8-13-2015 12:00 AM

Fibulin-3 Promotes Triple Negative Breast Cancer Cell Invasion

Michelle M. Noonan
The University of Western Ontario

Supervisor
Dr. Moshmi Bhattacharya
The University of Western Ontario

Graduate Program in Physiology and Pharmacology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

FIBULIN-3 PROMOTES TRIPLE NEGATIVE BREAST CANCER CELL INVASION

(Thesis format: Monograph)

By

Michelle Marie Noonan

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment

of the requirements for the degree of

Master of Science

The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

ABSTRACT

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer, and metastasis is a leading cause of mortality in these patients. Fibulin-3, a secreted extracellular matrix protein, has a pro-invasive role in other cancers. However, a role for fibulin-3 in TNBC invasion is unknown. We have previously shown that KISS1R signaling promotes TNBC cell invasion through EGFR and MMP-9 activity, *via* β-arrestin2. Thus, we hypothesized that KISS1R signaling promotes TNBC cell invasion *via* fibulin-3. Our clinical data suggests that plasma fibulin-3 levels are elevated in metastatic TNBC patients. Additionally, we found that invasive breast cancer cells have increased expression of fibulin-3 and treatment with kisspeptin, the KISS1R ligand, further increased fibulin-3 expression and secretion. Also, depletion of β-arrestins in TNBC cells decreased fibulin-3 expression. Furthermore, fibulin-3 depletion in TNBC cells inhibited cell invasiveness through decreased MMP-9 activity. These results identify fibulin-3 as a new signaling partner of KISS1R and as a potential anti-metastasis target in TNBC.

Keywords: Metastasis, triple negative breast cancer, fibulin-3, EFEMP1, invasion, KISS1R, kisspeptin, GPCR, EGFR, MMP-9, β-arrestin, MAPK

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisor, Dr. Moshmi Bhattacharya, for her expertise, enthusiasm and patient guidance that has been invaluable to me throughout my graduate studies. Thank you for challenging me to always think critically, and encouraging me to strive for excellence. Without your supervision and constant help this thesis would not have been possible.

I would also like to thank the members of my advisory committee, Dr. Nica Borradaile, Dr. Lina Dagnino and Dr. Rommel Tirona for providing useful insight and suggestions along the way. Your guidance has been tremendously appreciated.

In addition, I would like to thank all past and present members of the Bhattacharya lab, especially Cameron Goertzen, Alexandra Blake and Magda Dragan. Cameron, thank you for sharing your technical expertise and infectious laughter with me, you made joining the lab an easy transition. Alexandra, you have become a cherished friend over the past two years. Thank you for the stimulating discussions, both science and otherwise, and for all the fun we have had in and out of the lab. Magda, your technical assistance, calm words and encouragement have been unbelievably helpful over the last two years. I have truly appreciated you every step of the way, as both a mentor and friend.

I would also like to extend my gratitude to our neighbours, the DiGuglielmo lab. Special thanks go to Eddie Chan for his wise advice and friendship.

Last, but certainly not least, I would like to thank my family and friends for their unwavering support and motivation throughout my studies. To the Carmichaels, thank you for your kindhearted thoughts and encouragement, and for being my home away from home. Finally, my deepest gratitude goes to my parents and my brother, whose confidence in me and unconditional love have allowed me to succeed in this endeavor.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

3D	Three-dimensional
ANOVA	Analysis of Variance
CAF	Cancer-Associated Fibroblast
DAG	Diacylglycerol
DAN	Differential screening-selected gene Aberrative in Neuroblastoma
DCIS	Ductal Carcinoma *in situ*
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EFEMP1	EGF-containing Fibulin-like Extracellular Matrix Protein 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-to-Mesenchymal Transition
ER	Estrogen Receptor
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FRET	Fluorescence Resonance Energy Transfer
FSH	Follicle-Stimulating Hormone
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GnRH	Gonadotropin-Releasing Hormone

GPCR	G-Protein Coupled Receptor
GRK2	G-Protein Coupled Receptor Kinase 2
HBSS	Hank's Balanced Salt Solution
HER	Human Epidermal Growth Factor Receptor
HIF-1	Hypoxia Inducible Factor-1
HRP	Horseradish Peroxidase
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
IP_3	Inositol Triphosphate
IQGAP1	IQ-motif-containing GTPase Activating Protein 1
KISS1R	Kisspeptin 1 Receptor
KP	Kisspeptin
KRAS	Kristen Rat Sarcoma Viral Oncogene Homolog
LCIS	Lobular Carcinoma *in situ*
LH	Luteinizing Hormone
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVD	Microvessel Density
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of activated B Cells
PBS	Phosphate Buffered Saline
PEX	Hemopexin

PKC	Protein Kinase C
PLC	Phospholipase C
PR	Progesterone Receptor
PyMT	Polyoma Virus Middle T Antigen
RPMI	Roswell Park Memorial Institute
RTK	Receptor Tyrosine Kinase
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
shRNA	Small Hairpin Ribonucleic Acid
Src	Proto-oncogene Tyrosine-Protein Kinase Src
TCGA	The Cancer Genome Atlas
TGF-β	Transforming Growth Factor-β
TIMP-3	Tissue Inhibitor of Metalloproteinases-3
TNBC	Triple Negative Breast Cancer
TNM	Tumor-Node-Metastasis
uPA	Urokinase-type plasminogen activators
VEGF	Vascular Endothelial Growth Factor

Chapter 1 – Introduction

1.1 Breast Cancer

Breast cancer is the most common cancer diagnosed in Canadian women over the age of 20 and it is estimated that approximately 1 in 9 women will develop breast cancer in her lifetime[†]. In 2015, it is estimated that 25 000 Canadian women will be diagnosed with breast cancer[†]. Additionally, breast cancer is the second leading cause of cancer deaths in Canadian women with approximately 5 000 deaths estimated for 2015, representing 14% of all cancer deaths[†]. It is evident that breast cancer is a very prevalent disease with devastating consequences, and therefore it is imperative that the molecular processes of breast cancer development and progression be better understood so that early diagnostics and clinical therapies can be further advanced.

1.1.1 Breast Cancer Staging

Breast cancer staging is a means of determining the extent to which the cancer has developed and spread to other tissues in the body [1]. Clinically, breast cancer staging is utilized by physicians to estimate patient prognosis, since patient 10 year survival decreases with increasing cancer stage [2]. The tumor-node-metastasis (TNM) staging system is the clinical standard that takes into account the size of the primary tumor (T), the extent of lymph node involvement (N), and the presence of metastases in distant tissues (M) [1]. Breast cancer commonly develops from the epithelium that lines the ducts or lobules of the mammary glands and is defined as ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS). At this point, the cancer would be classified as stage 0, since the tumor has not invaded into the surrounding tissue and remains confined by the basement membrane to the ducts or lobules of the breast. However, as the cancer progresses these abnormal epithelial cells will undergo further molecular alterations promoting their survival and invasion into the

surrounding tissue [3]. Thus, the aforementioned cancers become known as invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). These cancers would be within the range of stage I – III, which are classified based on the size of the primary tumor and the extent that breast cancer cells have spread to lymph nodes in close proximity to the breast (**Figure 1.1**). The final stage, stage IV, involves breast cancer that has metastasized to distant tissues, such as the bone, lung, liver and brain [4].

1.1.2 Breast Cancer Classification: Hormone Receptor Status

Although the TNM staging system is an important tool in predicting patient prognosis, a limitation of the system is that it only takes into account tumor burden and excludes tumor biology [5]. It has been suggested that biomarkers and hormone receptor status should be included in the staging system of breast cancer to assist clinicians in optimizing treatments and to better predict survival [5]. Specifically, triple negative breast cancer (TNBC), which represents 15-20% of all breast cancer cases, consists of tumor cells that lack the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER)2 [6]. Consequently, this breast cancer subtype does not effectively respond to established hormone or HER2-targeted therapies. TNBC patients have poorer prognosis, a high recurrence rate, and decreased survival compared to other breast cancer subtypes [7,8,9]. TNBC patients are often younger than 50 years of age, African American, and are more likely to have *BRCA1* mutations [10,11,12,13]. Moreover, approximately 60% of TNBC patients display overexpression of the epidermal growth factor receptor (EGFR) [14].

EGFR (HER1), is a member of the EGF receptor family of receptor tyrosine kinases, which also comprises HER2, HER3 and HER4. This receptor family regulates a number of

important cellular functions such as cell proliferation, survival and cell motility [15]. Activation of these receptors involves ligand binding, which induces homo- or heterodimerization of EGFR with another receptor of the family, most frequently HER2 [15]. This dimerization promotes the autophosphorylation of the intracellular tyrosine residues, thereby allowing for interactions with intracellular signaling effectors and the initiation of signaling cascades [16]. Additionally, dysregulated expression and activity of EGFR has been demonstrated to promote tumorigenesis and metastasis in several cancers, including breast cancer [15].

Heitz and colleagues reported that women with TNBC displayed a higher rate and earlier occurrence of cerebral metastases compared to ER-positive patients [17]. Currently, chemotherapy is the only available pharmacological treatment used to treat TNBC; however, due to the aggressive nature and genomic instability of the tumors, TNBC patients develop chemoresistance [18]. Therefore, it is important that targeted therapies be further investigated and developed for use in TNBC patients. Recently, the purportedly normal cells of the tumor microenvironment have emerged as potential targets due to their ability to regulate the growth and function of the tumor itself [19].

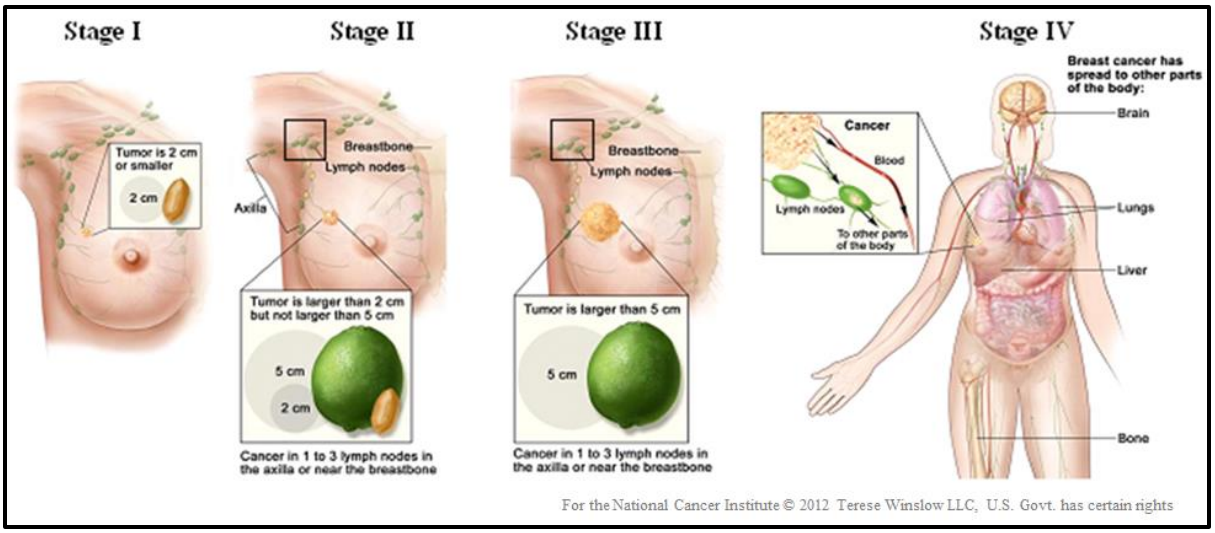

Figure 1.1. Breast cancer staging. Stage I- III are classified based on the size of the primary tumor and presence of cancer in regional lymph nodes. Stage I involves a tumor less than 2 cm in size and no lymph node involvement. Typically, stage II is a primary tumor of 2-5 cm in size and presence of cancer cells in the lymph nodes surrounding the breast. Stage III is a primary tumor greater than 5 cm in addition to regional lymph node involvement. Lastly, stage IV involves distant metastases in other tissues of the body such as the brain, lungs, liver and bone. *Adapted from the website of the National Cancer Institute (http://www.cancer.gov).*

1.2 Breast Cancer Metastasis

Metastasis is the process by which cancer cells detach from the primary tumor and travel mainly through the blood stream or lymphatic system to distant parts of the body [21]. When breast cancer remains confined to the breast and does not undergo metastasis, 5-year survival rates exceed 90% [22]. However, breast cancer patient prognosis is adversely affected by the extent and site of metastases [23].

1.2.1 Metastatic Cascade

The metastatic cascade comprises a series of complex steps (**Figure 1.2**), the first step in breast carcinoma development begins with cellular transformation of non-malignant epithelial cells to a malignant phenotype, which has been suggested to be a result of acquired genetic instability [24]. Cellular transformation involves uncontrolled growth regulation and increased cell survival, due to evasion of apoptotic signaling [25].

