in 5% FBS **(Figure 2.5B)**. To exclude the confounding effects of FBS on cell proliferation, cell growth assays were performed to determine the doubling time of these cells. Both MCF10A pFLAG-A1 and MCF10A FLAG-KISS1R had a doubling time that was greater than 72 hours **(Figure 2.5C)**. Thus, this suggests that the effects of KP-10 on scratch closure are due to motility and not due to proliferation. Therefore, KP-10 significantly enhances motility of the ER-negative non-malignant MCF10A cells and stables.

To investigate whether the KISS1R is localized to the leading edge of cells, which is consistent with a role in cell migration, we examined the distribution of KISS1R in migrating MCF10A and MCF10A FLAG-KISS1R cells. A well established marker for the leading edge of migratory cells is the actin scaffolding protein IQGAP1, which interacts with actin filaments to cross-link them (Mataraza *et al.*, 2003; Noritake *et al.*, 2005; Brown and Sacks, 2006). After wounding a confluent monolayer of cells,

immunofluorescence microscopy showed that endogenous KISS1R was localized to the leading edge of migrating MCF10A cells, where it co-localized with IQGAP1 in lamellipodia **(Figure 2.6A)**. Furthermore, in MCF10A FLAG-KISS1R cells, KISS1R was also localized to the leading edge, where it co-localized with IQGAP1 **(Figure 2.6B)**. Taken together, these results suggest that KISS1R activation not only induces MCF10A and MCF10A FLAG-KISS1R cells to invade, but also plays a role in cell motility.

We have previously shown that KP-10 enhances cell motility of MDA-MB-231 breast cancer cells (Zajac *et al.*, 2011). To verify a role of endogenous KISS1R signaling in regulating cell motility, I have performed cell motility assays in the presence of a KISS1R antagonist, P-234. KP-10-enhanced motility of MDA-MB-231 cells was significantly blocked in the presence of P-234 **(Figure 2.7A)**. Furthermore, treatment of these cells with the antagonist did not affect cell viability **(Figure 2.7B)**. These results validate a role for endogenous KISS1R signaling in KP-10-induced breast cancer cell migration.

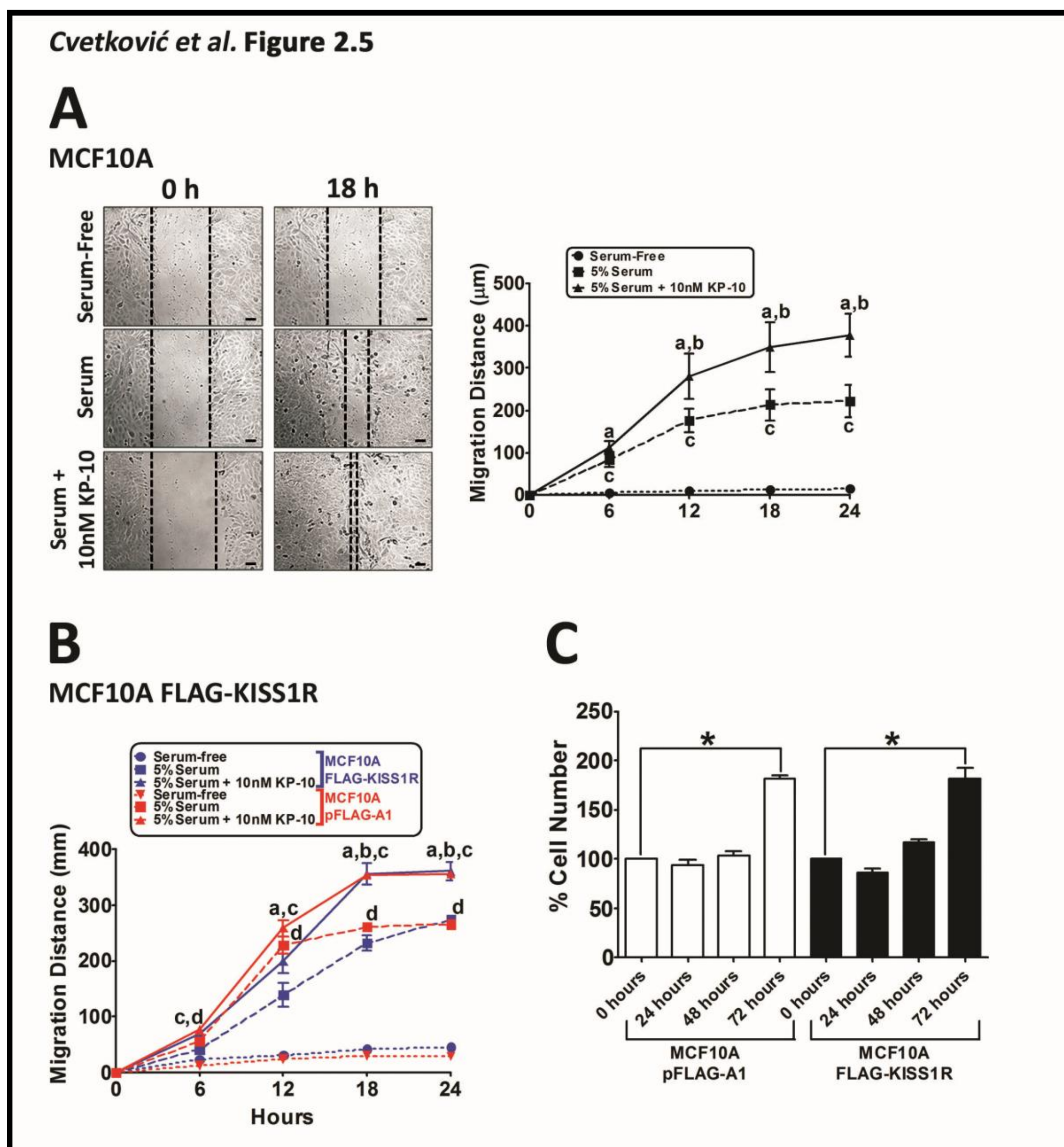


**Figure 2.5. KISS1R stimulates motility of mammary epithelial cells**. **(A)** Treatment with 10nM KP-10 significantly enhances the distance closed of MCF10A cells in a scratch assay over a 24 hour period (n=7). Two-way ANOVA followed by Bonferroni post-hoc test: *a*, $P<0.05$ for serum-free when compared to 5% serum and 10nM KP-10; *b*, $P<0.05$ for 5% serum when compared to 5% serum and 10nM KP-10; *c*, $P<0.05$ for serum-free when compared to 5% serum. *Scale bar*, 100 µm. **(B)** Treatment of 10nM

KP-10 significantly enhances the motility of MCF10A FLAG-KISS1R and MCF10A pFLAG-A1 (vector control) cells in scratch assay (n=3). Two-way ANOVA followed by Bonferroni post-hoc test: *a*, $P<0.05$ for 5% serum (MCF10A FLAG-KISS1R) when compared to 5% serum and 10nM KP-10 (MCF10A FLAG-KISS1R); *b*, $P<0.05$ for 5% serum (MCF10A pFLAG-A1) when compared to 5% serum and 10nM KP-10 (MCF10A pFLAG-A1); *c*, $P<0.05$ for serum-free (MCF10A pFLAG-A1 or MCF10A FLAG-KISS1R) when compared to 5% serum and 10nM KP-10 (MCF10A pFLAG-A1/MCF10A FLAG-KISS1R); *d*, $P<0.05$ for serum-free (MCF10A pFLAG-A1 or MCF10A FLAG-KISS1R) when compared to 5% serum (MCF10A pFLAG-A1/MCF10A FLAG-KISS1R). *Scale bar*, 100 µm. **(C)** MCF10A pFLAG-A1 and MCF10A FLAG-KISS1R cell proliferation at 0, 24, 48, 72 hours. $4.0 \times 10^5$ cells were seeded, and counted every 24 hours using hemocytometer. Cell proliferation is expressed as % Cell Number (set as 100% at the time of seeding (0 hours)). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$. Bars represent % Cell Number ± SEM.

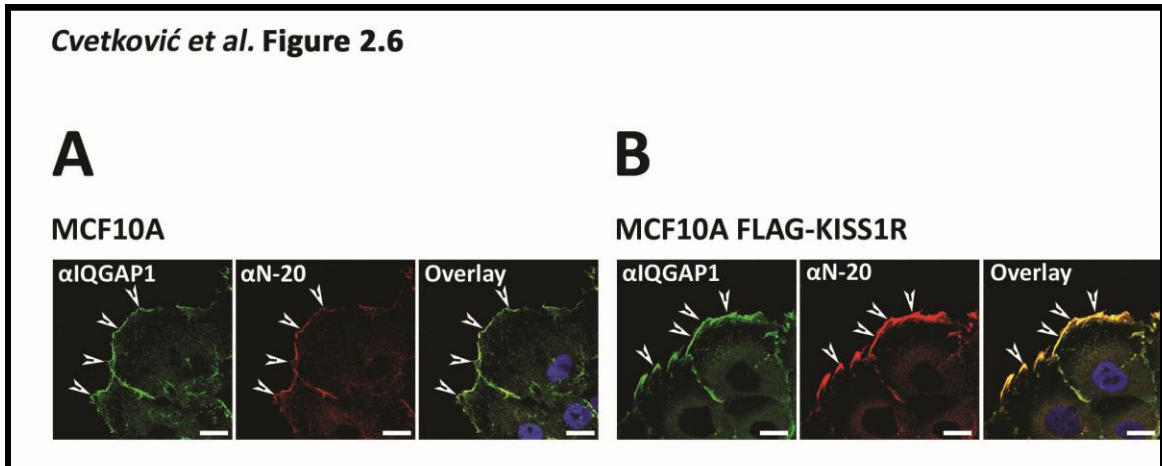


**Figure 2.6. KISS1R co-localizes with IQGAP1 in MCF10A and MCF10A FLAG-KISS1R cells.** KISS1R is localized to the leading edge of **(A)** MCF10A and **(B)** MCF10A FLAG-KISS1R cells where it is co-localized with IQGAP1 in lamellipodia (white arrowheads). Confocal micrographs showing KISS1R immunofluorescence using a goat polyclonal N-20 KISS1R antibody followed by an anti-goat Alexa Fluor 555 (red); IQGAP1 detected using a monoclonal anti-IQGAP1 antibody followed by an anti-mouse Alexa Fluor 488 (green). Areas of co-localization are shown in overlay (yellow). Nuclei stained using Hoechst (blue) (n=3). *Scale bar*, 20 µm.