Initial separation and spreading of cancer cells from the primary tumor involves a process known as epithelial-to-mesenchymal transition (EMT), where cells lose expression of epithelial markers and gain expression of mesenchymal markers, introducing a more migratory and invasive phenotype [26]. Normally, epithelial cells are tightly organized in continuous sheets of polarized cells, forming a barrier that is necessary to maintain homeostasis and protect the body. Epithelial cells display an apical-basolateral polarity, with distinct expression profiles at each plasma membrane [27]. Cell-cell adhesion and communication is accomplished through the use of gap and tight junctions, desmosomes, and adherens junctions [27]. However, during EMT cells lose polarity and cell-cell attachment due to the loss of important adhesion molecules, such as E-cadherin [28]. At the same time, cells also acquire a mesenchymal phenotype through increased expression of mesenchymal

markers, including N-cadherin, vimentin and matrix metalloproteases (MMPs) [29]. Cells which have undergone EMT are capable of migrating away from the primary site through cell-matrix interactions and cytoskeletal reorganization [30].

In addition to migration, cancer cells must invade through the basement membrane that borders the tumor and into the surrounding extracellular matrix (ECM)(**Figure 1.2**) [31]. Thus, ECM remodeling must occur to allow cancer cell migration and invasion through the tissue. This is accomplished by several proteolytic enzymes that are produced and secreted by cancer cells [28]. The most common proteases involved in cancer metastasis are urokinase-type plasminogen activators (uPA), cathepsins and MMPs [32,33,34]. Secretion and activation of these proteolytic enzymes results in uncontrolled degradation of the basement membrane and surrounding ECM, presenting the cancer cells with an avenue for migration and metastasis. Additionally, the degradation of the ECM releases growth factors, such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), which can subsequently act on cancer cells to further promote angiogenesis and metastatic progression [35].

The next step in the metastatic cascade is angiogenesis, the process by which new blood vessels are formed from pre-existing vessels (**Figure 1.2**) [25]. Angiogenesis occurs in normal physiological conditions, such as wound healing, the menstrual cycle and placentation during pregnancy [36]. The process of angiogenesis is carefully controlled by a balance of pro- and anti-angiogenic factors. However, in cancer, this balance is disrupted and angiogenesis-inducing factors are abundantly released from tumor cells and the surrounding stromal cells [36]. As the primary tumor continues to grow in size its need for oxygen and nutrients exceeds what the present vessels can sufficiently deliver and hypoxic

conditions are introduced. Hypoxia increases factors such as hypoxia inducible factor -1 (HIF-1), which has been shown to be increased in invasive breast carcinoma compared to *in situ* tumors [37]. HIF-1 stimulates the release of pro-angiogenic factors, such as VEGF, from tumor cells and from the degradation of the ECM [38]. VEGF subsequently binds to VEGF receptors on endothelial cells of pre-existing vessels in the surrounding tissue, stimulating the proliferation of endothelial cells and the development of a new vessel network within the tumor itself and the surrounding tissue [37]. Furthermore, VEGF expression has been inversely correlated to breast cancer patient survival [39]. Due to incomplete formation, these new blood vessels have abnormal architecture and consequently there is increased fenestration, enhancing the permeability and likelihood that cancer cells will enter the circulation [36].

Once the tumor cells have invaded through the epithelial basement membrane and surrounding ECM they will reach the newly formed tumor-associated vasculature. The tumor cells must then transverse the endothelial cell basement membrane to reach the vascular endothelial cells. It has been reported that blood vessel-associated macrophages produce chemoattractants, such as EGF, to attract breast cancer cells to the vessel [40]. The next step in the metastatic cascade is intravasation, the process by which these cancer cells will enter the vessels through leaky endothelial cell junctions (**Figure 1.2**) [41]. Initially, cancer cells adhere to endothelial cells, and then through endothelial cell retraction and cancer cell migration, the cancer cell enters the vasculature by transendothelial migration [41]. Additionally, cancer cells may also enter the circulation *via* the lymphatic system. In contrast to the cardiovascular system, cells of the lymphatic system lack tight intracellular junctions, and therefore lymph node metastases are more common earlier in tumor progression and generally indicate poor prognosis [36]. Cancer cells that survive in the

circulation arrive in capillary beds in distant tissues, adhere to endothelial cells, and enter the surrounding tissue by extravasation (**Figure 1.2**) [42]. Many of the metastasized cells undergo apoptosis, however a subset of metastasized cells may survive and enter dormancy or proliferation [43]. Like the primary tumor, the secondary tumor also requires angiogenesis to occur to supply the growing tumor with oxygen and nutrients.

Paget's century-old "seed and soil" theory proposes that the interaction between the cancer cells or "seeds" and the organ microenvironment or "soil" explains the preference for metastatic cells for certain organs [44]. This theory suggests that metastasis is multifactorial and that the processes are influenced by factors independent from the tumor cell itself [44]. Additionally, it has been suggested that the organ microenvironment at the site of metastasis can distinctly modify the gene-expression profile of cancer cells and consequently alter their behaviour and growth [43]. Therefore, it is imperative that the tumor microenvironment and its potential effects on the development of aggressive tumors be further investigated. Specifically, new therapeutic strategies targeting the molecular changes that occur in regards to expression of cell-cell and cell-ECM adhesion molecules and cell signaling cascades are worth considering for the development of anti-metastatic drugs [19].

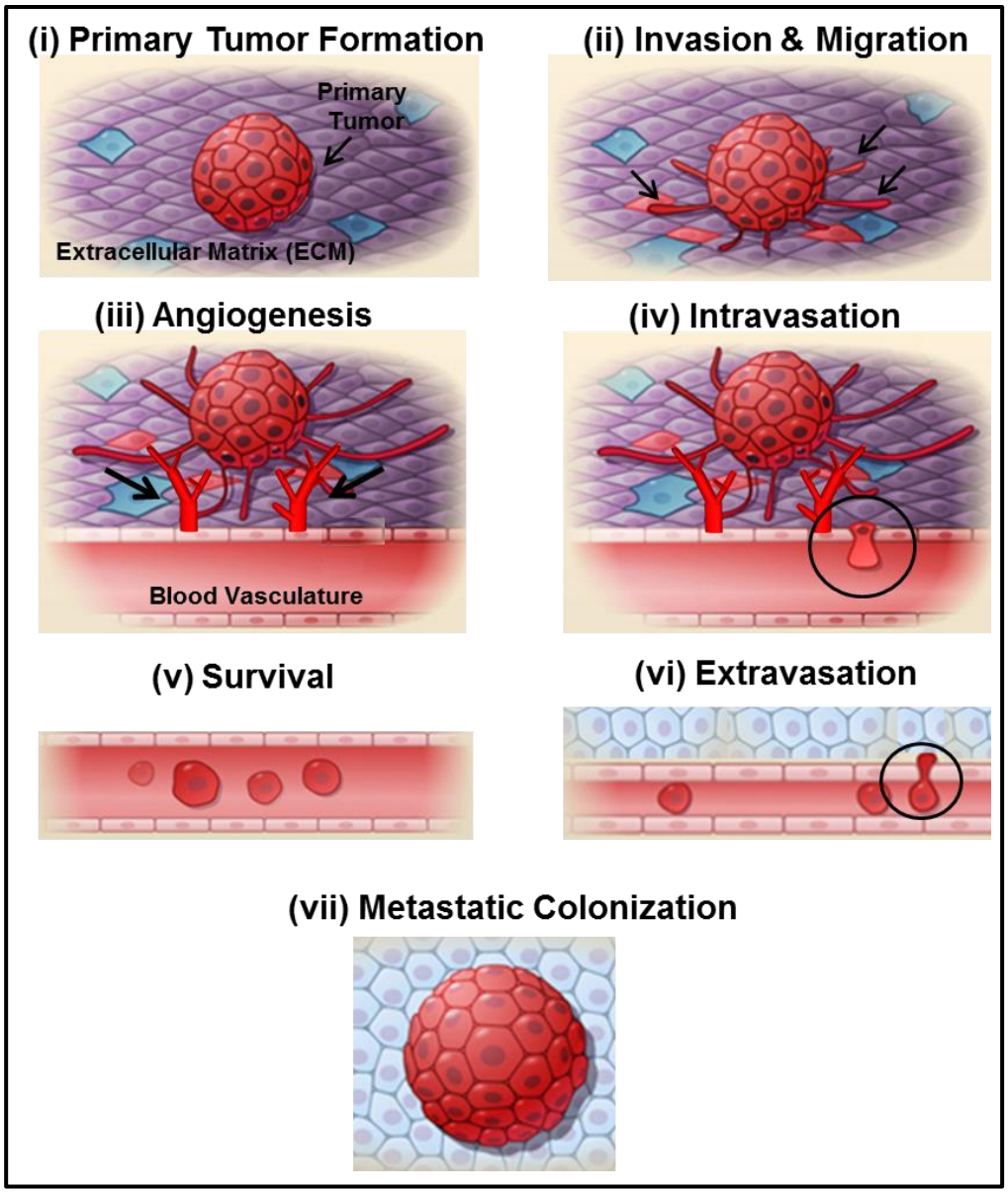

Figure 1.2. The metastatic cascade. The process of metastasis, when cancer cells spread from a primary tumor to distant tissues, requires several processes to occur. Initially, tumor cells undergo proliferation to form a primary tumor mass (i). As the tumor progresses, some tumor cells will exit their primary sites of growth and enter the surrounding tissue through invasion and migration (ii). Next, to supply the growing tumor with oxygen and nutrients, new blood vessels are formed through a process known as angiogenesis (iii). Some cancer cells will enter the newly formed blood vessels *via* intravasation (iv) and survive in the circulation (v). At distant tissues, cancer cells will exit the circulation through extravasation (vi) and undergo metastatic colonization (vii). *Adapted from Valastyan & Weinberg (2011) [45].*

1.3 Tumor Microenvironment

The tumor microenvironment consists of the ECM, soluble factors and altered cell types, including fibroblasts, endothelial cells and leukocytes [46]. These cells can interact directly or through paracrine signaling with surrounding cells [47]. Like any organ, the development and function of a tumor is the result of unique communication between epithelial cells and surrounding stromal cells [47]. Evidence suggests that disruptions to the ECM composition and structure may pave the way for tumor development or lead to cancer-causing mutations [48]. In addition to degrading the normal ECM, stromal cells of the microenvironment, primarily cancer-associated fibroblasts (CAFs), will deposit a new tumor-associated ECM that consists of molecules such as fibronectin, tenascins and stiffer collagen that will facilitate tumor cell motility [49]. Furthermore, studies have demonstrated that the gene expression profile of the tumor-associated ECM is dramatically altered, including upregulation of MMPs in invasive stroma compared to *in situ* stroma [50]. Additionally, due to their altered signaling pathways, cancer cells may fail to interpret cues from the microenvironment, and this can result in inappropriate cellular organization and growth [48]. For example, in three-dimensional (3D) culture, mammary epithelial cells will form polarized acini-like structures, but malignant cells in 3D culture form disorganized colonies [51]. These malignant cells will revert to organized polarized structures when cell-ECM β1 integrin signaling is inhibited [51]. Therefore, the influence of the tumor microenvironment, especially cell-cell and cell-ECM interactions, has become an emerging target for therapeutic strategies.

1.3.1 Fibulins

The fibulin family consists of seven secreted glycoproteins associated with basement membranes, elastic fibers and the ECM [52]. The fibulin family name is derived from the Latin word *fibula* meaning clasp or buckle. It is fitting then, that fibulins function as intramolecular bridges within ECM components, such as elastic fibres and basement membranes [53]. Interestingly, the fibulins are highly conserved in species as evolutionarily diverse as worms and humans [53]. Structural features of the fibulins include repeated EGF-like domains and a unique C-terminal fibulin-type module (**Figure 1.3**), and these proteins have been implicated in cell signaling and protein-protein interactions involved in cell growth, adhesion and motility [54]. The physiological and pathological roles of many of the fibulins have been extensively studied, however only within the last decade have roles for fibulin-3 been discovered [54].

1.3.2 Fibulin-3

Fibulin-3, also known as EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), interacts with tropoelastin and plays an important role in elastic fibre assembly during development [52]. Fibulin-3 is strongly expressed in human heart and placenta and weakly expressed in skeletal muscle, lung, brain and pancreas [55]. Fibulin-3 was also shown to be expressed in the bone and cartilage of murine embryos, suggesting that fibulin-3 may play a role in skeletal development [56]. Additionally, fibulin-3 is highly expressed in epithelial and endothelial cells [55]. The fibulin-3 promoter contains Sp1 transcription factor binding sites, in addition to an estrogen response element, and estrogen has been shown to supress fibulin-3 transcription in retinal cells [57]. Similarly, estrogen stimulation of ERα-

positive MCF7 breast cancer cells repressed the expression of the gene for fibulin-3, referred to as *EFEMP1* [58].