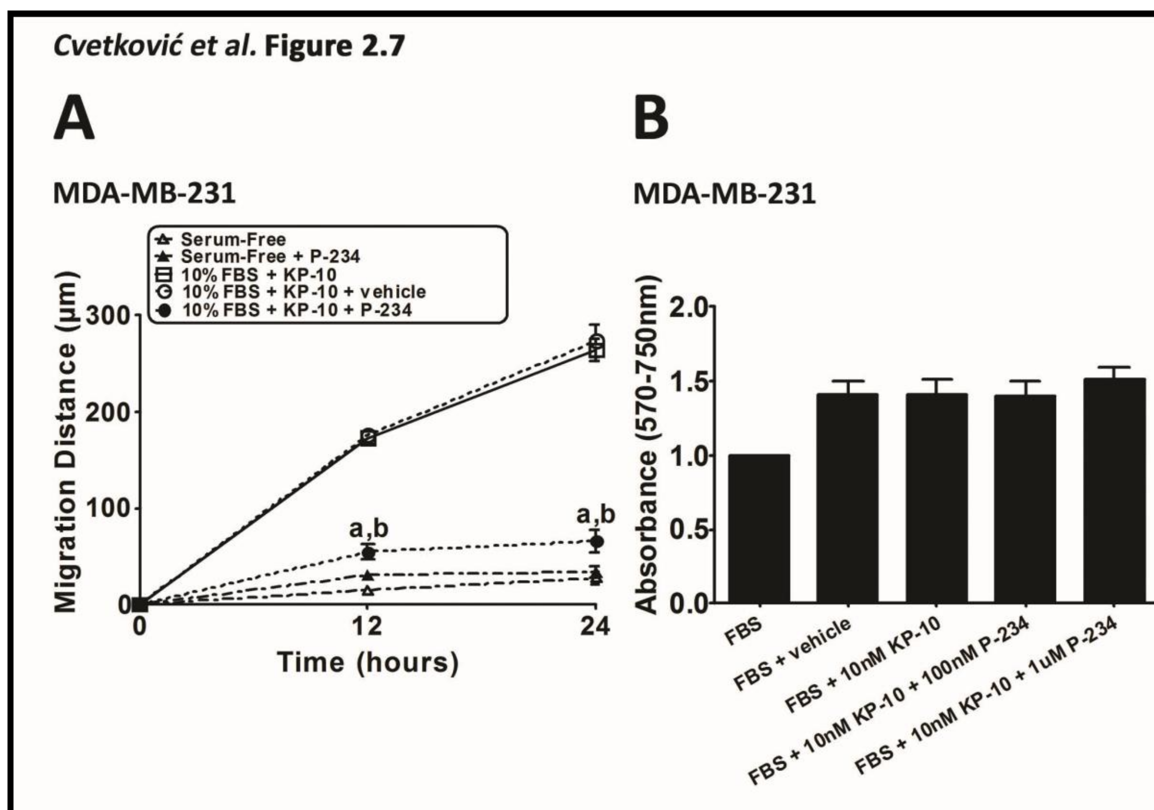


**Figure 2.7. P-234 (KISS1R antagonist) inhibits KP-10-enhanced cell motility**. **(A)** MDA-MB-231 were treated with 1μM P-234 and subjected to a scratch assay over a 24 hour period (n=3). Two-way ANOVA followed by Bonferroni post-hoc test: *a*, *P<0.05* for 10% FBS and 10nM KP-10 and 1μM P-234 when compared to 10% FBS and 10nM KP-10, *b*, *P<0.05* for 10% FBS and KP-10 and P-234 when compared to 10% FBS and KP-10 and vehicle. **(B)** P-234 does not affect the viability of MDA-MB-231 cells. 5.0 x $10^4$ cells were plated in 96-well plates and treated as indicated. Cells were then incubated with 0.5mg/mL of MTT labeling agent for 4 h and subsequently solubilized for 24 hours (n=3).

**KP-10 induces IP formation.** KISS1R is a canonical GPCR, which signals *via* $G_{q/11}$ G-protein, leading to production of second messengers DAG and $IP_3$ (Kotani *et al.*, 2001; Muir *et al.*, 2001). Since I found that KISS1R signaling stimulates MCF10A cells to migrate and invade, I also assessed whether activation of KISS1R stimulated IP formation in MCF10A, MCF10A pFLAG-A1, MCF10A FLAG-KISS1R and MCF10A FLAG-KISS1R mixed cells. I found that stimulation of cells with 100nM KP-10 (in media with 5% FBS) for one hour increased IP formation in each MCF10A cell line, compared to cells incubated only in media with 5% FBS **(Figure 2.8)**. However, treatment of cells with KP-10 in the absence of FBS did not increase IP formation in the MCF10A. These results reveal that a functionally active KISS1R receptor is present in parental MCF10A cells, and that both endogenously and exogenously expressed KISS1R signal in a similar fashion.

**KP-10 stimulates EGFR transactivation in non-malignant mammary epithelial cells and stable transfectants.** We have previously shown that KP-10 stimulates migration and invasion of the ER-negative breast cancer cells MDA-MB-231 and Hs578T *via* transactivation of EGFR (Zajac *et al.*, 2011). Therefore, I assessed whether the ER-negative MCF10A cells also undergo KP-10-stimulated EGFR transactivation. Following stimulation with 100nM KP-10, there was a significant increase in EGFR phosphorylation levels, compared to non-stimulated MCF10A cells **(Figure 2.9)**. EGF (positive control) caused a significant phosphorylation of EGFR in these cells. Moreover, similar observations were made in MCF10A pFLAG-A1 **(Figure 2.10A)**, MCF10A FLAG-KISS1R **(Figure 2.10B)** and MCF10A FLAG-KISS1R mixed cells **(Figure**

**2.10C**), whereby KP-10 stimulated EGFR transactivation. Furthermore, AG1478, an inhibitor of EGFR, blocked KP-10-stimulated EGFR transactivation **(Figure 2.10C)**. KP-10 stimulated EGFR transactivation was also blocked upon treatment of cells with AG1478 in the ER-negative SKBR3 cells **(Figure 2.10D)**. These data suggest that KP signaling transactivates EGFR in the ER-negative mammary epithelial cells and the ER-negative breast cancer cells.

**KP-10 stimulates EGFR transactivation in malignant MCF10Ca1h cells.** In a 2001 study, Santner and colleagues reported on the derivation of fully malignant MCF10CA1 lines generated upon Ras-transforming the MCF10A cells that complete the spectrum of progression from MCF10A to malignant breast cancer cells (Santner *et al.*, 2001). This well established MCF10 model provides a convenient tool for the investigation of molecular changes during progression of human breast neoplasia on a common genetic background (Santner *et al.*, 2001). Since, MCF10A cells undergo KP-10-stimulated EGFR transactivation **(Figure 2.9)**, I next sought to determine if the MCF10Ca1h cell do the same. KP-10 stimulated EGFR transactivation in MCF10Ca1h cells which was subsequently shown to be blocked with the EGFR inhibitor, AG1478 **(Figure 2.11)**. These data reveal that KP signaling *via* KISS1R transactivates EGFR not only in MCF10A cells, but also in Ras-transformed MCF10Ca1h cells. These data indicate that breast epithelial cells appear to undergo KP-10-stimulated EGFR transactivation irrespective of their aggressiveness.

**P-234 inhibits KP-10-stimulated EGFR transactivation.** To further assess the role of KISS1R in mediating KP-10-stimulated EGFR transactivation, the KISS1R antagonist was used to determine the effects on KP-10-stimulated EGFR transactivation. Pretreatment of MDA-MB-231 breast cancer cells and MCF10A FLAG-KISS1TR cells with 1μM P-234 inhibited KP-10-stimulated EGFR transactivation. These data suggest that KP signaling *via* KISS1R transactivates EGFR in the ER-negative MDA-MB-231 breast cancer **(Figure 2.12A)** and MCF10A FLAG-KISS1R cells **(Figure 2.12B)**.

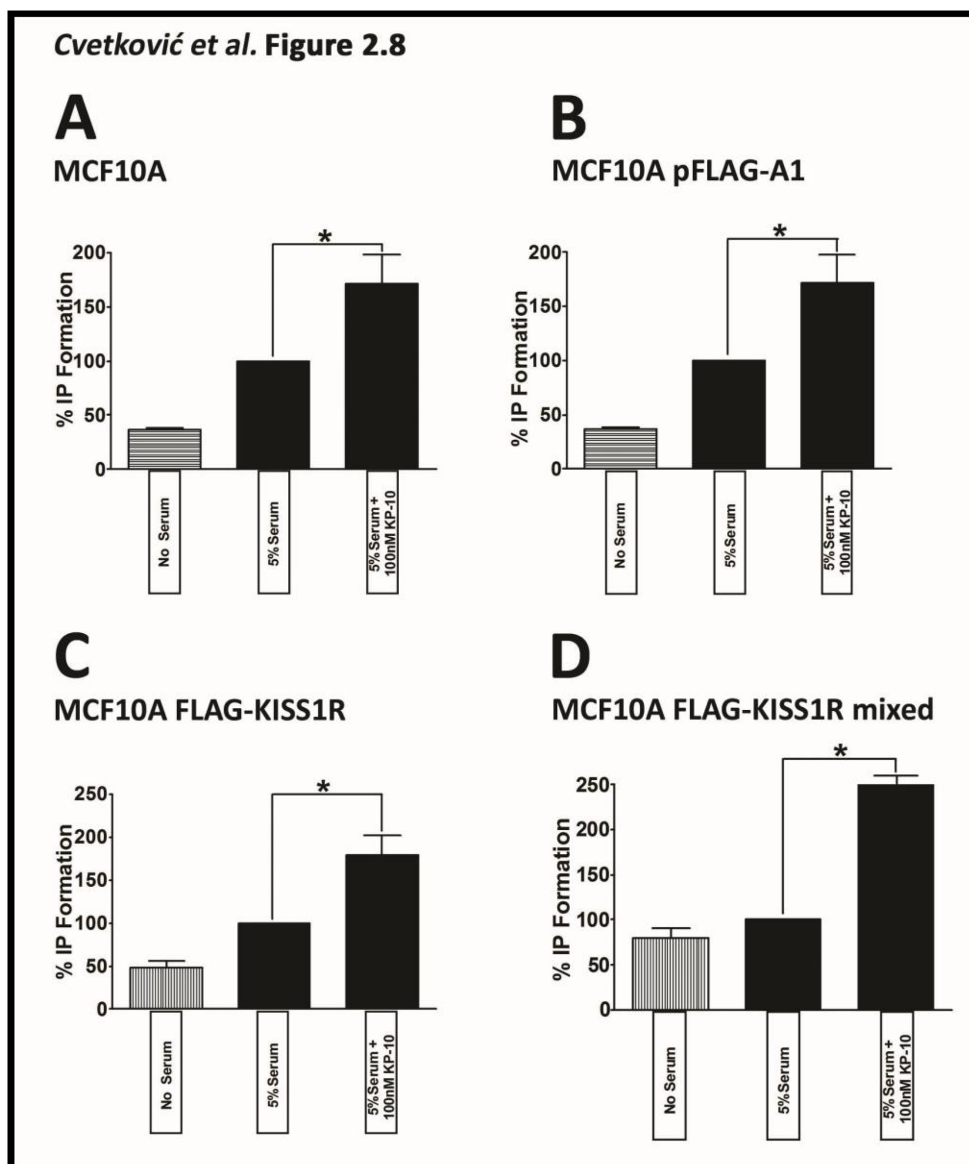


**Figure 2.8. KP-10 induces IP production in MCF10A and stable transfectants.** IP formation in MCF10A (**A**), MCF10A pFLAG-A1 (**B**), MCF10A FLAG-KISS1R **(C)** and MCF10A FLAG-KISS1R mixed cells **(D)** in the presence or absence of KP-10 (n=5). Cells were incubated overnight with 1 μCi/mL [$^3$H]myo-inositol in DMEM to radiolabel inositol lipids. Cells were incubated in either the absence or the presence of 100nM KP-10 for one hour at 37°C. Total [$^3$H]inositol incorporated into the cells was determined by counting the radioactivity present in 50μL of the cell lysate. Total IP was purified from

the cell extracts by anion exchange chromatography using AG® 1-X8 (analytical grade, 200-400 mesh, formate form) resin (Bio-Rad). [$^{3}$H]IP formation was determined by LS 6500 Scintillation Counter (Beckman). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$. Bars represent % IP Formation ± SEM.