The physiological roles of fibulin-3 in development and disease have been elucidated by studying animal knockout models and heritable human disorders [54]. The phenotype of the fibulin-3 knockout mouse involves reduced reproductivity, early aging, herniation and lack of support for pelvic organs [59,60]. These observations are likely a result of reduced elastic fibers in the fascia, a thin layer of connective tissue that separates and protects the body's organs [59]. Furthermore, a missense mutation in an EGF domain of fibulin-3 has been discovered as the cause of early-onset macular degenerative diseases including Malattia Leventinese and Doyne's honeycomb retinal dystrophy [61]. These inherited macular degenerative diseases resemble the most common cause of incurable blindness, age-related macular degeneration, and are characterized by yellow-white deposits of ECM, called drusen, that accumulate between Bruch's membrane and the retinal pigment epithelium [52]. Interestingly, mutations in tissue inhibitor of metalloproteinases-3 (TIMP-3), an inhibitor of MMPs, are also associated with macular degenerative diseases, and TIMP-3 has been shown to bind fibulin-3 [62]. Lastly, fibulin-3 has been described as an indirect regulator of cell growth through indirect protein interactions with the tumor suppressor differential screening-selected gene aberrative in neuroblastoma (DAN) [63]. Fibulin-3 has also been shown to promote cell proliferation by stimulating deoxyribonucleic acid (DNA) synthesis *in vitro* through autocrine or paracrine signaling [64].

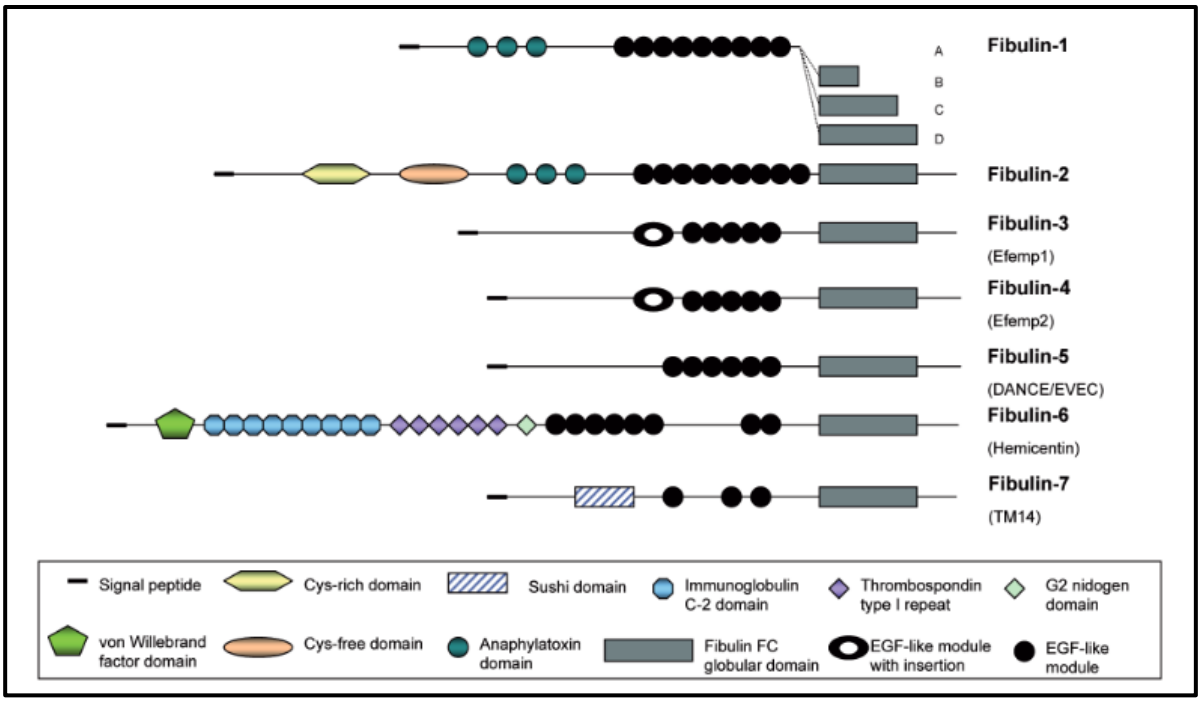

Figure 1.3. Structure of the fibulins. The fibulins contain a collection of epidermal growth factor (EGF)-like motifs and a C-terminal fibulin-type module, unique to the fibulins and fibrillins. Additionally, the N-terminal domains differ between the fibulin family members. *Adapted from de Vega et al. (2009) [52].*

1.3.3 Fibulin-3 in Cancer

In the last five years, several studies have shown that fibulin-3 plays a role in tumorigenesis. Fibulin-3 is differentially expressed in human cancers, and its effects have been described as both tumor suppressive and oncogenic (**Table 1.1**) [65]. Fibulin-3 expression is decreased in colorectal [66], lung [67], prostate [68], nasopharyngeal [69] and hepatocellular carcinomas [65]. In lung cancer, fibulin-3 downregulation promotes invasion and metastasis through activation of Wnt/β -catenin signaling and MMP-7 [70]. Similarly, Xu and colleagues showed that fibulin-3 negatively regulated lung cancer cell invasiveness *via* p38-mitogen-activated protein kinase (MAPK) and MMP-2 and -9 [71]. In nasopharyngeal carcinoma, fibulin-3 was shown to inhibit cell migration and invasion through suppression of AKT phosphorylation [69]. In a fibulin-3 overexpression model in pancreatic cancer cells, cancer stem cell markers were decreased, cells were resensitized to cytotoxic agents and EMT was inhibited [72]. Thus, numerous studies have demonstrated that fibulin-3 can have tumor suppressive roles in various human cancers.

Conversely, fibulin-3 has been reported to be upregulated in pancreatic [73], ovarian[74] and cervical [75] carcinomas, as well as malignant gliomas [76] and malignant mesothelioma [77]. Plasma and effusion fibulin-3 levels were reported to be increased in patients with pleural mesothelioma compared to patients without pleural mesothelioma, and therefore fibulin-3 has been proposed as a clinical biomarker for the disease [78]. Interestingly, fibulin-3 has also been shown to bind cell surface receptors, specifically EGFR in pancreatic carcinoma cells (**Figure 1.4**) [79]. Camaj and colleagues demonstrated that fibulin-3 competes with EGF for binding to EGFR, and activates downstream extracellular signal-regulated kinase (ERK1/2) and AKT signaling cascades involved in cancer cell

growth [79]. Fibulin-3 has pro-angiogenic roles in pancreatic [73] and ovarian [74] carcinomas and malignant gliomas [80]. Fibulin-3 was upregulated in human ovarian carcinoma tissues and positively correlated with microvessel density (MVD) and VEGF expression [74]. Serum fibulin-3 levels were also elevated in ovarian carcinoma patients compared to healthy controls and benign ovarian carcinoma patients, and were associated with low differentiation, high stage and lymph node metastasis [74]. Pancreatic cancer cells transfected with fibulin-3 displayed increased MVD, tumor growth and VEGF production *in vivo* [73]. Additionally, tumor cells that were transfected with fibulin-3 displayed a decrease in apoptosis and a shift from G_0-G_1 phase to S phase and mitosis, implicating fibulin-3 in tumor cell survival and cell cycle progression [73]. Similar results were observed in an *in vivo* model of malignant glioma in regards to VEGF secretion and MVD, and fibulin-3 was found to be localized around tumor blood vessels [80]. Also, fibulin-3 stimulated endothelial cell migration *via* a Notch-dependent signaling mechanism, a proliferative pathway dysregulated in many cancers [80]. Malignant glioma cells and tumors overexpressing fibulin-3 displayed increased expression and activity of MMP-2 and 9 [76]. Fibulin-3 has also been shown to promote glioma cell survival and invasion through activation of Notch signaling [81]. In contrast to Tong and colleagues, who suggested decreased fibulin-3 is associated with poor prognosis in colorectal cancer, a recent study reports that fibulin-3 promotes colorectal cancer cell migration and invasion and tumor growth *via* a p38 MAPK mechanism [82].

Although a role for fibulin-3 in breast cancer invasion is unknown, fibulin-3 has been reported to be overexpressed in breast cancer effusions [83]. However, whether fibulin-3 signaling regulates TNBC cell migration and invasion, important processes required for metastasis, is currently unknown and will be investigated in this study.

Table 1.1: Role of fibulin-3 in various cancers.

Cancer	Evidence	Ref
Tumor Suppressive		
Colorectal	• ↓ fibulin-3 protein in clinical tissue correlated with increased tumor stage, lymph node metastasis and poor survival.	[66]
Lung	• Promoter methylation in cancer cell lines and clinical tissue. • Fibulin-3 decreases invasion and metastasis *in vivo* by supressing Wnt/beta-catenin signaling and inhibition of MMP-7. • Overexpression of fibulin-3 inhibits cell growth, migration and invasion and decreased the expression and activity of MMP-2/9.	[67] [70] [71]
Prostate	• Promoter methylation in cell lines and clinical tissue.	[68]
Nasopharyngeal	• ↓ fibulin-3 mRNA and protein in clinical tissue correlated with tumor progression, lymph node metastasis and poor survival. • Fibulin-3 decreases cell migration and invasion through decreased pAKT activity.	[69]
Hepatocellular	• ↓ fibulin-3 mRNA and protein in cell lines and clinical tissue, correlated with poor survival. • Fibulin-3 knockdown increased cancer cell viability and invasion.	[65]
Oncogenic		
Pancreatic	• ↑ fibulin-3 mRNA in metastatic cancer cells and clinical tumor samples and overexpression results in enhanced tumor growth *in vivo*, and rescues tumor cells from apoptosis. • Fibulin-3 competitively binds and activates EGFR, leading to phosphorylation of AKT and ERK1/2.	[73] [79]
Ovarian	• ↑ fibulin-3 serum levels associated with high stage, low differentiation, lymph node metastasis and poor prognosis. • ↑ fibulin-3 mRNA and protein in clinical tissue positively correlated with MVD and VEGF expression.	[74]
Cervical	• Fibulin-3 protein expression was positively correlated with MVD and VEGF mRNA, and overexpression was associated with lymph node metastasis, vascular invasion and poor survival.	[75]
Malignant glioma	• ↑ fibulin-3 mRNA and protein in cell lines and clinical tissue and overexpression of fibulin-3 increases expression and activity of MMP-2/9 and tumor dispersion *in vivo*. • Overexpression of fibulin-3 increased tumor VEGF levels, MVD, and vessel permeability. • Fibulin-3 promotes tumor cell survival and invasion through activation of Notch signaling.	[76] [80] [81]
Malignant mesothelioma	• ↑ fibulin-3 plasma and effusion levels.	[78]

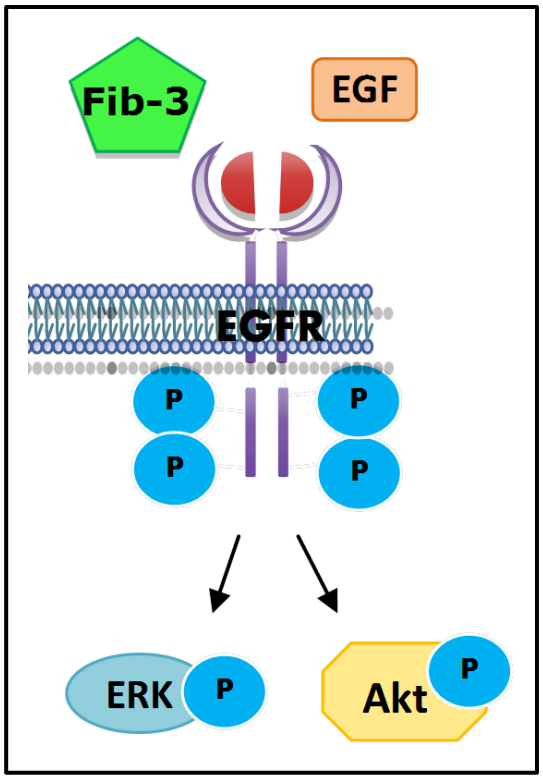

Figure 1.4. Fibulin-3 activates EGFR in pancreatic cancer. Camaj and colleagues demonstrated that fibulin-3 competes with epidermal growth factor (EGF) for binding to epidermal growth factor receptor (EGFR) in pancreatic carcinoma cells, resulting in autophosphorylation of EGFR. Additionally, downstream cell growth signaling cascades are activated through phosphorylation of AKT and extracellular signal-regulated kinase (ERK1/2) [79].

1.4 Matrix Metalloproteinases

MMPs are a family of zinc-dependent endopeptidases that are capable of degrading other proteinases, proteinase inhibitors, cell surface receptors, cell-cell adhesion molecules and essentially all ECM proteins [84]. Thus, since tumor cells must cross multiple ECM barriers to metastasize to distant sites, and upregulation of MMPs is associated with invasive ability in many cancers, MMPs can promote metastasis [85]. Additionally, a role for MMPs has been demonstrated in EMT, angiogenesis and intravasation [86,87,88].

1.4.1 Matrix Metalloproteinase-9 in Cancer

MMP-9 is secreted as an inactive pro-MMP that can be activated by plasmin, trypsin-2, MMP-2, MMP-13 and MMP-3 [89]. There are numerous substrates for MMP-9, a few of which include collagens, elastin, plasminogen and kisspeptins [90]. MMP-9 is primarily produced and secreted by stromal cells, however cancer cells may stimulate stromal cell production through paracrine signaling [34]. Increased MMP-9 expression and activity has been demonstrated in many cancers, including brain, urogenital, lung, skin, colorectal and breast [91]. Data from transgenic mice expressing an MMP-9 promoter-driven β-galactosidase transgene demonstrate that the MMP-9 promoter activity is induced during invasive cancer, but not *in situ* tumors [92]. MMP-9 has also been proposed as a prognostic biomarker in breast cancer due to its significant association with aggressive subtypes of breast cancer, such as TNBC, and its correlation with metastasis and relapse [93]. It has been demonstrated that MMP-9 regulates cell invasion downstream of ERK signaling in glioma [94] and breast cancer (ERα-positive MCF7 and TNBC MDA-MB-231) cells [95]. Moreover, it has been reported that EGF stimulates MMP-9 expression and activity in an ERK-dependent manner in ERα-negative SKBR3 breast cancer cells [96].

Despite its role in invasion, MMP-9 does more than just degrade ECM components, MMP-9 can also regulate signaling pathways such as proliferation, survival and angiogenesis [90]. The hemopexin (PEX) domain of both, inactive and active MMP-9 allows it to dock to the hyaluronan receptor CD44 and initiate intracellular signaling cascades (**Figure 1.5**) [97]. Thus, in normal keratinocytes and malignant cells, proteolytically active MMP-9 binds to CD44 on the cell surface and cleaves latent transforming growth factor-β (TGF-β) into an active form, allowing it to activate its receptor and lead to enhanced tumor growth [97]. Catalytically inactive MMP-9 has been shown to increase leukemia cell survival after docking to integrin and CD44 and activating intracellular anti-apoptosis pathways [98]. Both active and pro-MMP-9 have been implicated in breast cancer cell migration through interaction with CD44 [99,100]. Bourguignon and colleagues showed that CD44 is closely associated with active MMP-9 in invadopodia, actin-rich protrusions of the plasma membrane that are associated with degradation of the ECM [99]. Similarly, Dufour and colleagues demonstrated that pro-MMP-9 interacts with CD44 on tumor epithelial cells, leading to EGFR activation and phosphorylation of downstream signaling molecules, such as ERK, AKT, and focal adhesion kinase (FAK) to increase cell migration [100]. MMP-9 has also been shown to induce angiogenesis in pancreatic islet cells by stimulating VEGF release, and MMP inhibitors and MMP-9 knockout decreased the number of angiogenic islets and tumor burden [101]. MMP-9 has also been implicated in the process of intravasation, since MMP inhibition significantly reduced intravasation and only cancer cells expressing MMP-9 intravasated [88].

Thus, due to the ability of MMPs to facilitate invasion and metastasis, inhibitors have been developed to target their proteolytic activity in the hopes of preventing metastasis. Unfortunately, these therapies have failed clinically, and it has been suggested that it may be

a result of their lack of selectivity towards MMPs [90]. However, our knowledge of MMPs has grown considerably, particularly in regards to their non-proteolytic abilities, interactions with cell surface proteins and resulting downstream signaling cascades. New pharmacological inhibitors are focusing on blocking the PEX domain from binding to cell surface proteins and therefore preventing subsequent effects on cell survival, migration and angiogenesis [102]. This strategy has already been used to target MMP-9 [89,100]. Synthetic peptides that bind specific sites within the MMP-9 PEX domain were shown to inhibit cell motility in both human fibrosarcoma and breast cancer cell lines [100]. Similarly, a small inhibitor that prevents PEX from binding to cell surface integrins decreases cell migration and tumor growth [103]. These studies suggest that pharmacologically targeting the PEX domain of MMP-9 may be clinically beneficial alone or in combination with other therapeutics to effectively prevent tumor growth and metastasis.

MMP-9 serum levels have been shown to be high in breast cancer compared to benign breast disease and healthy controls [104]. Additionally, high MMP-9 serum levels were associated with lymph node metastasis, increased tumor stage, and lower overall survival [104]. Interestingly, fibulin-3 has been shown to regulate the expression and activity of MMP-9 in malignant glioma, however the role of fibulin-3 in regulating MMP-9 in breast cancer remains unknown and will be investigated by the current study.

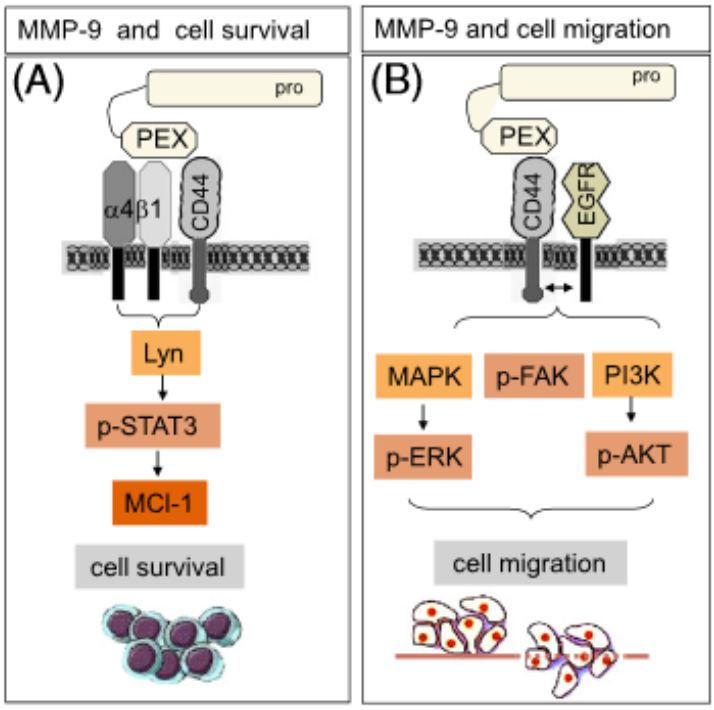

Figure 1.5. Signaling cascades induced by pro-MMP-9 interaction with cell surface proteins. (**A**) Pro-matrix metalloproteinase (MMP)-9 interacts with α4β1 integrin and CD44 *via* its hemopexin (PEX) domain to activate Lyn, phosphorylate signal transducer and activator of transcription 3 (STAT3) and upregulate the anti-apoptosis protein MCl-1 in chronic lymphocytic leukemia cells. (**B**) Similarly, the PEX domain of pro-MMP-9 facilitates binding to CD44 on tumor epithelial cells (COS-1 kidney cells, HT1080 fibrosarcoma cells and MDA-MB-435 melanoma cells) resulting in the activation of epidermal growth factor receptor (EGFR) and the phosphorylation of intracellular signaling molecules extracellular signal-regulated kinase (ERK), AKT and focal adhesion kinase (FAK) that stimulate cell migration. *Adapted from Bauvois (2012) [90].*

1.5 Metastasis Suppressor Genes

Recently, new groups of genes have been classified as metastasis activator or metastasis suppressor genes. These genes differ from oncogenes and tumor suppressor genes in that they do not influence primary tumor growth, but rather are involved in metastasis of cancer cells to distant tissues [105]. Metastasis requires cancer cells to undergo a series of processes to successfully colonize secondary sites, therefore a gene that inhibits even one of these processes would effectively block metastasis [106]. There have been over 150 genes discovered to be involved in breast cancer tumor development and progression, however only 6 metastasis suppressor genes have been shown to function *in vivo*, in metastasis assays [22]. These include *NME1*, *KAI1*, *BRMS1*, *MKK4*, *E-cadherin* and *KISS1* [22]. Evidently, metastasis suppressor genes provide a promising target for pharmacological interventions to prevent metastasis in cancer patients.

1.5.1 KISS1R Signaling

The metastasis suppressor gene, *KISS1*, encodes a 145-residue kisspeptin protein that is rapidly cleaved into shorter secreted peptides termed kisspeptin-10 (KP-10), kisspeptin-13, kisspeptin-14 and kisspeptin-54 (**Figure 1.6A**) [107]. The shorter kisspeptins are N-terminally truncated peptides of kisspeptin-145 cleaved by furin, MMP-2, -9, and -14 or prohormone convertases [108]. KP-10 is the shortest biologically active kisspeptin, and is highly conserved among vertebrates [109]. Kisspeptins are the endogenous ligand for the kisspeptin receptor (KISS1R) [107,110,111]. KISS1R has a single high affinity binding site for kisspeptins, and all kisspeptins bind with similar affinities and potencies [107,110,112,113]. *KISS1* and KISS1R expression have been shown in similar tissues,

including the placenta, pituitary, pancreas, brain, small intestine, liver, lung, kidney, testis, ovary and breast [107,110,111].

KISS1R is a G-protein coupled receptor (GPCR) that signals *via* a $G_{q/11}$-coupled mechanism leading to activation of phospholipase C (PLC) (**Figure 1.6B**). PLC activation leads to increased production of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) which results in increased intracellular calcium and subsequent activation of protein kinase C (PKC) and ERK1/2 [107,110,111]. In addition, KISS1R has been shown to activate ERK1/2 through a G-protein independent and β-arrestin2-dependent pathway [114]. KISS1R contains residues in the second intracellular loop and cytoplasmic tail that allow it to constitutively interact with β-arrestin1 and β-arrestin2 [115]. Also, desensitization and internalization of KISS1R is mediated by G-protein-coupled receptor serine/threonine kinase 2 (GRK2) and β-arrestins [116]. Bianco and colleagues observed persistent expression of KISS1R at the plasma membrane, even after KP-10 stimulation, and there was no co-localization of KISS1R with lysosomal markers, indicating that ligand-induced KISS1R internalization does not result in lysosomal degradation [117]. Instead, KISS1R is recycled to the cell surface, prolonging KISS1R signaling, and suggesting that KISS1R may have constitutive activity [117].

KP/KISS1R signaling plays an important physiological role in the regulation of the reproductive axis and the initiation of puberty [118]. Studies have shown that loss-of-function mutations in KISS1R result in hypogonadotropic hypogonadism in humans [116]. Hypogonadotropic hypogonadism is characterized by diminished functional activity of the gonads and infertility due to the insufficiency of the pituitary to secrete gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [116]. KP/KISS1R

signaling regulates the reproductive axis by stimulating the release of gonadotropin-releasing hormone (GnRH) [118]. Pulsatile GnRH secretion then stimulates the pituitary to synthesize and secrete FSH and LH [119]. KP/KISS1R signaling has also been implicated in pregnancy and placentation. The circulating serum levels of KP increase approximately 1000 fold in the first trimester and an amazing 10,000 fold in the third trimester, signifying an important role for KP throughout pregnancy [120]. Moreover, KP has been shown to inhibit trophoblast migration and invasion through decreased MMP-2 [121]. Consequently, KP has been proposed to play a critical role in regulating placental invasion of the uterine wall during implantation [121]. Due to the emergence of KISS1R as a clinically relevant GPCR, it is vital that the molecular pathways regulating KISS1R signaling be further investigated in other diseases and conditions.