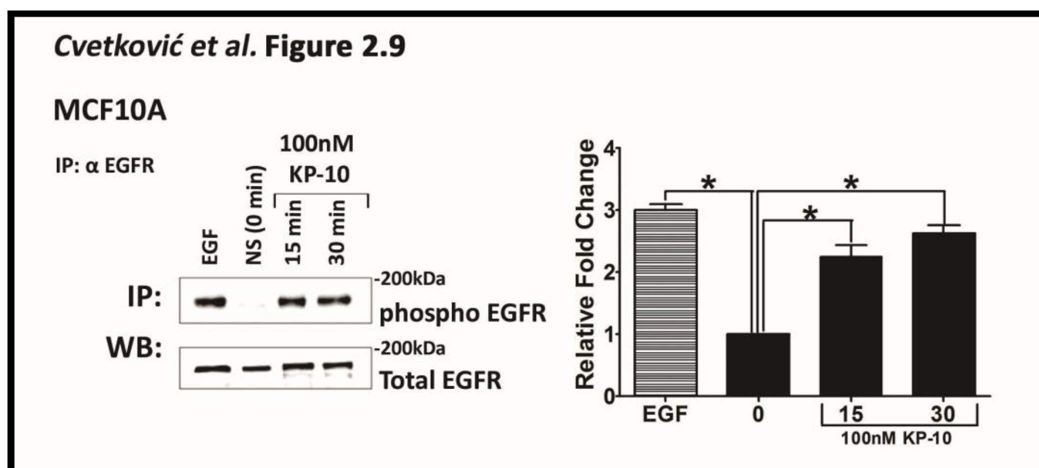


**Figure 2.9. KP-10 stimulates EGFR transactivation in non-malignant mammary epithelial cells.** MCF10A cells were serum-starved for 24 hours, and then left unstimulated (NS), treated with 10ng/mL EGF (positive control) or 100 nM KP-10 for the indicated time points and then solubilized in lysis buffer. KP-10 treatment results in increase of EGFR phosphorylation (n=4). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$.

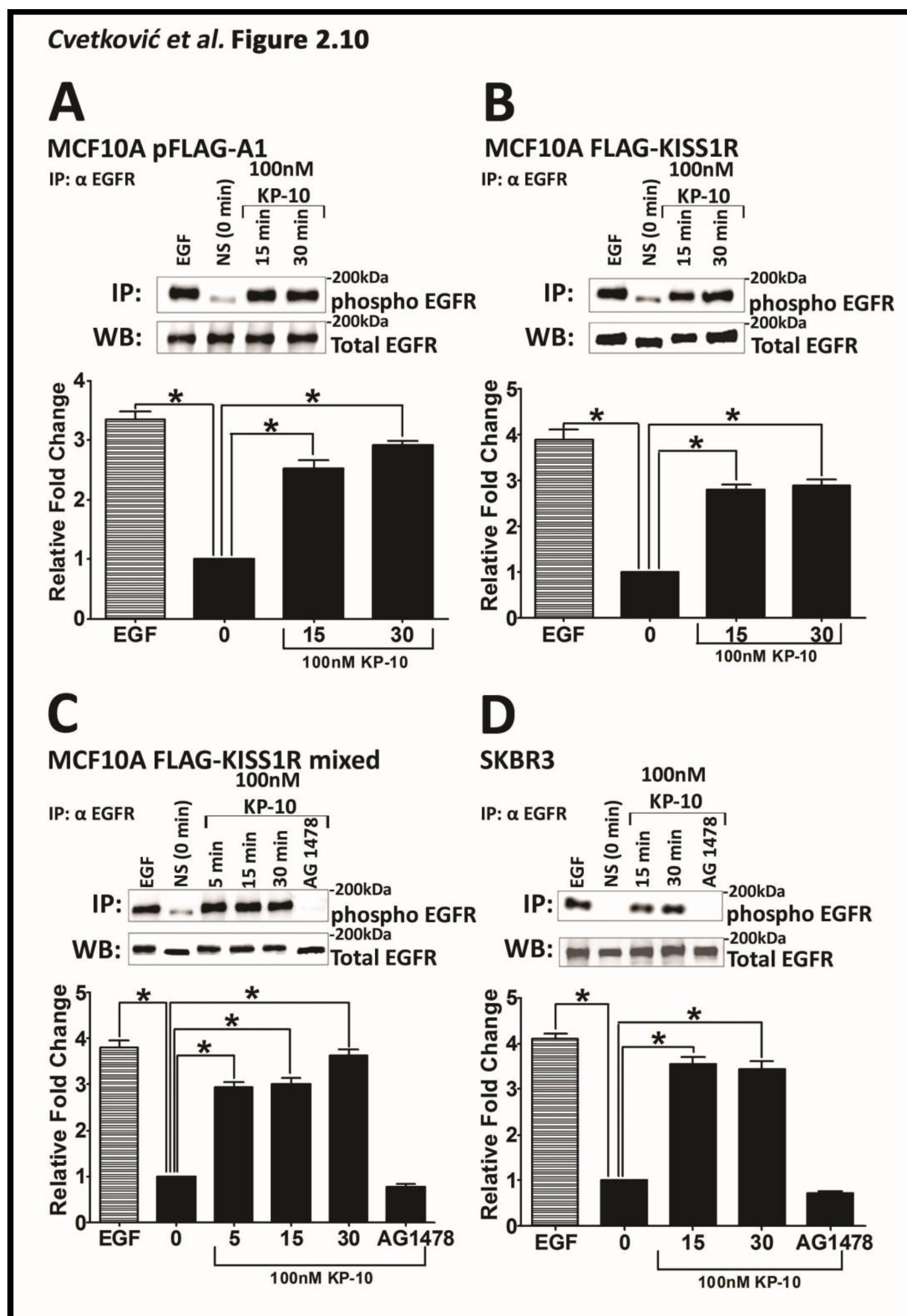


**Figure 2.10. KP-10 stimulates EGFR transactivation in ER-negative cells. (A)** MCF10A pFLAG-A1, **(B)** MCF10A FLAG-KISS1R, **(C)** MCF10A FLAG-KISS1R

mixed and **(D)** SKBR3 cells were serum-starved for 24 hours, then either left in serum-free medium or pretreated with 500nM AG1478 for one hour, and then left un-stimulated (NS), treated with 10ng/mL EGF (positive control) or 100 nM KP-10 for the indicated time points and then solubilized in lysis buffer. KP-10 increases EGFR phosphorylation (n=3). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$.

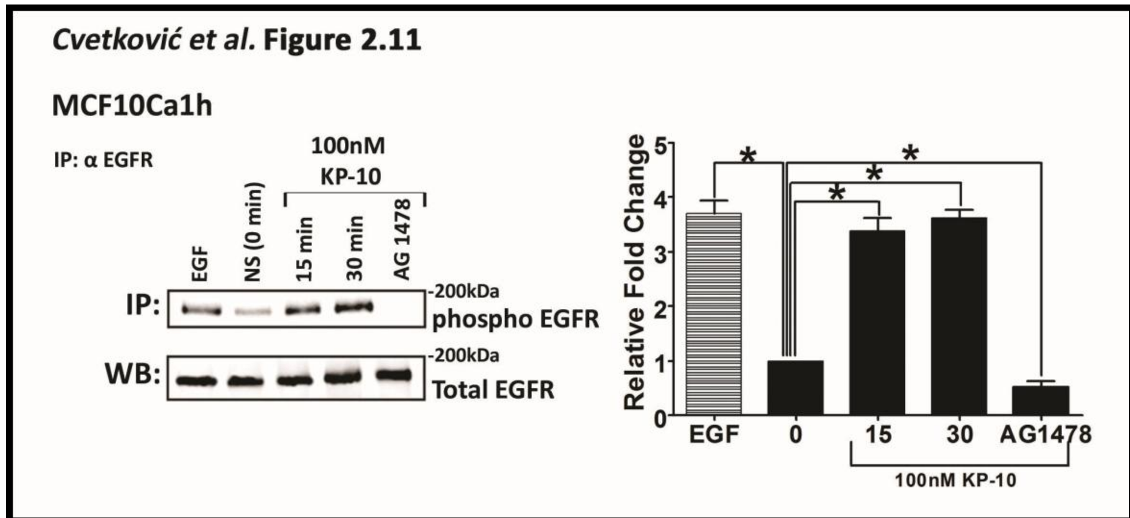


**Figure 2.11. KP-10 stimulates EGFR transactivation in malignant MCF10Ca1h cells.** Cells were serum-starved for 24 hours, then either left in serum-free medium or pretreated with 500nM AG1478 for one hour, and then left un-stimulated (NS), treated with 10ng/mL EGF (positive control) or 100 nM KP-10 for the indicated time points and then solubilized in lysis buffer. KP-10 increases EGFR phosphorylation (n=3). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$.

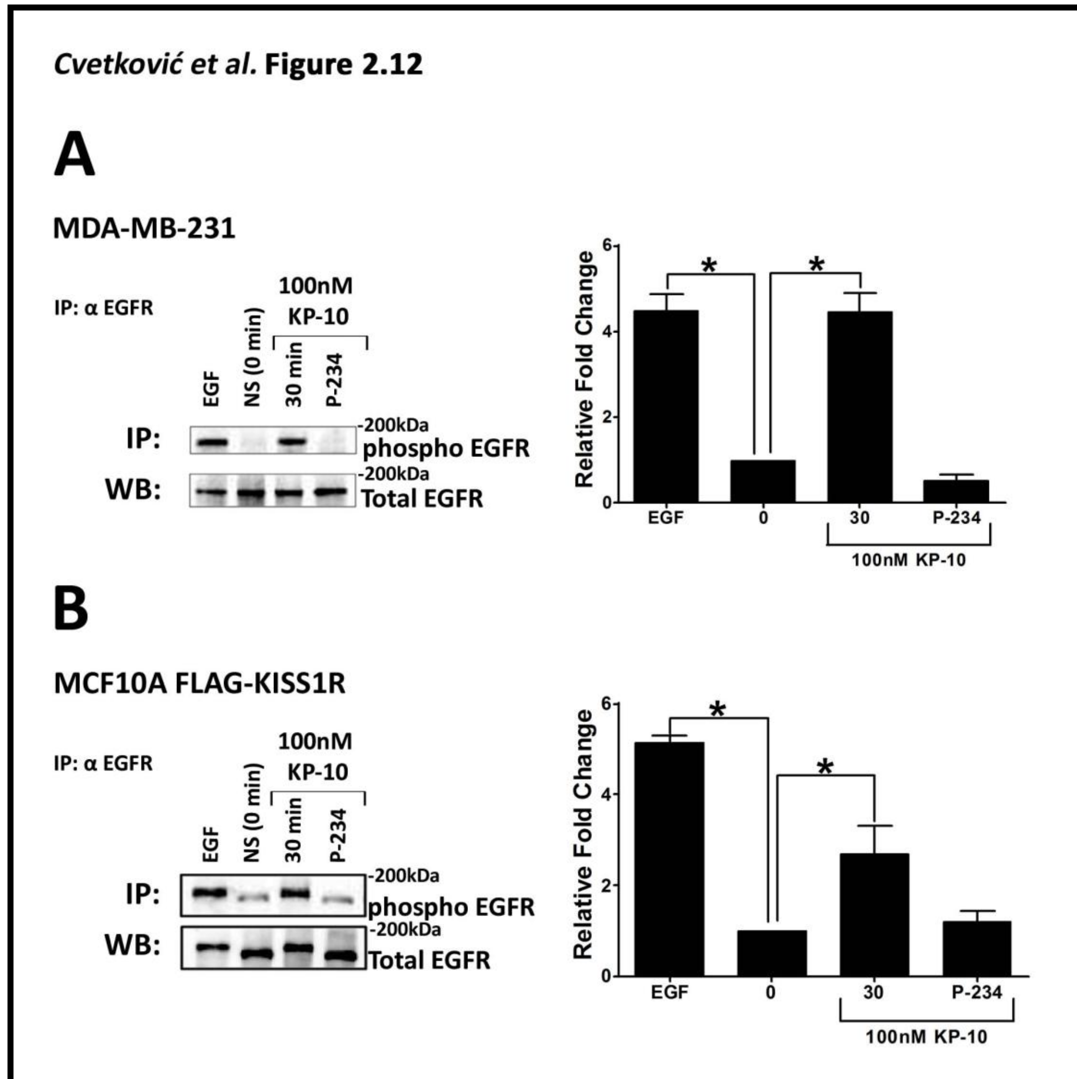


**Figure 2.12. P-234 inhibits KP-10-stimulated EGFR transactivation. (A)** MDA-MB-231 or **(B)** MCF10A FLAG-KISS1R cells were serum-starved for 24 hours or pretreated with 1μM P-234, and then either left untreated (NS), treated with 10ng/mL EGF (positive control) or 100 nM KP-10 for the indicated time points and then solubilized in lysis buffer. P-234 inhibits KP-10-stimulated EGFR phosphorylation (n=3). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$.

**KP-10 does not stimulate invasiveness and EGFR transactivation of ER-positive breast cancer cells.** Thus far, my data indicated that KP-10 stimulates invasiveness and EGFR transactivation in the ER-negative breast epithelia. Next, I sought to determine if KP-10 induces invasion, motility and EGFR transactivation in the ER-positive, MCF7 and T47D breast cancer cells. KP-10 did not stimulate MCF7 cell invasion in 3D cultures **(Figure 2.13A)** or cell migration, using Transwell chambers **(Figure 2.13B)**. To visualize the effect of KP-10 on cell motility, scratch assays were performed and although EGF significantly stimulated MCF7 cell motility, KP-10 (10nM or 100nM) did not enhance scratch closure, when compared to cells in the presence of 10% FBS **(Figure 2.13C)**. Additionally, KP-10 did not transactivate EGFR in MCF7 cells, while stimulation of cells with EGF (positive control) resulted in EGFR phosphorylation **(Figure 2.13D)**. We have previously reported that KP-10 does not stimulate migration of T47D cells (Zajac *et al.*, 2011). Furthermore, KP-10 did not stimulate T47D cells to invade in 3D cultures **(Figure 2.14A)** or migrate using scratch assays **(Figure 2.14B)**. Additionally, T47D cells did not undergo KP-10-stimulated EGFR transactivation **(Figure 2.14C)**. Thus, these data suggest that the ER status of the cells critically regulates whether KISS1R signaling stimulates invasiveness and EGFR transactivation, suggesting that KP-10 may influence metastatic potential of the breast cancer cells that are deficient of ERα.

**ERα expression in the ER-negative MDA-MB-231 attenuates KP-10-induced migration, invasion and EGFR transactivation.** To further ascertain the correlation between ERα expression and KISS1R signaling, ERα was stably expressed in the ER-

negative MDA-MB-231 cells **(Figure 2.15A)**. I found that stable expression of ERα blocked both KP-10-induced migration and invasion **(Figure 2.15B)**, and KP-10-stimulated EGFR transactivation **(Figure 2.15C)**, compared to cells expressing the vector control. Thus, these data suggest that KP-10-stimulated EGFR transactivation depends on the ER status of breast epithelia.

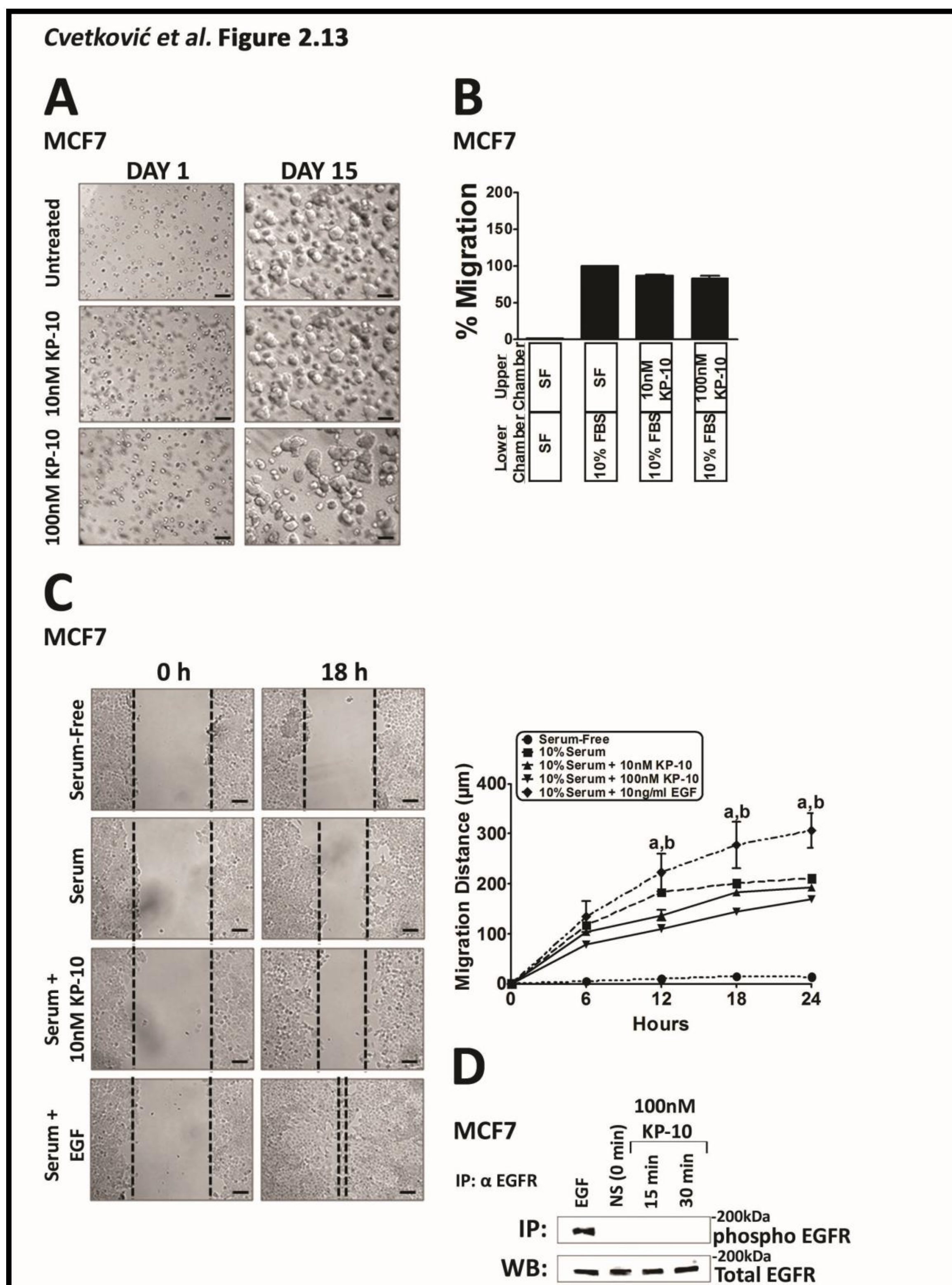


**Figure 2.13. KP-10 does not stimulate invasion, migration or EGFR transactivation in the ER-positive MCF7 breast cancer cells. (A)** Treatment of MCF7 cells with either

10 or 100nM KP-10 does not result in invasive stellate structure formation (n=3). **(B)** KP-10 does not stimulate migration of MCF7 cells. Results are presented as a ratio of cells that migrated relative to cells seeded in serum-free medium and migrating towards 10% (v/v) fetal bovine serum-supplemented medium, as was measured by Transwell chamber assay (n=6). On average 200-300 cell migrated towards 10% FBS per field. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$. **(C)** KP-10 does not stimulate the motility of MCF7 cells using a scratch assay (n=3). Two-way ANOVA followed by Bonferroni post-hoc test: *a*, $P<0.05$ for 10ng/mL EGF and 10% serum when compared to untreated cells; *b*, $P<0.05$ for 10ng/mL EGF and 10% serum when compared to 10% serum. *Scale bar*, 100 µm. **(D)** MCF7 cells were serum-starved for 24 hours, and then left untreated (NS), treated with 10ng/mL EGF (positive control) or 100 nM KP-10 for the indicated time points and then solubilized in lysis buffer. KP-10 treatment does not lead to EGFR phosphorylation (n=3).

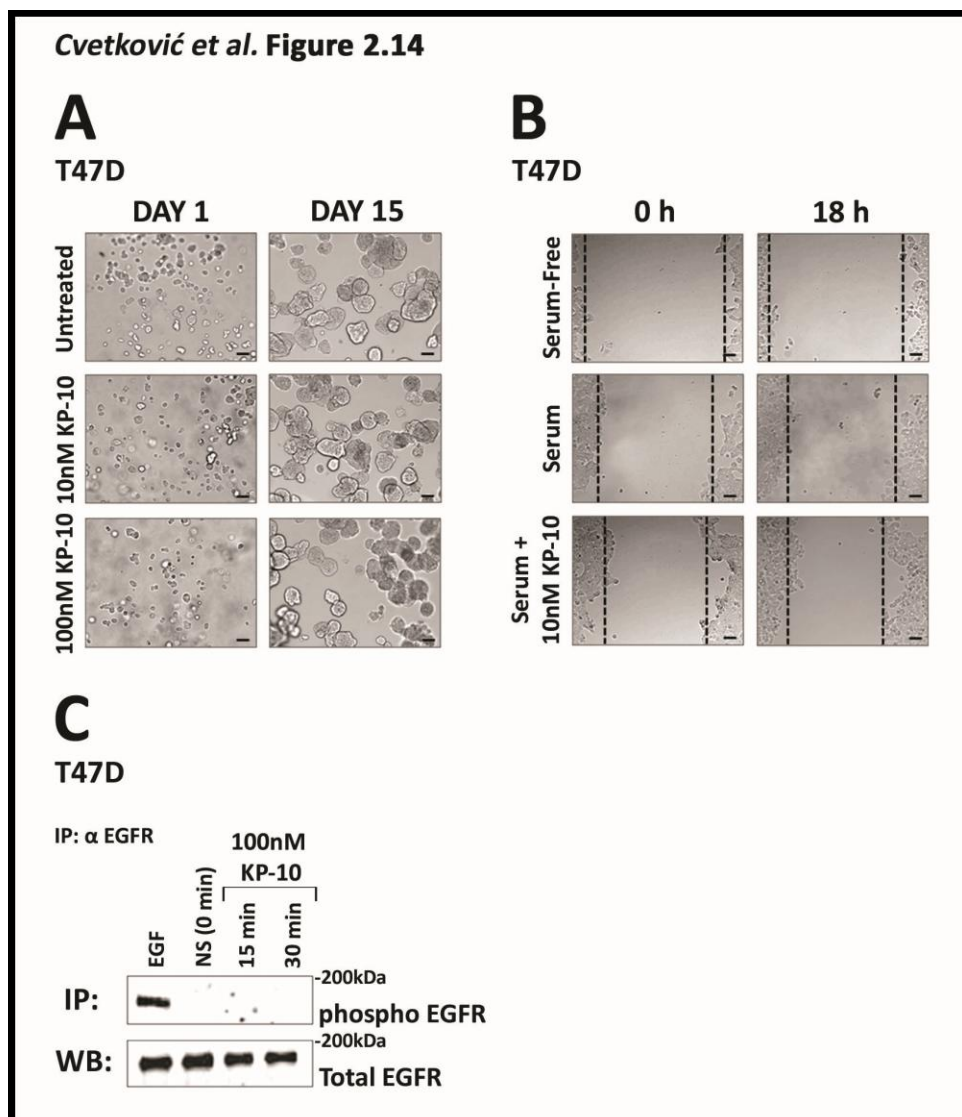


**Figure 2.14. KP-10 does not stimulate invasive stellate structure formation, motility or EGFR transactivation in the ER-positive T47D breast cancer cells.** **(A)** Treatment of T47D cells with either 10 or 100nM KP-10 does not result in a stellate structure formation (n=3). **(B)** KP-10 does not affect the motility of T47D cells in a scratch assay (n=3). *Scale bar*, 100 μm. **(C)** Cells were serum-starved for 24 hours, and then left untreated (NS), treated with 10ng/mL EGF (positive control) or 100 nM KP-10 for the indicated time points and then solubilized in lysis buffer. KP-10 treatment does not lead to EGFR phosphorylation (n=3).