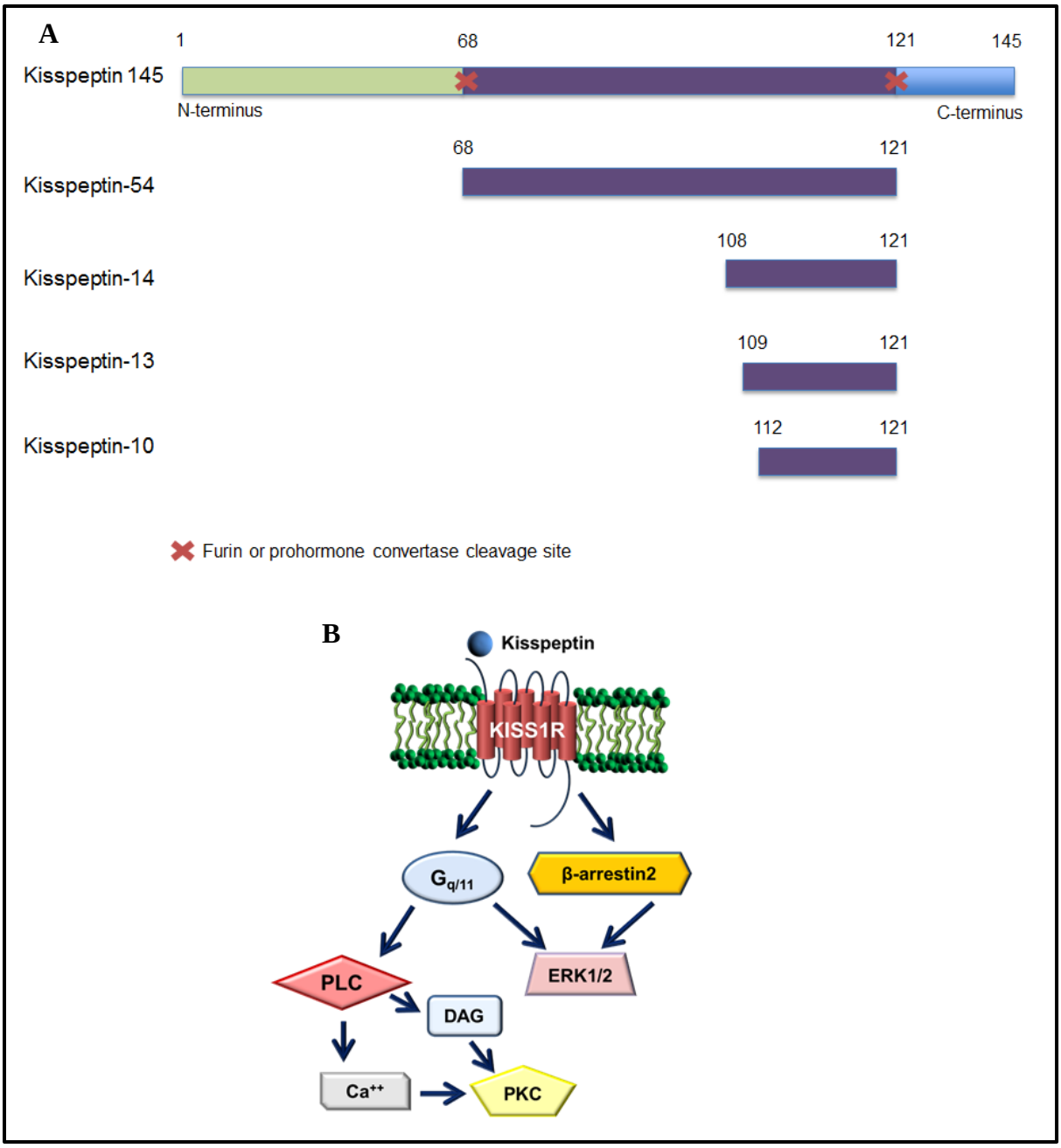

Figure 1.6. Kisspeptins (KP) and KISS1R signaling cascades. (**A**) Cleavage of kisspeptin-145 results in the production of smaller peptides, termed KP-54, KP-14, KP-13 and KP-10. (**B**) The kisspeptin receptor (KISS1R) is a $G_{q/11}$-coupled receptor that upon binding of its ligand, KP, will activate phospholipase C (PLC) and extracellular signal-regulated kinase (ERK1/2). Activation of PLC will lead to production of diacylglycerol (DAG) and increased intracellular calcium which will result in activation of protein kinase C (PKC). Additionally, KISS1R can also signal independently of G-proteins to activate ERK1/2 in a β-arrestin2 dependent mechanism.

1.5.2 KISS1R Signaling in Cancer

The KP/KISS1R system functions as a metastasis suppressor in melanoma [105], pancreatic [122], bladder [123], ovarian [124], endometrial [124], esophageal [125], lung [126] and gastric cancers [127]. These cancers displayed decreased expression of *KISS1* and /or *KISS1R* in tumor tissue compared to non-malignant tissue, and this observation correlated with decreased patient prognosis. *KISS1* signaling was first studied in melanoma and was found to be expressed in non-metastatic cells, but was absent in metastatic melanoma cell lines [105]. *KISS1* mRNA expression was also lower in pancreatic carcinoma tissue in comparison to non-malignant tissue [122]. When stimulated with exogenous KP, pancreatic cancer cells exhibited ERK1 activation and decreased migration, however no effect on cell proliferation was observed [122]. In gastric cancer, KP-145 protein expression was reduced in metastatic tissues from the lymph nodes and liver compared to the primary tumor [127]. In bladder [123] and ovarian [128] carcinomas, decreased expression of *KISS1* mRNA was associated with poor patient survival. In a fibrosarcoma cell line, exogenous expression of *KISS1* resulted in decreased MMP-9 expression and activity, as well as reduced invasion [129]. The observed decrease in MMP-9 expression was attributed to diminished nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription factor binding to the promoter of MMP-9 as a result of *KISS1* expression [129]. Thus, KISS1R undeniably plays a role in metastasis and may serve as a potential therapeutic target in cancer.

1.5.3 KISS1R Signaling in Breast Cancer

In contrast to its role as a metastasis suppressor in numerous cancers, KP/KISS1R signaling has been shown to play detrimental roles in breast cancer. The first study looked at clinical *KISS1* and *KISS1R* mRNA expression in breast tumor tissue and discovered that *KISS1* expression was elevated in breast cancer tissue compared to non-malignant mammary tissue [130]. However, this study did not examine hormone receptor status. Additionally, breast tumors positive for lymph node metastasis displayed increased *KISS1* mRNA compared to lymph node negative tumors [130]. However, no significant difference in *KISS1R* mRNA expression was observed [130]. Exogenous expression of *KISS1* in the human breast cancer cell line MDA-MB-231 enhanced motility, invasive ability and reduced cell adhesion, therefore *KISS1* overexpression induced a more aggressive phenotype [130]. Lastly, this study demonstrated the positive correlation between *KISS1* mRNA expression and poor patient prognosis [130].

The second study examined ERα status and *KISS1* expression in breast tumors. Marot and colleagues demonstrated that ERα-positive breast tumors expressed sevenfold less *KISS1* than ERα-negative breast tumors [131]. This study showed that treatment of ERα-positive MCF7 and T47D breast cancer cells with the estrogen receptor antagonist, tamoxifen, stimulated *KISS1* and *KISS1R* mRNA expression [131]. Exogenous expression of ERα in the ERα-negative breast cancer cell line MDA-MB-231 resulted in diminished *KISS1* mRNA expression [131]. Similarly, ERα-positive breast tumors in postmenopausal women treated with tamoxifen also displayed high *KISS1* and *KISS1R* mRNA levels that was associated with shorter relapse-free survival [131]. Furthermore, *KISS1* mRNA expression in breast tumors was shown to increase with grade [131]. The results of this study suggest that

KISS1 and *KISS1R* mRNA levels in breast tumors may be a prognostic marker for anti-estrogen therapy resistance [131]. Therefore clinical data suggests that KP/KISS1R signaling may play an influential role in breast cancer progression.

A 2011 study investigated a role for mouse KP/KISS1R signaling in regulating breast cancer metastasis. Breast cancer was induced in mice expressing the polyoma middle T antigen (PyMT) under the control of the mouse mammary tumor virus (MMTV) promoter to explore the role of KISS1R expression in breast cancer progression and metastasis [132]. Results from this study indicated that PyMT-induced breast tumor development and lung metastases were delayed in the KISS1R heterozygous (PyMT/Kiss1r$^{+/-}$) mouse in comparison to the wild-type (PyMT/Kiss1r$^{+/+}$) mouse [132]. Interestingly, KISS1R heterozygous (PyMT/Kiss1r$^{+/-}$) tumors displayed a significant reduction in *MMP-9* mRNA compared to wild-type (PyMT/Kiss1r$^{+/+}$) tumors [132]. Furthermore, heterozygous mouse primary breast cancer cells exhibited decreased cellular invasion *in vitro* [132]. Accordingly, this study proposes that KP/KISS1R signaling regulates breast tumor development, progression and metastasis in a mouse model.

Studies from our laboratory have investigated the underlying molecular mechanisms regulated by KP/KISS1R in breast cancer metastasis (**Figure 1.7**) [133,134]. Our first study demonstrated that treatment of human TNBC cell lines, MDA-MB-231 and Hs578T, with KP-10, stimulated migration and invasion [133]. Interestingly, KP-10 stimulation also increased MMP-9 secretion and activity [133]. We found that activation of KISS1R signaling promoted the activation of EGFR *via* a β-arrestin2 dependent pathway [133]. Lastly, fluorescence resonance energy transfer (FRET) analysis determined that KISS1R directly associates with EGFR under basal conditions and KP-10 treatment enhanced this

interaction [133]. In our second study, we found that exogenous KISS1R expression or KP-10 treatment induced EMT and cell invasion in ERα-negative non-malignant mammary epithelial MCF10A cells [134]. This demonstrates that KP signaling stimulates malignant transformation. KISS1R overexpression in ERα-negative SKBR3 breast cancer cells stimulated extravasation *in vivo* using the chick chorioallantoic membrane assay [134]. Most interestingly, KP-10 failed to stimulate invasion and EGFR activation in ERα-positive MCF7 and T47D breast cancer cells, leading us to conclude that ERα negatively regulates KISS1R signaling in breast cancer [134]. This concept was validated by exogenously expressing ERα in the ERα-negative MDA-MB-231 breast cancer cells, and upon estradiol stimulation KP-10-induced invasion and EGFR activation was blocked, and KISS1R protein expression was decreased [134]. This demonstrates that ERα negatively regulates KISS1R expression and signaling. Finally, we discovered that KISS1R tranactivation of EGFR is dependent upon an actin cytoskeletal binding protein, IQ-motif-containing GTPase activating protein 1 (IQGAP1), and that IQGAP1 is a novel binding partner of KISS1R [134]. However, the molecular mechanism by which KP/KISS1R signaling stimulates TNBC cell invasion is still largely unknown and will be further investigated by this thesis.

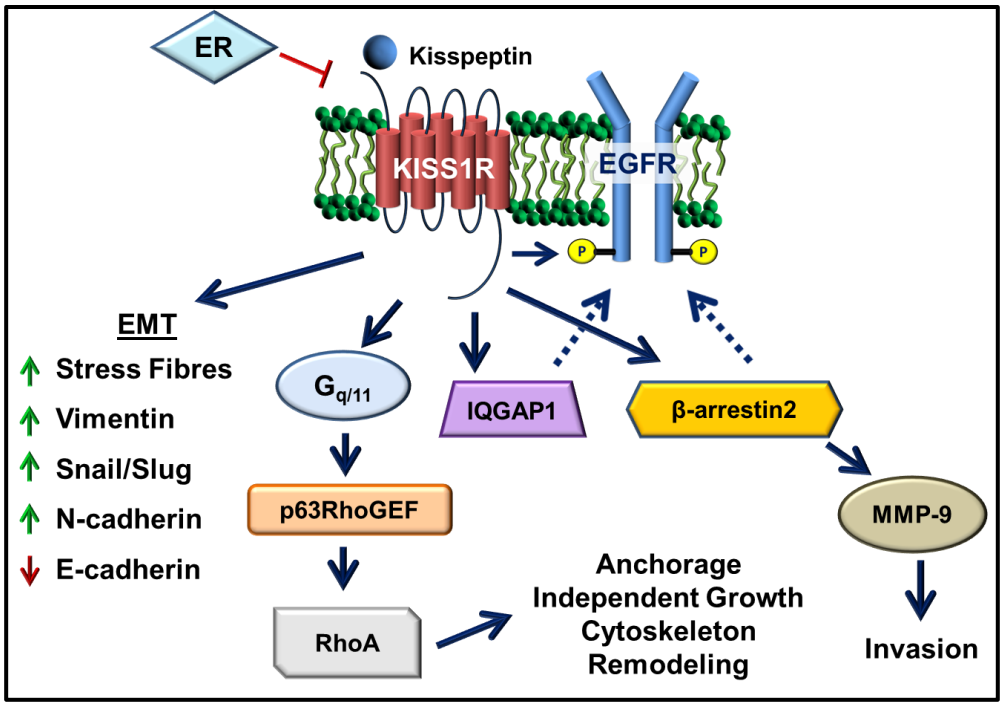

Figure 1.7. KISS1R signaling in ERα-negative breast cancer. Kisspeptin receptor (KISS1R) signaling induces epithelial-to-mesenchymal transition (EMT). Also, KISS1R activation leads to anchorage independent growth and cytoskeletal reorganization through the small GTPase, RhoA. Kisspeptin binds and activates KISS1R leading to epidermal growth factor receptor (EGFR) activation *via* β-arrestin2 and IQGAP1 dependent pathways. Additionally, β-arrestin2 is required for kisspeptin stimulated matrix metalloproteinase (MMP)-9 activity and breast cancer cell invasion. Estrogen receptor (ER)α negatively regulates KISS1R expression and signaling in breast cancer cells. *Adapted from Cvetkovic et al. (2013) [134].*

1.5.4 β-arrestins

β-arrestins are ubiquitously expressed proteins that function as signaling adaptors and molecular scaffolds [135], and play a crucial role in the phosphorylation of intracellular targets. The two β-arrestin isoforms (β-arrestin1 and β-arrestin2) display a high degree of homology (~70% of similar sequence identity) and may function similarly to regulate GPCR desensitization and internalization [136]. β-arrestins can also regulate the signaling and trafficking of other types of receptors, such as EGFR and the TGF-β receptor [137]. β-arrestins are capable of scaffolding numerous cell signaling molecules, including many that have oncogenic roles and regulate the cytoskeleton, such as proto-oncogene tyrosine-protein kinase Src (Src), ERK1/2 and AKT [138].

β-arrestins are also emerging regulators of breast cancer progression and metastasis. β-arrestin1 was shown to regulate phosphorylation of ERK1/2 in ERα-positive MCF7 breast cancer cells, and β-arrestin1 depletion resulted in decreased tumor cell proliferation [139]. β-arrestin1 has also been shown to promote survival of breast cancer cells in hypoxic conditions *via* increased expression of VEGF and positively regulate breast cancer metastatic spread in nude mice [136]. Furthermore, depletion of both β-arrestin1 and β-arrestin2 in MDA-MB-231 breast cancer cells decreased tumor growth and increased mouse survival rate in experimental metastasis assays [136]. Another breast cancer study has linked amplified stromal β-arrestin1 expression with poor patient prognosis and increases in HIF-1 expression, tumor growth and metastases [140]. Clinical data from our lab has shown that the expression of β-arrestin2 is elevated in invasive human breast cancer, and that β-arrestin2 regulates the invasion of ERα-negative breast cancer cells, upon binding to small GTPases, Ral and

Rap1A [141,142]. Lastly, we have shown that KP-10-induced EGFR transactivation and cell invasion is dependent on β-arrestin2 [133].

1.6 Rationale, Hypothesis and Objectives

1.6.1 Rationale

Fibulin-3 is overexpressed in breast cancer effusions [83], however, whether plasma fibulin-3 concentrations change in TNBC patients is currently unknown. Despite evidence that fibulin-3 is capable of binding and activating EGFR [79], as well as upregulating MMP-9 [76], a role for fibulin-3 in TNBC cell invasion has not yet been determined. Previous studies from our lab have demonstrated that in TNBC cells, KISS1R signaling stimulates cell invasion by stimulating EGFR activation and MMP-9 secretion and activity [133,134]. Here we will investigate whether fibulin-3 regulates KISS1R-induced TNBC cell invasion and the underlying mechanism(s).

1.6.2 Hypothesis

KISS1R stimulates TNBC cell invasion *via* a fibulin-3 dependent mechanism.

1.6.3 Objectives

To determine if:

1) Plasma fibulin-3 levels are increased in TNBC patients compared to healthy controls.
2) Fibulin-3 regulates KISS1R-induced TNBC cell invasion, and uncover the potential underlying mechanism(s).
3) Fibulin-3 regulates EGFR phosphorylation in TNBC cells.

1.7 References

† http://www.cbcf.org/ontario/AboutBreastCancerMain/FactsStats/Pages/Breast-Cancer-Canada.aspx

1. Singletary, S. E., & Connolly, J. L. (2006). Breast cancer staging: working with the sixth edition of the AJCC Cancer Staging Manual. *CA Cancer J Clin, 56*(1), 37-47; quiz 50-31.
2. Bland, K. I., Menck, H. R., Scott-Conner, C. E., Morrow, M., Winchester, D. J., & Winchester, D. P. (1998). The National Cancer Data Base 10-year survey of breast carcinoma treatment at hospitals in the United States. *Cancer, 83*(6), 1262-1273.
3. Sakorafas, G. H., & Tsiotou, A. G. (2000). Ductal carcinoma in situ (DCIS) of the breast: evolving perspectives. *Cancer Treat Rev, 26*(2), 103-125. doi: 10.1053/ctrv.1999.0149
4. Fantozzi, A., & Christofori, G. (2006). Mouse models of breast cancer metastasis. *Breast Cancer Res, 8*(4), 212. doi: 10.1186/bcr1530
5. Bagaria, S. P., Ray, P. S., Sim, M. S., Ye, X., Shamonki, J. M., Cui, X., & Giuliano, A. E. (2014). Personalizing breast cancer staging by the inclusion of ER, PR, and HER2. *JAMA Surg, 149*(2), 125-129. doi: 10.1001/jamasurg.2013.3181
6. Zhang, M., Zhang, X., Zhao, S., Wang, Y., Di, W., Zhao, G., . . . Zhang, Q. (2013). Prognostic value of survivin and EGFR protein expression in triple-negative breast cancer (TNBC) patients. *Target Oncol*. doi: 10.1007/s11523-013-0300-y
7. Barbieri, V., Sanpaolo, P., & Genovesi, D. (2011). Prognostic impact of triple negative phenotype in conservatively treated breast cancer. *Breast J, 17*(4), 377-382. doi: 10.1111/j.1524-4741.2011.01100.x
8. Brady-West, D. C., & McGrowder, D. A. (2011). Triple negative breast cancer: therapeutic and prognostic implications. *Asian Pac J Cancer Prev, 12*(8), 2139-2143.
9. Elnashar, A. T., Ali el, S. M., & Gaber, A. (2012). The prognostic value of triple negative in stage II/III breast cancer. *J Oncol Pharm Pract, 18*(1), 68-75. doi: 10.1177/1078155211398299
10. Carey, L. A., Perou, C. M., Livasy, C. A., Dressler, L. G., Cowan, D., Conway, K., . . . Millikan, R. C. (2006). Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA, 295*(21), 2492-2502. doi: 10.1001/jama.295.21.2492
11. Dent, R., Trudeau, M., Pritchard, K. I., Hanna, W. M., Kahn, H. K., Sawka, C. A., . . . Narod, S. A. (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res, 13*(15 Pt 1), 4429-4434. doi: 10.1158/1078-0432.ccr-06-3045
12. Ihemelandu, C. U., Leffall, L. D., Jr., Dewitty, R. L., Naab, T. J., Mezghebe, H. M., Makambi, K. H., . . . Frederick, W. A. (2007). Molecular breast cancer subtypes in premenopausal African-American women, tumor biologic factors and clinical outcome. *Ann Surg Oncol, 14*(10), 2994-3003. doi: 10.1245/s10434-007-9477-6
13. Atchley, D. P., Albarracin, C. T., Lopez, A., Valero, V., Amos, C. I., Gonzalez-Angulo, A. M., . . . Arun, B. K. (2008). Clinical and pathologic characteristics of patients with BRCA-positive and BRCA-negative breast cancer. *J Clin Oncol, 26*(26), 4282-4288. doi: 10.1200/jco.2008.16.6231
14. Siziopikou, K. P., Ariga, R., Proussaloglou, K. E., Gattuso, P., & Cobleigh, M. (2006). The challenging estrogen receptor-negative/ progesterone receptor-negative/HER-2-

negative patient: a promising candidate for epidermal growth factor receptor-targeted therapy? *Breast J, 12*(4), 360-362. doi: 10.1111/j.1075-122X.2006.00276.x

15. Yarden, Y. (2001). The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur J Cancer, 37 Suppl 4*, S3-8.
16. Pan, D., & Lin, X. (2013). Epithelial growth factor receptor-activated nuclear factor kappaB signaling and its role in epithelial growth factor receptor-associated tumors. *Cancer J, 19*(6), 461-467. doi: 10.1097/ppo.0000000000000001
17. Heitz, F., Harter, P., Lueck, H. J., Fissler-Eckhoff, A., Lorenz-Salehi, F., Scheil-Bertram, S., . . . du Bois, A. (2009). Triple-negative and HER2-overexpressing breast cancers exhibit an elevated risk and an earlier occurrence of cerebral metastases. *Eur J Cancer, 45*(16), 2792-2798. doi: 10.1016/j.ejca.2009.06.027
18. Peddi, P. F., Ellis, M. J., & Ma, C. (2012). Molecular basis of triple negative breast cancer and implications for therapy. *Int J Breast Cancer, 2012*, 217185. doi: 10.1155/2012/217185
19. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013
20. Greene FL, P. D., Fleming ID, Fritz AG, Balch CM, Haller DG, Morrow M (Ed.). (2002) (6th ed.). New York: Springer.
21. Mehlen, P., & Puisieux, A. (2006). Metastasis: a question of life or death. *Nat Rev Cancer, 6*(6), 449-458. doi: 10.1038/nrc1886
22. Welch, D. R., Steeg, P. S., & Rinker-Schaeffer, C. W. (2000). Molecular biology of breast cancer metastasis. Genetic regulation of human breast carcinoma metastasis. *Breast Cancer Res, 2*(6), 408-416.
23. Solomayer, E. F., Diel, I. J., Meyberg, G. C., Gollan, C., & Bastert, G. (2000). Metastatic breast cancer: clinical course, prognosis and therapy related to the first site of metastasis. *Breast Cancer Res Treat, 59*(3), 271-278.
24. Fidler, I. J. (2003). The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer, 3*(6), 453-458. doi: 10.1038/nrc1098
25. Liotta, L. A., Steeg, P. S., & Stetler-Stevenson, W. G. (1991). Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell, 64*(2), 327-336.
26. Tse, J. C., & Kalluri, R. (2007). Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. *J Cell Biochem, 101*(4), 816-829. doi: 10.1002/jcb.21215
27. Marchiando, A. M., Graham, W. V., & Turner, J. R. (2010). Epithelial barriers in homeostasis and disease. *Annu Rev Pathol, 5*, 119-144. doi: 10.1146/annurev.pathol.4.110807.092135
28. Meyer, T., & Hart, I. R. (1998). Mechanisms of tumour metastasis. *Eur J Cancer, 34*(2), 214-221.
29. Micalizzi, D. S., Farabaugh, S. M., & Ford, H. L. (2010). Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia, 15*(2), 117-134. doi: 10.1007/s10911-010-9178-9
30. Gupta, G. P., & Massague, J. (2006). Cancer metastasis: building a framework. *Cell, 127*(4), 679-695. doi: 10.1016/j.cell.2006.11.001
31. Hunter, K. W., Crawford, N. P., & Alsarraj, J. (2008). Mechanisms of metastasis. *Breast Cancer Res, 10 Suppl 1*, S2. doi: 10.1186/bcr1988
32. Dano, K., Behrendt, N., Hoyer-Hansen, G., Johnsen, M., Lund, L. R., Ploug, M., & Romer, J. (2005). Plasminogen activation and cancer. *Thromb Haemost, 93*(4), 676-681. doi: 10.1267/thro05040676

33. Price, J. T., Bonovich, M. T., & Kohn, E. C. (1997). The biochemistry of cancer dissemination. *Crit Rev Biochem Mol Biol, 32*(3), 175-253. doi: 10.3109/10409239709082573
34. Egeblad, M., & Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer, 2*(3), 161-174. doi: 10.1038/nrc745
35. Blood, C. H., & Zetter, B. R. (1990). Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochim Biophys Acta, 1032*(1), 89-118.
36. Brooks, S. A., Lomax-Browne, H. J., Carter, T. M., Kinch, C. E., & Hall, D. M. (2010). Molecular interactions in cancer cell metastasis. *Acta Histochem, 112*(1), 3-25. doi: 10.1016/j.acthis.2008.11.022
37. Schneider, B. P., & Miller, K. D. (2005). Angiogenesis of breast cancer. *J Clin Oncol, 23*(8), 1782-1790. doi: 10.1200/jco.2005.12.017
38. Scully, O. J., Bay, B. H., Yip, G., & Yu, Y. (2012). Breast cancer metastasis. *Cancer Genomics Proteomics, 9*(5), 311-320.
39. Gasparini, G., Toi, M., Gion, M., Verderio, P., Dittadi, R., Hanatani, M., . . . Tominaga, T. (1997). Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. *J Natl Cancer Inst, 89*(2), 139-147.
40. Roussos, E. T., Condeelis, J. S., & Patsialou, A. (2011). Chemotaxis in cancer. *Nat Rev Cancer, 11*(8), 573-587. doi: 10.1038/nrc3078
41. Reymond, N., d'Agua, B. B., & Ridley, A. J. (2013). Crossing the endothelial barrier during metastasis. *Nat Rev Cancer, 13*(12), 858-870. doi: 10.1038/nrc3628
42. Bersini, S., Jeon, J. S., Moretti, M., & Kamm, R. D. (2014). In vitro models of the metastatic cascade: from local invasion to extravasation. *Drug Discov Today, 19*(6), 735-742. doi: 10.1016/j.drudis.2013.12.006
43. Chambers, A. F., Groom, A. C., & MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer, 2*(8), 563-572. doi: 10.1038/nrc865
44. Weber, C. E., & Kuo, P. C. (2012). The tumor microenvironment. *Surg Oncol, 21*(3), 172-177. doi: 10.1016/j.suronc.2011.09.001
45. Valastyan, S., & Weinberg, R. A. (2011). Tumor metastasis: molecular insights and evolving paradigms. *Cell, 147*(2), 275-292. doi: 10.1016/j.cell.2011.09.024
46. Nelson, C. M., & Bissell, M. J. (2006). Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. *Annu Rev Cell Dev Biol, 22*, 287-309. doi: 10.1146/annurev.cellbio.22.010305.104315
47. Kenny, P. A., Lee, G. Y., & Bissell, M. J. (2007). Targeting the tumor microenvironment. *Front Biosci, 12*, 3468-3474.
48. Ghajar, C. M., & Bissell, M. J. (2008). Extracellular matrix control of mammary gland morphogenesis and tumorigenesis: insights from imaging. *Histochem Cell Biol, 130*(6), 1105-1118. doi: 10.1007/s00418-008-0537-1
49. Boudreau, A., van't Veer, L. J., & Bissell, M. J. (2012). An "elite hacker": breast tumors exploit the normal microenvironment program to instruct their progression and biological diversity. *Cell Adh Migr, 6*(3), 236-248. doi: 10.4161/cam.20880
50. Ma, X. J., Dahiya, S., Richardson, E., Erlander, M., & Sgroi, D. C. (2009). Gene expression profiling of the tumor microenvironment during breast cancer progression. *Breast Cancer Res, 11*(1), R7. doi: 10.1186/bcr2222
51. Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C. A., Briand, P., Damsky, C., & Bissell, M. J. (1997). Reversion of the malignant phenotype of human breast cells in

three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol, 137*(1), 231-245.