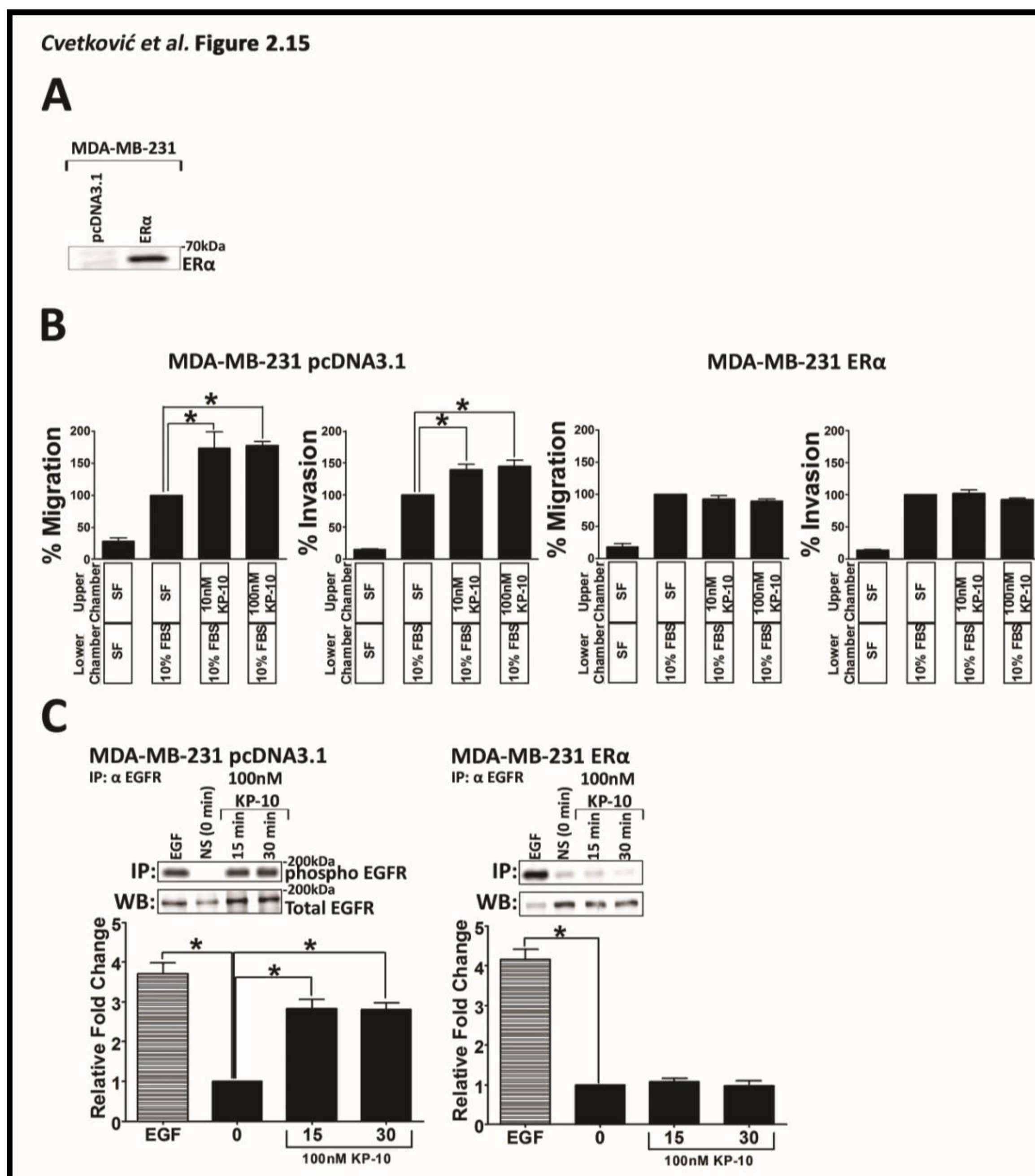


**Figure 2.15. ERα expression in ER-negative MDA-MB-231 cells abrogates KP-10-enhanced migration and EGFR transactivation. (A)** A representative Western blot showing stable expression of ERα in MDA-MB-231 cells. **(B)** KP-10 (10nM and 100nM) significantly stimulates cell migration and invasion in MDA-MB-231 pcDNA3.1 (vector control) cells. ERα expression in MDA-MB-231 cells blocks KP-10-enhanced migration

and invasion observed in MDA-MB-231 pcDNA3.1 (vector control) cells. Results are presented as a ratio of cells that migrated relative to cells seeded in serum-free medium and migrating towards 10% (v/v) fetal bovine serum-supplemented medium, as was measured by Transwell chamber assay (n=3). On average 400-500 cell migrated towards 10% FBS per field in both MDA-MB-231 pcDNA3.1 and MDA-MB-231 ERα cells. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$. Bars represent % migration ± SEM. **(C)** KP-10-mediated EGFR transactivation in MDA-MB-231 pcDNA3.1, is ablated in MDA-MB-231 ERα cells. Cells were serum-starved for 24 hours, and then left untreated (NS), treated with 10ng/mL EGF (positive control) or 100 nM KP-10 for the indicated time points. (n=3). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P <0.05$. Bars represent % migration ± SEM.

**Exogenous expression of KISS1R induces a partial EMT-like phenotype.** Two of the most important pathways in human breast cancer involve estradiol ($E_2$)/ERα and E-cadherin/Snail/Slug signaling, the latter leading to EMT (Micalizzi *et al.*, 2010). The E-cadherin-Snail-Slug cascade is an important pathway implicated in tumor progression, invasion and metastasis in human breast cancer (Hajra *et al.*, 2002; Catalano *et al.*, 2004). The Snail transcription family consisting of members, Snai1 (Snail) and Snai2 (Slug), is thought to repress E-cadherin expression, leading to EMT (Hajra *et al.*, 2002). EMT is characterized by a loss of epithelial morphology and acquisition of a more migratory, spindle-shaped phenotype (Micalizzi *et al.*, 2010). The non-malignant MCF10A cells have been used as models to study EMT and cell transformation as reported in several studies (Hugo *et al.*, 2007; Micalizzi *et al.*, 2010).

Since the results thus far have shown that stable expression of KISS1R in the non-malignant MCF10A and moderately invasive breast cancer cells induces an invasive phenotype, even in the absence of KP-10 stimulation **(Figure 2.3B, Figure 2.4B)**, I sought to determine if MCF10A FLAG-KISS1R and SKBR3 FLAG-KISS1R cells have undergone EMT. MCF10A FLAG-KISS1R and SKBR3-KISS1R cells exhibited a mesenchymal phenotype, compared to their respective vector controls **(Figure 2.16A)**. I found that the epithelial marker, E-cadherin was strikingly absent from cell-cell junctions in MCF10A FLAG-KISS1R cells, compared to MCF10A pFLAG-A1 (vector controls) cells, where E-cadherin was localized to the cell surface **(Figure 2.16B)**. MCF10A FLAG-KISS1R cells also exhibited stress fibre formation **(Figure 2.16B)**. Furthermore, Western blot analysis of EMT markers in MCF10A FLAG-KISS1R cells revealed that there was an increase in expression of the mesenchymal markers Snail/Slug and N-

cadherin compared to vector controls **(Figure 2.16C)**. However, we did not observe a change in E-cadherin expression levels by Western blot analysis in MCF10A FLAG-KISS1R cells compared to vector control cells **(Figure 2.16C)**. Western blot analysis of SKBR3 FLAG-KISS1R cells revealed an increase in mesenchymal markers vimentin and N-cadhein **(Figure 2.16D)**. Thus, these data suggest that stable expression of KISS1R in the ER-negative MCF10A and SKBR3 cells induces a partial EMT-like phenotype.

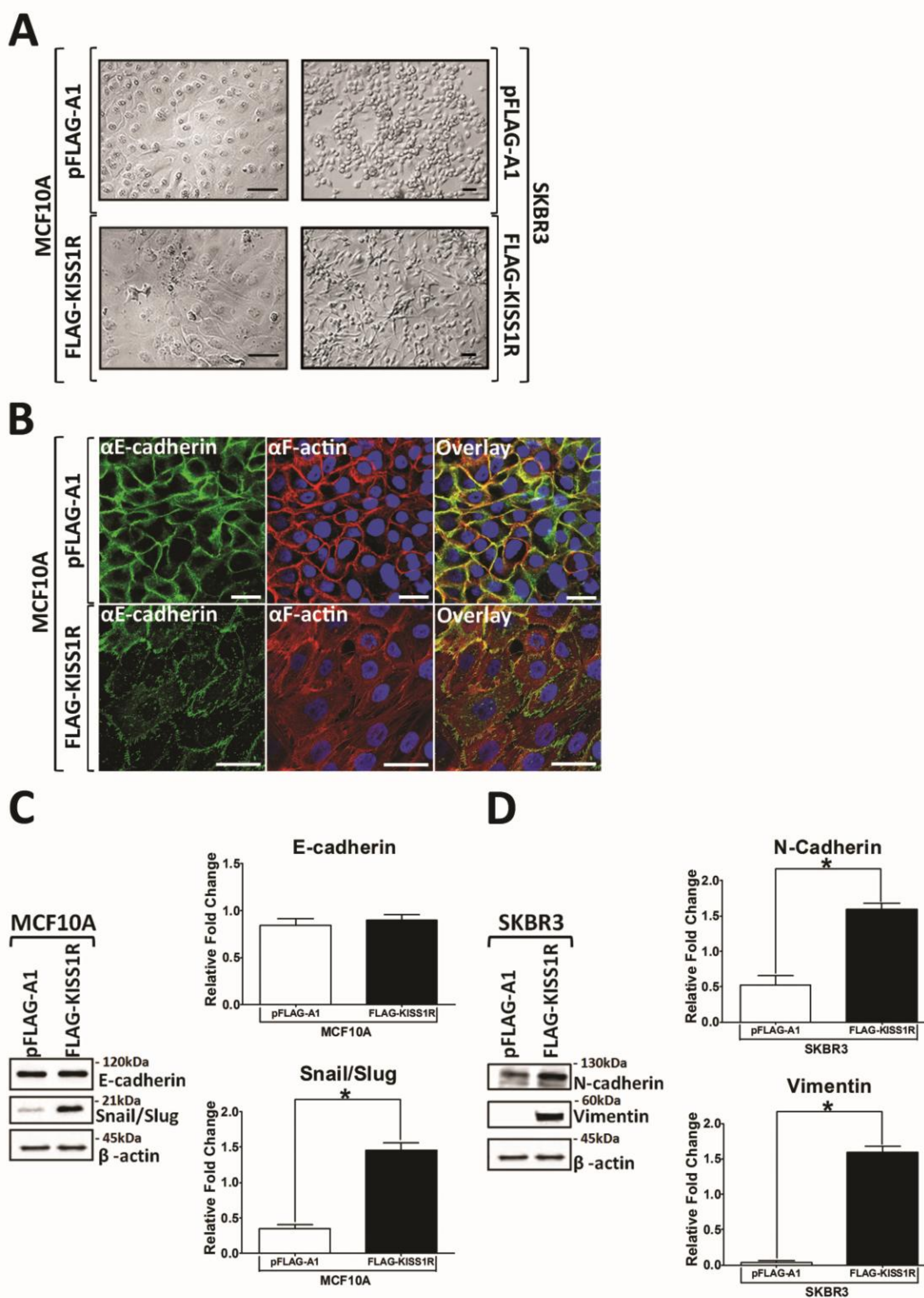
Cvetković et al. Figure 2.16
A
MCF10A
pFLAG-A1
FLAG-KISS1R
SKBR3
pFLAG-A1
FLAG-KISS1R
B
MCF10A
pFLAG-A1
FLAG-KISS1R
αE-cadherin
αF-actin
Overlay
C
MCF10A
pFLAG-A1
FLAG-KISS1R
- 120kDa
E-cadherin
- 21kDa
Snail/Slug
- 45kDa
β -actin
E-cadherin
Relative Fold Change
pFLAG-A1
FLAG-KISS1R
MCF10A
Snail/Slug
*
D
SKBR3
pFLAG-A1
FLAG-KISS1R
- 130kDa
N-cadherin
- 60kDa
Vimentin
- 45kDa
β -actin
N-Cadherin
Vimentin
SKBR3

**Figure 2.16. KISS1R expression induces a partial EMT-like phenotype in mammary epithelial cells.** **(A)** Representative DIC images of MCF10A pFLAG-A1 and FLAG-KISS1R, and SKBR3 pFLAG-A1 and FLAG-KISS1R cells. *Scale bar*, 100 μm. **(B)** KISS1R expression in MCF10A cells results in intracellular translocation of E-cadherin, and stress fiber formation compared to vector controls (n=5). E-cadherin immunostaining detected using an anti-mouse E-cadherin antibody, followed by Alexa Fluor 488 secondary antibody. F-actin staining using Phalloidin conjugated to Alexa Fluor 555 (red); nuclei stained using Hoechst (blue). **(C)** Representative Western blots showing expression levels of E-cadherin and Snail/Slug in MCF10A FLAG-KISS1R cells in comparison to MCF10A pFLAG-A1 (vector control) cells. Expression levels are normalized to β-Actin, *loading control* (n=3). **(D)** Representative Western blots showing expression levels of N-cadherin and Vimentin in SKBR3 FLAG-KISS1R cells in comparison to SKBR3 pFLAG-A1 (vector control) cells. Expression levels are normalized to β-Actin, *loading control* (n=3).

**IQGAP1 associates with KISS1R and regulates KP-10-induced breast cancer cell invasion.** IQGAP1 binds to a diverse array of signaling and structural proteins to regulate various processes including cell polarization, cell invasion, cytoskeleton structure, cell-cell adhesion and cell motility (Briggs and Sacks, 2003; Brown and Sacks, 2006). A study has shown that IQGAP1 protein expression is higher in human invasive ductal carcinoma relative to normal breast tissue (Briggs and Sacks, 2003; Brown and Sacks, 2006). Since I found that IQGAP1 co-localizes with KISS1R at the leading edge of motile cells, I next sought to determine whether or not IQGAP1 binds KISS1R and plays a role in transducing its signals to the cytoskeleton, to thereby stimulate breast cancer cell invasion. I found that endogenous IQGAP1 associates with FLAG-KISS1R stably expressed in MDA-MB-231 under basal conditions, and KP-10 treatment did not change the amount of interaction between these two proteins **(Figure 2.17)**.

I then sought to determine a role for IQGAP1 in KP-10-induced breast cancer invasiveness. IQGAP1 expression was stably depleted in MDA-MB-231 cells, which express the highest level of this protein amongst the cell lines tested (Jadeski *et al.*, 2008) with two different siRNA constructs (Mataraza *et al.*, 2003) **(Figure 2.18A)**. I found that depletion of IQGAP1 in MDA-MB-231 cells significantly blocked KP-10-induced migration and invasion **(Figure 2.18B)**. These results implicate IQGAP1 as a novel regulator of KISS1R-mediated breast cancer cell migration and invasion.

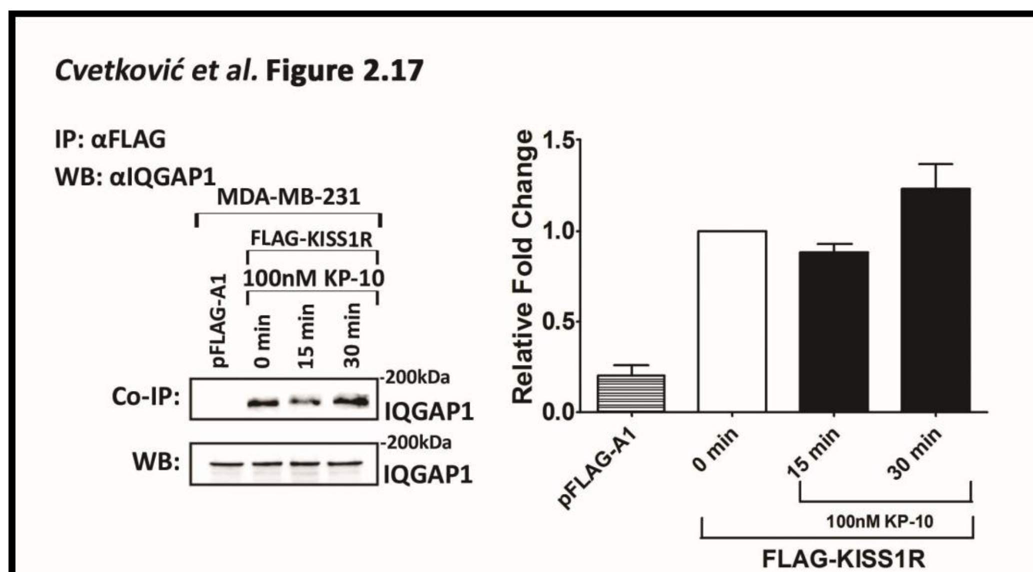


**Figure 2.17. KISS1R associates with IQGAP1 in breast cancer cells.** FLAG-KISS1R interacts with IQGAP1 both in presence and absence of KP-10 in MDA-MB-231 cells (n=3).

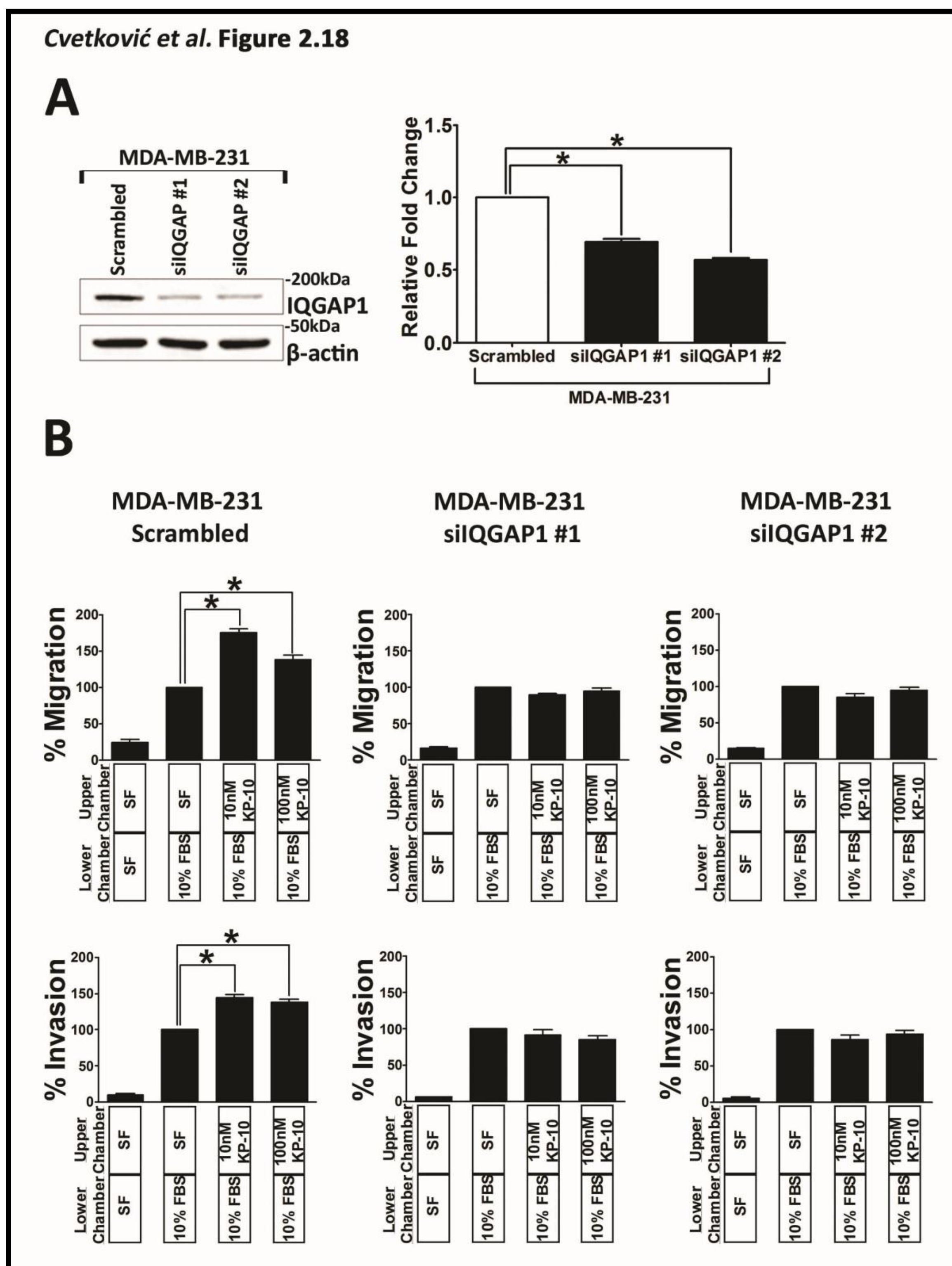


**Figure 2.18. Depletion of IQGAP1 blocks KP-10-induced MDA-MB-231 breast cancer cell migration and invasion. (A)** Western blot analysis of IQGAP1 expression in

MDA-MB-231 cells stably expressing IQGAP1 siRNA constructs (n=5). One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. **(B)** MDA-MB-231 scrambled, MDA-MB-231 siIQGAP1 #1 and siIQGAP1 #2 cells were subjected to Transwell chamber migration and invasion studies. Results are presented as a ratio of cells that migrated or invaded relative to cells seeded in serum-free medium and migrating or invading towards 10% (v/v) fetal bovine serum-supplemented medium, as was measured by Transwell chamber assay (n=3). On average 500-600 MDA-MB-231 scrambled cells migrated towards 10% FBS; 300-400 MDA-MB-231 siIQGAP1 #1 and siIQGAP1 #2 cells migrated towards 10% FBS. On average 300-400 MDA-MB-231 scrambled cells invaded towards 10% FBS; 100-200 MDA-MB-231 siIQGAP1 #1 and siIQGAP1 #2 cells invaded toward 10% FBS. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$. Bars represent % migration or invasion ±SEM.

# CHAPTER 3: DISCUSSION

## 3.1. Discussion

Although studies indicate that KISS1R signaling may correlate positively with breast tumor progression and metastatic potential (Ulasov *et al.*, 2002; Marot *et al.*, 2007; Cho *et al.*, 2011), the effect of KISS1R signaling on non-malignant breast epithelia is currently unknown. KISS1R has been shown to be expressed in normal breast tissue (Ohtaki *et al.*, 2001) and it has been postulated that KISS1R may to be involved in development of mammary tissue, although how this occurs is presently unknown. Additionally, the underlying mechanism by which KISS1R stimulates cell migration and invasion, processes required for metastasis is largely unknown. Contrary to the initial hypothesis KP-10 signaling *via* KISSIR will not stimulate invasion and motility of non-malignant mammary epithelial MCF10A cells, here I demonstrate for the first time that KP-10/KISS1R signaling is pro-migratory and pro-invasive in MCF10A cells and breast cancer cells that are ER-negative. I found that KP-10 transactivates EGFR only in the ER-negative non-malignant and malignant breast cell lines, but fails to stimulate migration, invasion or EGFR transactivation in the ER-positive breast cancer cell lines. Hence, the ER status of breast epithelia critically regulates the ability of KISS1R to induce an invasive phenotype. Furthermore, KISS1R was found to be localized to the cell front of motile cells migrating into a wound, where it co-localized with the leading edge marker, IQGAP1. Furthermore, I identified IQGAP1 as a novel binding partner for KISS1R in MDA-MB-231 breast cancer cells and showed that KISS1R stimulates breast cancer cell migration and invasion in an IQGAP1-dependent manner. Progress made in the understanding of the signaling of the KP-10/KISS1R in this study is summarized in **Table 3.1**.