52. de Vega, S., Iwamoto, T., & Yamada, Y. (2009). Fibulins: multiple roles in matrix structures and tissue functions. *Cell Mol Life Sci, 66*(11-12), 1890-1902. doi: 10.1007/s00018-009-8632-6
53. Argraves, W. S., Greene, L. M., Cooley, M. A., & Gallagher, W. M. (2003). Fibulins: physiological and disease perspectives. *EMBO Rep, 4*(12), 1127-1131. doi: 10.1038/sj.embor.7400033
54. Gallagher, W. M., Currid, C. A., & Whelan, L. C. (2005). Fibulins and cancer: friend or foe? *Trends Mol Med, 11*(7), 336-340. doi: 10.1016/j.molmed.2005.06.001
55. Giltay, R., Timpl, R., & Kostka, G. (1999). Sequence, recombinant expression and tissue localization of two novel extracellular matrix proteins, fibulin-3 and fibulin-4. *Matrix Biol, 18*(5), 469-480.
56. Ehlermann, J., Weber, S., Pfisterer, P., & Schorle, H. (2003). Cloning, expression and characterization of the murine Efemp1, a gene mutated in Doyne-Honeycomb retinal dystrophy. *Gene Expr Patterns, 3*(4), 441-447.
57. Blackburn, J., Tarttelin, E. E., Gregory-Evans, C. Y., Moosajee, M., & Gregory-Evans, K. (2003). Transcriptional regulation and expression of the dominant drusen gene FBLN3 (EFEMP1) in mammalian retina. *Invest Ophthalmol Vis Sci, 44*(11), 4613-4621.
58. Hayashi, S. I., Eguchi, H., Tanimoto, K., Yoshida, T., Omoto, Y., Inoue, A., . . . Yamaguchi, Y. (2003). The expression and function of estrogen receptor alpha and beta in human breast cancer and its clinical application. *Endocr Relat Cancer, 10*(2), 193-202.
59. McLaughlin, P. J., Bakall, B., Choi, J., Liu, Z., Sasaki, T., Davis, E. C., . . . Marmorstein, L. Y. (2007). Lack of fibulin-3 causes early aging and herniation, but not macular degeneration in mice. *Hum Mol Genet, 16*(24), 3059-3070. doi: 10.1093/hmg/ddm264
60. Rahn, D. D., Acevedo, J. F., Roshanravan, S., Keller, P. W., Davis, E. C., Marmorstein, L. Y., & Word, R. A. (2009). Failure of pelvic organ support in mice deficient in fibulin-3. *Am J Pathol, 174*(1), 206-215. doi: 10.2353/ajpath.2009.080212
61. Cooley, M., & Argraves, W. S. (2011). The Fibulins. In R. P. Mecham (Ed.), *The Extracellular Matrix: an Overview* (pp. 337-367): Springer Berlin Heidelberg.
62. Klenotic, P. A., Munier, F. L., Marmorstein, L. Y., & Anand-Apte, B. (2004). Tissue inhibitor of metalloproteinases-3 (TIMP-3) is a binding partner of epithelial growth factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1). Implications for macular degenerations. *J Biol Chem, 279*(29), 30469-30473. doi: 10.1074/jbc.M403026200
63. Ozaki, T., Kondo, K., Nakamura, Y., Ichimiya, S., Nakagawara, A., & Sakiyama, S. (1997). Interaction of DA41, a DAN-binding protein, with the epidermal growth factor-like protein, S(1-5). *Biochem Biophys Res Commun, 237*(2), 245-250. doi: 10.1006/bbrc.1997.7122
64. Lecka-Czernik, B., Lumpkin, C. K., Jr., & Goldstein, S. (1995). An overexpressed gene transcript in senescent and quiescent human fibroblasts encoding a novel protein in the epidermal growth factor-like repeat family stimulates DNA synthesis. *Mol Cell Biol, 15*(1), 120-128.
65. Luo, R., Zhang, M., Liu, L., Lu, S., Zhang, C. Z., & Yun, J. (2013). Decrease of fibulin-3 in hepatocellular carcinoma indicates poor prognosis. *PLoS One, 8*(8), e70511. doi: 10.1371/journal.pone.0070511

66. Tong, J. D., Jiao, N. L., Wang, Y. X., Zhang, Y. W., & Han, F. (2011). Downregulation of fibulin-3 gene by promoter methylation in colorectal cancer predicts adverse prognosis. *Neoplasma, 58*(5), 441-448.
67. Yue, W., Dacic, S., Sun, Q., Landreneau, R., Guo, M., Zhou, W., . . . Zhang, L. (2007). Frequent inactivation of RAMP2, EFEMP1 and Dutt1 in lung cancer by promoter hypermethylation. *Clin Cancer Res, 13*(15 Pt 1), 4336-4344. doi: 10.1158/1078-0432.ccr-07-0015
68. Kim, Y. J., Yoon, H. Y., Kim, S. K., Kim, Y. W., Kim, E. J., Kim, I. Y., & Kim, W. J. (2011). EFEMP1 as a novel DNA methylation marker for prostate cancer: array-based DNA methylation and expression profiling. *Clin Cancer Res, 17*(13), 4523-4530. doi: 10.1158/1078-0432.ccr-10-2817
69. Hwang, C. F., Chien, C. Y., Huang, S. C., Yin, Y. F., Huang, C. C., Fang, F. M., . . . Chen, C. H. (2010). Fibulin-3 is associated with tumour progression and a poor prognosis in nasopharyngeal carcinomas and inhibits cell migration and invasion via suppressed AKT activity. *J Pathol, 222*(4), 367-379. doi: 10.1002/path.2776
70. Chen, X., Meng, J., Yue, W., Yu, J., Yang, J., Yao, Z., & Zhang, L. (2014). Fibulin-3 suppresses Wnt/beta-catenin signaling and lung cancer invasion. *Carcinogenesis, 35*(8), 1707-1716. doi: 10.1093/carcin/bgu023
71. Xu, S., Yang, Y., Sun, Y. B., Wang, H. Y., Sun, C. B., & Zhang, X. (2014). Role of fibulin-3 in lung cancer: in vivo and in vitro analyses. *Oncol Rep, 31*(1), 79-86. doi: 10.3892/or.2013.2799
72. Kim, I. G., Lee, J. H., Kim, S. Y., Kim, J. Y., & Cho, E. W. (2014). Fibulin-3 negatively regulates ALDH1 via c-MET suppression and increases gamma-radiation-induced sensitivity in some pancreatic cancer cell lines. *Biochem Biophys Res Commun, 454*(3), 369-375. doi: 10.1016/j.bbrc.2014.10.084
73. Seeliger, H., Camaj, P., Ischenko, I., Kleespies, A., De Toni, E. N., Thieme, S. E., . . . Bruns, C. J. (2009). EFEMP1 expression promotes in vivo tumor growth in human pancreatic adenocarcinoma. *Mol Cancer Res, 7*(2), 189-198. doi: 10.1158/1541-7786.mcr-08-0132
74. Chen, J., Wei, D., Zhao, Y., Liu, X., & Zhang, J. (2013). Overexpression of EFEMP1 correlates with tumor progression and poor prognosis in human ovarian carcinoma. *PLoS One, 8*(11), e78783. doi: 10.1371/journal.pone.0078783
75. En-lin, S., Sheng-guo, C., & Hua-qiao, W. (2010). The expression of EFEMP1 in cervical carcinoma and its relationship with prognosis. *Gynecol Oncol, 117*(3), 417-422. doi: 10.1016/j.ygyno.2009.12.016
76. Hu, B., Thirtamara-Rajamani, K. K., Sim, H., & Viapiano, M. S. (2009). Fibulin-3 is uniquely upregulated in malignant gliomas and promotes tumor cell motility and invasion. *Mol Cancer Res, 7*(11), 1756-1770. doi: 10.1158/1541-7786.mcr-09-0207
77. Kaya, H., Demir, M., Taylan, M., Sezgi, C., Tanrikulu, A. C., Yilmaz, S., . . . Senyigit, A. (2015). Fibulin-3 as a diagnostic biomarker in patients with malignant mesothelioma. *Asian Pac J Cancer Prev, 16*(4), 1403-1407.
78. Pass, H. I., Levin, S. M., Harbut, M. R., Melamed, J., Chiriboga, L., Donington, J., . . . Goparaju, C. (2012). Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. *N Engl J Med, 367*(15), 1417-1427. doi: 10.1056/NEJMoa1115050
79. Camaj, P., Seeliger, H., Ischenko, I., Krebs, S., Blum, H., De Toni, E. N., . . . Bruns, C. J. (2009). EFEMP1 binds the EGF receptor and activates MAPK and Akt pathways in pancreatic carcinoma cells. *Biol Chem, 390*(12), 1293-1302. doi: 10.1515/bc.2009.140

80. Nandhu, M. S., Hu, B., Cole, S. E., Erdreich-Epstein, A., Rodriguez-Gil, D. J., & Viapiano, M. S. (2014). Novel paracrine modulation of Notch-DLL4 signaling by fibulin-3 promotes angiogenesis in high-grade gliomas. *Cancer Res, 74*(19), 5435-5448. doi: 10.1158/0008-5472.can-14-0685
81. Hu, B., Nandhu, M. S., Sim, H., Agudelo-Garcia, P. A., Saldivar, J. C., Dolan, C. E., . . . Viapiano, M. S. (2012). Fibulin-3 promotes glioma growth and resistance through a novel paracrine regulation of Notch signaling. *Cancer Res, 72*(15), 3873-3885. doi: 10.1158/0008-5472.can-12-1060
82. Arechederra, M., Priego, N., Vazquez-Carballo, A., Sequera, C., Gutierrez-Uzquiza, A., Cerezo-Guisado, M. I., . . . Porras, A. (2015). p38 MAPK down-regulates fibulin 3 expression through methylation of gene regulatory sequences: role in migration and invasion. *J Biol Chem, 290*(7), 4383-4397. doi: 10.1074/jbc.M114.582239
83. Davidson, B., Stavnes, H. T., Holth, A., Chen, X., Yang, Y., Shih Ie, M., & Wang, T. L. (2011). Gene expression signatures differentiate ovarian/peritoneal serous carcinoma from breast carcinoma in effusions. *J Cell Mol Med, 15*(3), 535-544. doi: 10.1111/j.1582-4934.2010.01023.x
84. Sternlicht, & Bergers, G. (2000). Matrix metalloproteinases as emerging targets in anticancer therapy: status and prospects. *Emerging Therapeutic Targets, 4*(5), 609-633.
85. Sternlicht, & Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol, 17*, 463-516. doi: 10.1146/annurev.cellbio.17.1.463
86. Lochter, A., Galosy, S., Muschler, J., Freedman, N., Werb, Z., & Bissell, M. J. (1997). Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol, 139*(7), 1861-1872.
87. Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H., & Itohara, S. (1998). Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res, 58*(5), 1048-1051.
88. Kim, J., Yu, W., Kovalski, K., & Ossowski, L. (1998). Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell, 94*(3), 353-362.
89. Bjorklund, M., & Koivunen, E. (2005). Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta, 1755*(1), 37-69. doi: 10.1016/j.bbcan.2005.03.001
90. Bauvois, B. (2012). New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression. *Biochim Biophys Acta, 1825*(1), 29-36. doi: 10.1016/j.bbcan.2011.10.001
91. Klein, T., & Bischoff, R. (2011). Physiology and pathophysiology of matrix metalloproteases. *Amino Acids, 41*(2), 271-290. doi: 10.1007/s00726-010-0689-x
92. Kupferman, M. E., Fini, M. E., Muller, W. J., Weber, R., Cheng, Y., & Muschel, R. J. (2000). Matrix metalloproteinase 9 promoter activity is induced coincident with invasion during tumor progression. *Am J Pathol, 157*(6), 1777-1783. doi: 10.1016/s0002-9440(10)64815-8
93. Yousef, E. M., Tahir, M. R., St-Pierre, Y., & Gaboury, L. A. (2014). MMP-9 expression varies according to molecular subtypes of breast cancer. *BMC Cancer, 14*, 609. doi: 10.1186/1471-2407-14-609