**Table 3.1. Progress made in the understanding of the KP-10/KISS1R signaling in this study.**

<table>
<tr><th colspan="2">MAJOR FINDINGS</th></tr>
<tr><th>ZAJAC ET AL. 2011</th><th>CVETKOVIĆ ET AL. 2012<br>(current findings)</th></tr>
<tr><td>• KP-10 promotes migration and invasion of the ERα-negative MDA-MB-231 and Hs578T breast cancer cells via EGFR transactivation, concominant with MMP-9 secretion and activitity, and have implicated β-arrestin 2.</td><td>Findings from the ERα-negative cells:<br>• KP-10 increases invasiveness of the ERα-negative MCF10A cells and stimulates EGFR transactivation.<br>• KISS1R is localized to the leading edge of migratory cells, and co-localizes with IQGAP1.<br>• Stable expression of KISS1R in MCF10A and SKBR3 cells stimulates a partial epithelial-to-mesenchymal transition-like phenotype (EMT).<br><br>Finding fromt the ERα-positive breast cancer cells:<br>• KP-10 does not affect invasiveness or EGFR transactivation in the T47D and MCF7 breast cancer cells.<br>• Stable expression of ERα in MDA-MB-231 cells abolishes KP-10-stimulated migration, invasion and EGFR transactivation.<br><br>Mechanism by which KISS1R induces invasiveness:<br>• Depletion of IQGAP1 in MDA-MB-231 cells blocks KP-10-stimulated migration and invasion.</td></tr>
</table>

Over the last decade, significant progress has been made in understanding the complexity of GPCR-RTK signaling. Once seen as isolated receptors connecting extracellular signals to the activation of G proteins, GPCRs are now regarded as complex receptors capable of initiating a vast array of signaling pathways, including G protein-dependent and -independent signaling, involvement with scaffolding molecules, and interacting both directly and indirectly with other receptor families. RTKs such as the EGFR are overexpressed in numerous cancers including breast cancer where signaling through this receptor contributes to cell survival, proliferation, and invasion (Thomas *et al.*, 2006; Rodland *et al.*, 2008). Inhibition of EGFR alone using receptor tyrosine kinase inhibitors, although highly promising clinically, has resulted in limited anti-metastatic effects due to acquired resistance to these agents (Engelman and Janne, 2008). In addition to RTKs, GPCRs regulate the responsiveness of cancer cells to external stimuli (Thomas *et al.*, 2006). GPCRs have been shown to transactivate EGFR *via* the serine/threonine kinase PKC (Slack, 2000), the non-RTKs of the Src family (Luttrell and Lefkowitz, 2002), increased intracellular calcium levels (Zwick *et al.*, 1997) and *via* β-arrestins (Zajac *et al.*, 2011). It is well established that transactivation of EGFR results in increased proliferation and invasion, two hallmarks of the metastatic process (Thomas *et al.*, 2006). Our previous work has shown that KP-10, the most potent KP, stimulates breast cancer cell invasion *via* transactivation of EGFR, concomitant with MMP-9 secretion and activity, and have implicated β-arrestin 2 in this process (Zajac *et al.*, 2011). Furthermore, we discovered that KISS1R directly complexes with EGFR, and that stimulation of breast cancer cells with either KP-10 or EGF regulates the endocytosis of KISS1R and EGFR (Zajac *et al.*, 2011). Because KISS1R appears to signal *via* EGFR-

dependent mechanisms to result in increased invasiveness of breast cancer cells, targeting both receptors simultaneously could potentially result in increased efficacy compared with inhibiting either receptor alone.

We have previously shown that KISS1R induces EGFR transactivation in the ER-negative invasive breast cancer cells, namely MDA-MB-231, Hs578T (Zajac *et al.*, 2011). Here, I show that KISS1R activation by KP-10 also transactivates EGFR in ER-negative moderatvely invasive SKBR3 breast cancer cells and ER-negative non-malignant mammary epithelial MCF10A cells. MCF10A cells are spontaneously immortalized mammary epithelial cells, which harbor a basal-like phenotype and are capable of undergoing EMT spontaneously when plated under sparse conditions (Sarrio *et al.*, 2008). Therefore, all of the studies with MCF10A cells and the derived stable lines were performed with cells of high density, to eliminate the influence of confluency on EMT. Interestingly, we observed that stable exogenous expression of KISS1R in MCF10A or SKBR3 cells induced a partial EMT-like phenotype in the FLAG-KISS1R expressing cells, in comparison to vector controls. Additionally, exogenous KISS1R expression stimulated these cells to acquire an invasive phenotype both in the presence as well as absence of the ligand. This maybe due to the constitutive activity exhibited by KISS1R as was previously reported (Pampillo *et al.*, 2009). At the present time we cannot confirm the existence of constitutive activity because an inverse agonist is not yet available to test this. But given the observations that have been made and the fact that constitutive activity has been described for more than 60 wild-type GPCRs (Smit *et al.*, 2007), it should not be surprising to find that KISS1R does indeed display constitutive activity. Further support of constitutive KISS1R activity comes from *in vivo* studies

performed on the *Kiss1*$^{-/-}$ and *Kiss1r*$^{-/-}$ animals (Lapatto *et al.*, 2007). In this study, the authors document a phenotypic variability observed among *Kiss1* knockout female mice and suggest that one likely explanation for it can be as a result of modest constitutive KISS1R activity. Moreover, Pampillo and others reported that maximum basal activity of KISS1R is approximately 5% of the maximum KP-10-induced IP formation in HEK 293 cells transiently expressing KISS1R (Pampillo *et al.*, 2009). Previous studies have shown that *KISS1 and KISS1R* mRNA is elevated in breast tumor tissue compared to normal mammary tissue, and these high levels correlate with the shortest relapse-free survival (Martin *et al.*, 2005). Hence, it is possible that under pathological conditions such as breast cancer, upregulation of KP and/or KISS1R may stimulate the mammary epithelial cells to undergo EMT-like events, acquiring mesenchymal-like phenotypes, ultimately resulting in enhanced migration and invasion implicated in promoting metastasis (Hugo *et al.*, 2007).

Spontaneously immortalized non-malignant mammary epithelial MCF10A cell line arose in culture from MCF10 cells which were originally derived from a thirty-six year old Caucasian female patient with fibrocystic disease (Soule *et al.*, 1990). MCF10 cells are diploid, while the MCF10A line has a stable, near-diploid karyotype (Soule *et al.*, 1990; Yoon *et al.*, 2002) with modest genetic modifications typical of culture-adapted breast epithelial cells (Yaswen and Stampfer, 2002) including loss of the p16 locus (Debnath *et al.*, 2003). The cells express normal p53 (Merlo *et al.*, 1995; Debnath *et al.*, 2003), they do not form colonies in anchorage-independent growth assay, and they do not form tumors in immunocompromised mice (Heppner and Wolman, 1999). MCF10A cells grown in 3D reconstituted basement membrane culture (rBM) develop important features

of normal breast tissue *via* a well described progression of proliferation, cell cycle arrest, apical-basolateral polarization, and finally, apoptosis to create a luminal space (Debnath *et al.*, 2002; Debnath *et al.*, 2003; Underwood *et al.*, 2006). Furthermore, the appearance of cell nuclei of MCF10A cells forming acini in 3D cultures more closely resemble those of mammary epithelial cells in tissue than those cultured in monolayer (Lelievre *et al.*, 1998). Cancer arises from a complex interaction of factors including both genetic changes as well as changes in the microenvironment (Ingber, 2002; Bissell and Labarge, 2005). The development of 3D culture systems which more closely recapitulate the tissue microenvironment have allowed for a more detailed investigation of the dynamic and reciprocal crosstalk between the ECM and nuclear gene expression that may play a critical role in breast tumorigenesis (Weaver *et al.*, 1995; Lelievre *et al.*, 1998; Weaver *et al.*, 2002; Underwood *et al.*, 2006).

In order to visualize whether or not KP-10 stimulates motility of non-malignant mammary epithelial MCF10A cells in real-time, we performed scratch assays as described (Zajac *et al.*, 2011). We observed that 10nM KP-10 (dissolved in 5% FBS) significantly enhanced the distance travelled by the MCF10A cells over time, when compared to cells seeded only in 5% FBS. Similar observations were made for the MCF10A pFLAG-A1 vector control and MCF10A FLAG-KISS1R cells, and furthermore we did not observe any significant difference in scratch closure efficiencies between these cell lines. Previously, we've shown that KP-10 (dissolved in 10% FBS) also enhances motility of MDA-MB-231 cells, when compared to cells seeded only in 10% FBS (Zajac *et al.*, 2011). Previously, studies from our laboratory have shown that KP-10 does not act as a chemoattractant (Zajac *et al.*, 2011). When MCF10A, MCF10A

pFLAG-A1 or MCF10A FLAG-KISS1R cells were treated with 10nM KP-10 in the presence of 10% FBS, I did not observe a difference in cell motility, when compared to cells seeded in 10% FBS alone. Significant difference in cell motility only occurred in the presence of 5% FBS. The failure to observe a significant difference between the aforementioned treatment groups with higher concentrations of FBS could be due to greater levels of EGF found with the higher FBS. We've previously shown that KP-10-enhanced motility of MDA-MB-231 cells can be blocked with EGFR inhibitor, AG1478 (Zajac *et al.*, 2011), suggesting the EGFR signaling is responsible for KP-10-enhanced motility. We propose that greater amount of EGF present in 10% FBS *versus* 5% FBS may result in saturation of the EGFR response, and therefore addition of KP-10 does not result in any further increase in motility. Furthermore, treating cells with only KP-10 (dissolved in media lacking FBS, i.e. serum-free media) did not induce motility of MDA-MB-231 cells (Zajac *et al.*, 2011) or MCF10A, MCF10A pFLAG-A1, MCF10A FLAG-KISS1R (data not shown), suggesting that FBS is required to stimulate these cells to migrate.

Accumulating evidence strongly supports a role for scaffolding protein IQGAP1 in tumorigenesis (Johnson *et al.*, 2009; White *et al.*, 2011). IQGAP1 binds to a diverse array of signaling and structural proteins to participate in multiple cellular functions including cell polarization, cell motility, cell invasion, cytoskeletal architecture, and E-cadherin-mediated cell-cell adhesion (Briggs and Sacks, 2003). IQGAP1 is localized to sites of cell-cell adhesion and regulates adherens junction stability (Briggs and Sacks, 2003). IQGAP1 has been shown to promote tumorigenesis of breast cancer cells (Mataraza *et al.*, 2003; Jadeski *et al.*, 2008) and is considered to be an oncogene (Johnson *et al.*, 2009;

White *et al.*, 2011). Depletion of IQGAP1 by siRNA resulted in smaller and less invasive tumors *in vivo* (Brown and Sacks, 2006). White and colleagues have shown that IQGAP1 is overexpressed in trastuzumab-resistant human breast epithelial cells and that specific knockdown of IQGAP1 both enhances the inhibitory effects of trastuzumab *in vitro* and abrogates trastuzumab resistance (White *et al.*, 2011). Thus, these findings imply that IQGAP1 is a potential target for the development of additional therapeutic strategies for patients with HER2-positive breast cancers.

Here, I have shown that KISS1R is localized to the leading edge of cell membranes where it co-localizes with IQGAP1 in lamellipodia in motile MCF10A and MCF10A FLAG-KISS1R cells. This suggests that KISS1R may play a dynamic role in cell migration and indeed, treatment of cells with the KISS1R antagonist, P-234 inhibits MDA-MB-231 cell motility, thus, demonstrating the necessity of KISS1R signaling in this process. P-234 has been shown to inhibit the firing of GnRH neurons in the brain of the mouse and to reduce pulsatile GnRH secretion in female pubertal monkeys (Roseweir *et al.*, 2009). Additionally, P-234 inhibited the KP-10-induced release of LH in rats and mice and blocked the postcastration rise in LH in sheep, rats, and mice (Roseweir *et al.*, 2009). Therefore, the development of KISS1R antagonists such as P-234 provides a valuable tool for investigating the physiological and pathophysiological roles of KP/KISS1R signaling and could offer a unique therapeutic agent for treating cancers and reproductive disorders.

As previously mentioned, stable expression of KISS1R in non-malignant MCF10A cells causes abnormal localization of E-cadherin, whereby E-cadherin was no longer decorating the cell membrane, but rather appeared to be translocated to the intracellular

compartment of the MCF10A FLAG-KISS1R cells. This finding is supported by Li and colleagues who have shown that the translocation of IQGAP1 from the cytoplasm to the cell membrane, inhibits E-cadherin-mediated cell-cell adhesion (Li *et al.*, 1999), and correlates with E-cadherin dysfunction and tumor dedifferentiation in gastric carcinoma (Takemoto *et al.*, 2001). These data suggest that IQGAP1 promotes EMT, at least in part, by reducting E-cadherin-mediated cell-cell adhesion.

MDA-MB-231 cells were previously reported to express the highest amount of IQGAP1 of the breast cancer cells tested (Jadeski *et al.*, 2008). I found that endogenous IQGAP1 associates with KISS1R in MDA-MB-231 cells. Furthemore, depletion of IQGAP1 levels in MDA-MB-231 cells inhibited KP-10-induced cell migration and invasion, indicating that IQGAP1 plays a key role in KP-10-stimulated migration and invasion of MDA-MB-231 breast cancer cells. We have previously shown that KISS1R signals *via* β-arrestin 2 to regulate breast cancer cell invasion, and have shown that depletion of β-arrestin 2 blocks invasion, MMP-9 secretion and EGFR transactivation in MDA-MB-231 breast cancer cells (Zajac *et al.*, 2011). Both IQGAP1 and β-arrestin 2 have been reported to facilitate the scaffolding of the MAPK signaling components (Brown and Sacks, 2006) suggesting that the association of β-arrestin 2 and IQGAP1 may bring together individual signaling complexes within the same area of the cell to allow for the spatial regulation of multiple processes including cell migration. Future studies will investigate the mechanism by which KISS1R signaling *via* IQGAP1 regulates breast cancer migration, invasion and metastasis and to further characterize the interactions between KISS1R and IQGAP1.

KPs have since been identified as major regulators of the hypothalamic-pituitary-gonadal axis, *via* tight modulation of GnRH secretion (Huijbregts and de Roux, 2010). Regulation of GnRH secretion by estrogen is crucial to normal fertility. Several mechanisms have been proposed to explain both negative and positive feedback effect of estrogen on the gonadotropic axis. One of them involves modulation of GnRH secretion by estrogen, that was found to diminish *KISS1* expression in the arcuate nucleus of gonadectomized mice, suggesting a role for *KISS1* downregulation in the negative feedback effect of $E_2$ (Huijbregts and de Roux, 2010). Studies of knockin mice expressing an ERα variant that was unable to bind DNA showed that regulation of hypothalamic *KISS1* expression was directly involved in the negative feedback of estrogen on the gonadotropic axis *via* a nonclassical pathway (Huijbregts and de Roux, 2010).

ERα-mediated pathways play a crucial role in breast carcinogenesis, and thus, ERα level is used as a prognostic marker of breast tumors (Clarke *et al.*, 2004). For that reason, I wanted to determine whether the presence of ERα in breast cancer cells affects their response to KP-10. Here, for the first time I show that KP-10 does not increase migration, invasion and motility of the ER-positive T47D and MCF7 breast cancer cells. Furthermore, these cells do not undergo KP-10-stimulated EGFR transactivaton. These observations are supported by a previous study that has shown that tamoxifen treatment of ERα positive MCF7 and T47D cells increased *KISS1* and *KISS1R* levels (Marot *et al.*, 2007). My findings further support their claim, since the clinical relevance of this negative regulation of *KISS1* and *KISS1R* by estrogen maybe crucial for the understanding of breast cancer progression. I found that stable ERα expression in the ER-

negative MDA-MB-231 blocked KP-10-induced migration, invasion and EGFR transactivation, providing further support for the negative regulation of KP-10/KISS1R signaling by ERα. My studies extend onto the findings of Margot and colleagues (2007) who have observed a significant decrease in *KISS1* mRNA levels in ERα-negative MDA-MB-231 cells expressing recombinant ERα.

Here, I propose a model for ERα-mediated modulation of KP/KISS1R signaling (**Figure 3.1**). In normal healthy mammary epithelia, estrogen signaling through ER is responsible for maintaining normal breast epithelial growth and function, and thus preserving homeostatic balance, by keeping KP/KISS1R signaling in check through transcriptional regulation of *KISS1* (Huijbregts and de Roux, 2010). However, in certain cancers, where ER expression is lost or silenced *via* DNA methylation as is the case of ER-negative cancers, the brake keeping KP/KISS1R signaling in check is removed, and this disinhibition results in increased transcription of *KISS1*, and consequently increases signaling through KISS1R ultimately allowing for the non-malignant mammary epithelial cells to acquire a more migratory and invasive phenotype. To summarize, this study reveals for the first time that the ER status of mammary cells may dictate whether KISS1R signaling stimulates invasiveness, thus identifying a novel target for metastasis. Therefore, a better understanding of the mechanisms that regulate KISS1R signaling, particularly those that regulate activity immediately after receptor activation is required to evaluate its potential as a therapeutic target in cancer.

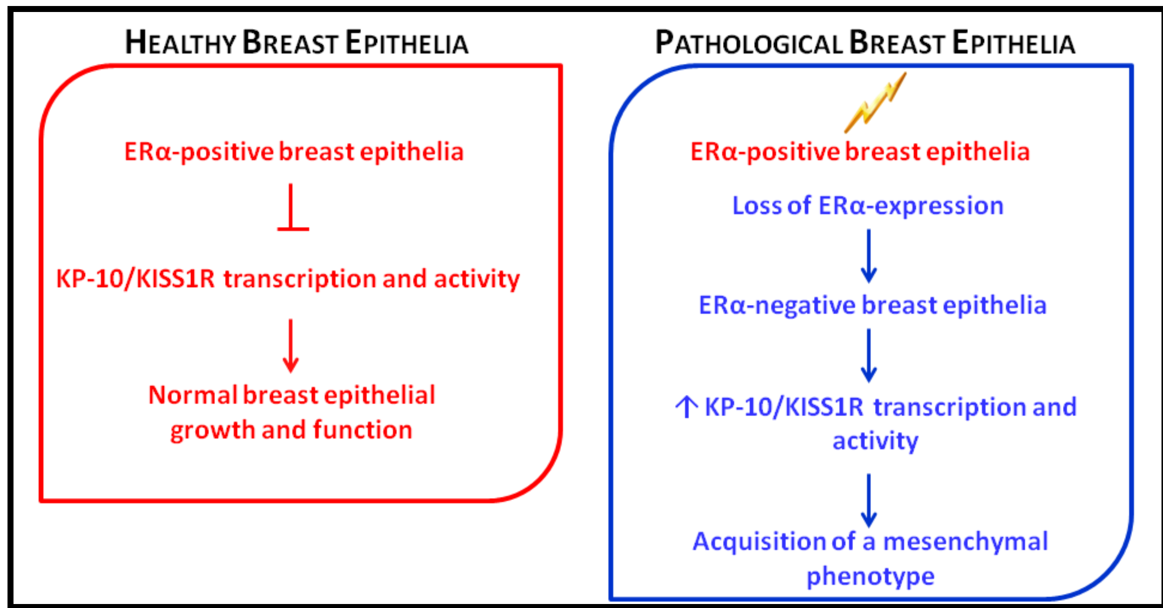


**Figure 3.1. Proposed model for ERα-dependent KP-10/KISS1R signaling.** In normal healthy mammary epithelia, estrogen signaling through ERα is responsible for maintaining normal breast epithelial growth and function, and thus preserving homeostatic balance, by keeping KP/KISS1R signaling in check through transcriptional regulation of *KISS*. However, in breast cancer, where ERα expression is lost or silenced as is the case of ERα-negative cancers, the brake keeping KP/KISS1R signaling in check is removed, and this disinhibition results in increased transcription of *KISS1* and *KISS1R*, and consequently increases signaling through KISS1R ultimately allowing for the non-malignant mammary epithelial cells to acquire a more migratory and invasive phenotype.

### 3.2. References

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## ACADEMIC BACKGROUND

| | |
|---|---|
| **2010 - Present:** | M.S.c., Department of Physiology and Pharmacology, University of Western Ontario, London, ON<br>*Supervisor:* Dr. Moshmi Bhattacharya |
| **2006 - 2010:** | B.M.S.c., (Western Scholar) Honors Specialization in Physiology with distinction, University of Western Ontario, London, ON |

## SCHOLARSHIPS AND AWARDS

| | |
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| **2011 - 2012:** | CIHR Strategic Training Program (STP) in Cancer Research and Technology Transfer (CaRTT) |
| **2010 - 2012:** | Translational Breast Cancer Research Unit (TBCRU) Studentship |
| **2010 - 2012:** | Schulich Graduate Scholarship |
| **2010 - 2007:** | UWO Dean's Honors List |
| **2009:** | Leadership and Mentorship Program: Letter of Accomplishment in Individual Student Leadership |
| **2006:** | Western Scholarship of Excellence (value $2000) |

## CERTIFICATIONS

| | |
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| **2011:** | UWO Biosafety Training |
| **2009:** | UWO Laboratory - Environment Waste Management Safety Training |
| **2009:** | UWO Comprehensive WHMIS Training |
| **2009:** | St. John Ambulance: Standard 1st Aid with CPR-C |

## PUBLISHED LITERATURE

Zajac M, Law J, **Cvetković D**, Pampillo M, McColl L, Pape C, Di Guglielmo GM, Postovit LM, Babwah AV, Bhattcharya M. GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness. PLoS ONE. 2011.

## POSTER PRESENTATIONS

Department of Oncology Research and Education Day (22June2012, London, Ontario) – ***Poster Presenter (winner)***

London Health Research Day (20March2012, London, Ontario) – ***Poster Presenter***

Department of Physiology and Pharmacology Research Day (08November2011, London, Ontario) – ***Poster Presenter***

Department of Oncology Research and Education Day (17June2011, London, Ontario) – ***Poster Presenter***

Cvetković D, Corneil B, Goonetilleke S. Testing surface electromyography of the activity of splenius capitis muscle evoked by transcranial magnetic stimulation of the human frontal eye fields. Physiology and Pharmacology 4th Year Student's Poster Day (29March2010, London, Ontario) – ***Poster Presenter***

## VOLUNTEERING EXPERIENCES

**2011 - 2007:** Volunteered at the University Hospital (London, ON) at the Operation Room information desk and Emergency Department

**2009:** Involved in the design and delivery of the Anaphylaxis Research Study, St. Joseph's Hospital, London, ON

**2003 - 2005:** Volunteered at the London Public Library and Carson Library, London, ON