94. Lakka, S. S., Jasti, S. L., Gondi, C., Boyd, D., Chandrasekar, N., Dinh, D. H., . . . Rao, J. S. (2002). Downregulation of MMP-9 in ERK-mutated stable transfectants inhibits glioma invasion in vitro. *Oncogene, 21*(36), 5601-5608. doi: 10.1038/sj.onc.1205646
95. Moulik, S., Pal, S., Biswas, J., & Chatterjee, A. (2014). Role of ERK in Modulating MMP 2 and MMP 9 with Respect to Tumour Invasiveness in Human Cancer Cell Line MCF-7 and MDA-MB-231. *Journal of Tumor, 2*(2).
96. Kim, S., Choi, J. H., Lim, H. I., Lee, S. K., Kim, W. W., Cho, S., . . . Yang, J. H. (2009). EGF-induced MMP-9 expression is mediated by the JAK3/ERK pathway, but not by the JAK3/STAT-3 pathway in a SKBR3 breast cancer cell line. *Cell Signal, 21*(6), 892-898.
97. Yu, Q., & Stamenkovic, I. (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev, 14*(2), 163-176.
98. Redondo-Munoz, J., Ugarte-Berzal, E., Terol, M. J., Van den Steen, P. E., Hernandez del Cerro, M., Roderfeld, M., . . . Garcia-Pardo, A. (2010). Matrix metalloproteinase-9 promotes chronic lymphocytic leukemia b cell survival through its hemopexin domain. *Cancer Cell, 17*(2), 160-172. doi: 10.1016/j.ccr.2009.12.044
99. Bourguignon, L. Y., Gunja-Smith, Z., Iida, N., Zhu, H. B., Young, L. J., Muller, W. J., & Cardiff, R. D. (1998). CD44v(3,8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *J Cell Physiol, 176*(1), 206-215. doi: 10.1002/(sici)1097-4652(199807)176:1<206::aid-jcp22>3.0.co;2-3
100. Dufour, A., Zucker, S., Sampson, N. S., Kuscu, C., & Cao, J. (2010). Role of matrix metalloproteinase-9 dimers in cell migration: design of inhibitory peptides. *J Biol Chem, 285*(46), 35944-35956. doi: 10.1074/jbc.M109.091769
101. Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., . . . Hanahan, D. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol, 2*(10), 737-744. doi: 10.1038/35036374
102. Kessenbrock, K., Wang, C. Y., & Werb, Z. (2015). Matrix metalloproteinases in stem cell regulation and cancer. *Matrix Biol*. doi: 10.1016/j.matbio.2015.01.022
103. Bjorklund, M., Heikkila, P., & Koivunen, E. (2004). Peptide inhibition of catalytic and noncatalytic activities of matrix metalloproteinase-9 blocks tumor cell migration and invasion. *J Biol Chem, 279*(28), 29589-29597. doi: 10.1074/jbc.M401601200
104. Wu, Z. S., Wu, Q., Yang, J. H., Wang, H. Q., Ding, X. D., Yang, F., & Xu, X. C. (2008). Prognostic significance of MMP-9 and TIMP-1 serum and tissue expression in breast cancer. *Int J Cancer, 122*(9), 2050-2056. doi: 10.1002/ijc.23337
105. Lee, J. H., Miele, M. E., Hicks, D. J., Phillips, K. K., Trent, J. M., Weissman, B. E., & Welch, D. R. (1996). KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst, 88*(23), 1731-1737.
106. Welch, D. R., & Rinker-Schaeffer, C. W. (1999). What defines a useful marker of metastasis in human cancer? *J Natl Cancer Inst, 91*(16), 1351-1353.
107. Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., . . . Fujino, M. (2001). Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature, 411*(6837), 613-617. doi: 10.1038/35079135
108. Harihar, S., Pounds, K. M., Iwakuma, T., Seidah, N. G., & Welch, D. R. (2014). Furin is the major proprotein convertase required for KISS1-to-Kisspeptin processing. *PLoS One, 9*(1), e84958. doi: 10.1371/journal.pone.0084958

109. Pasquier, J., Kamech, N., Lafont, A. G., Vaudry, H., Rousseau, K., & Dufour, S. (2014). Molecular evolution of GPCRs: Kisspeptin/kisspeptin receptors. *J Mol Endocrinol, 52*(3), T101-117. doi: 10.1530/jme-13-0224
110. Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J. M., Le Poul, E., . . . Parmentier, M. (2001). The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem, 276*(37), 34631-34636. doi: 10.1074/jbc.M104847200
111. Muir, A. I., Chamberlain, L., Elshourbagy, N. A., Michalovich, D., Moore, D. J., Calamari, A., . . . Harrison, D. C. (2001). AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J Biol Chem, 276*(31), 28969-28975. doi: 10.1074/jbc.M102743200
112. Kirby, H. R., Maguire, J. J., Colledge, W. H., & Davenport, A. P. (2010). International Union of Basic and Clinical Pharmacology. LXXVII. Kisspeptin receptor nomenclature, distribution, and function. *Pharmacol Rev, 62*(4), 565-578. doi: 10.1124/pr.110.002774
113. Mead, E. J., Maguire, J. J., Kuc, R. E., & Davenport, A. P. (2007). Kisspeptins are novel potent vasoconstrictors in humans, with a discrete localization of their receptor, G protein-coupled receptor 54, to atherosclerosis-prone vessels. *Endocrinology, 148*(1), 140-147. doi: 10.1210/en.2006-0818
114. Szereszewski, J. M., Pampillo, M., Ahow, M. R., Offermanns, S., Bhattacharya, M., & Babwah, A. V. (2010). GPR54 regulates ERK1/2 activity and hypothalamic gene expression in a Galpha(q/11) and beta-arrestin-dependent manner. *PLoS One, 5*(9), e12964. doi: 10.1371/journal.pone.0012964
115. Pampillo, M., Camuso, N., Taylor, J. E., Szereszewski, J. M., Ahow, M. R., Zajac, M., . . . Babwah, A. V. (2009). Regulation of GPR54 signaling by GRK2 and {beta}-arrestin. *Mol Endocrinol, 23*(12), 2060-2074. doi: 10.1210/me.2009-0013
116. Seminara, S. B. (2007). Kisspeptin in reproduction. *Semin Reprod Med, 25*(5), 337-343. doi: 10.1055/s-2007-984739
117. Bianco, S. D., Vandepas, L., Correa-Medina, M., Gereben, B., Mukherjee, A., Kuohung, W., . . . Kaiser, U. B. (2011). KISS1R intracellular trafficking and degradation: effect of the Arg386Pro disease-associated mutation. *Endocrinology*.
118. Seminara, S. B., Messager, S., Chatzidaki, E. E., Thresher, R. R., Acierno, J. S., Jr., Shagoury, J. K., . . . Colledge, W. H. (2003). The GPR54 gene as a regulator of puberty. *N Engl J Med, 349*(17), 1614-1627. doi: 10.1056/NEJMoa035322
119. Maeda, K., Ohkura, S., Uenoyama, Y., Wakabayashi, Y., Oka, Y., Tsukamura, H., & Okamura, H. (2010). Neurobiological mechanisms underlying GnRH pulse generation by the hypothalamus. *Brain Res, 1364*, 103-115. doi: 10.1016/j.brainres.2010.10.026
120. Horikoshi, Y., Matsumoto, H., Takatsu, Y., Ohtaki, T., Kitada, C., Usuki, S., & Fujino, M. (2003). Dramatic elevation of plasma metastin concentrations in human pregnancy: metastin as a novel placenta-derived hormone in humans. *J Clin Endocrinol Metab, 88*(2), 914-919. doi: 10.1210/jc.2002-021235
121. Bilban, M., Ghaffari-Tabrizi, N., Hintermann, E., Bauer, S., Molzer, S., Zoratti, C., . . . Desoye, G. (2004). Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. *J Cell Sci, 117*(Pt 8), 1319-1328. doi: 10.1242/jcs.00971

122. Masui, T., Doi, R., Mori, T., Toyoda, E., Koizumi, M., Kami, K., . . . Imamura, M. (2004). Metastin and its variant forms suppress migration of pancreatic cancer cells. *Biochem Biophys Res Commun, 315*(1), 85-92. doi: 10.1016/j.bbrc.2004.01.021
123. Sanchez-Carbayo, M., Capodieci, P., & Cordon-Cardo, C. (2003). Tumor suppressor role of KiSS-1 in bladder cancer: loss of KiSS-1 expression is associated with bladder cancer progression and clinical outcome. *Am J Pathol, 162*(2), 609-617. doi: 10.1016/s0002-9440(10)63854-0
124. Jiang, Y., Berk, M., Singh, L. S., Tan, H., Yin, L., Powell, C. T., & Xu, Y. (2005). KiSS1 suppresses metastasis in human ovarian cancer via inhibition of protein kinase C alpha. *Clin Exp Metastasis, 22*(5), 369-376. doi: 10.1007/s10585-005-8186-4
125. Ikeguchi, M., Yamaguchi, K., & Kaibara, N. (2004). Clinical significance of the loss of KiSS-1 and orphan G-protein-coupled receptor (hOT7T175) gene expression in esophageal squamous cell carcinoma. *Clin Cancer Res, 10*(4), 1379-1383.
126. Zohrabian, V. M., Nandu, H., Gulati, N., Khitrov, G., Zhao, C., Mohan, A., . . . Jhanwar-Uniyal, M. (2007). Gene expression profiling of metastatic brain cancer. *Oncol Rep, 18*(2), 321-328.
127. Guan-Zhen, Y., Ying, C., Can-Rong, N., Guo-Dong, W., Jian-Xin, Q., & Jie-Jun, W. (2007). Reduced protein expression of metastasis-related genes (nm23, KISS1, KAI1 and p53) in lymph node and liver metastases of gastric cancer. *Int J Exp Pathol, 88*(3), 175-183. doi: 10.1111/j.1365-2613.2006.00510.x
128. Hata, K., Dhar, D. K., Watanabe, Y., Nakai, H., & Hoshiai, H. (2007). Expression of metastin and a G-protein-coupled receptor (AXOR12) in epithelial ovarian cancer. *Eur J Cancer, 43*(9), 1452-1459. doi: 10.1016/j.ejca.2007.03.004
129. Yan, C., Wang, H., & Boyd, D. D. (2001). KiSS-1 represses 92-kDa type IV collagenase expression by down-regulating NF-kappa B binding to the promoter as a consequence of Ikappa Balpha -induced block of p65/p50 nuclear translocation. *J Biol Chem, 276*(2), 1164-1172. doi: 10.1074/jbc.M008681200
130. Martin, T. A., Watkins, G., & Jiang, W. G. (2005). KiSS-1 expression in human breast cancer. *Clin Exp Metastasis, 22*(6), 503-511. doi: 10.1007/s10585-005-4180-0
131. Marot, D., Bieche, I., Aumas, C., Esselin, S., Bouquet, C., Vacher, S., . . . de Roux, N. (2007). High tumoral levels of Kiss1 and G-protein-coupled receptor 54 expression are correlated with poor prognosis of estrogen receptor-positive breast tumors. *Endocr Relat Cancer, 14*(3), 691-702. doi: 10.1677/erc-07-0012
132. Cho, S. G., Wang, Y., Rodriguez, M., Tan, K., Zhang, W., Luo, J., . . . Liu, M. (2011). Haploinsufficiency in the prometastasis Kiss1 receptor Gpr54 delays breast tumor initiation, progression, and lung metastasis. *Cancer Res, 71*(20), 6535-6546. doi: 10.1158/0008-5472.can-11-0329
133. Zajac, M., Law, J., Cvetkovic, D. D., Pampillo, M., McColl, L., Pape, C., . . . Bhattacharya, M. (2011). GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness. *PLoS One, 6*(6), e21599. doi: 10.1371/journal.pone.0021599
134. Cvetkovic, D., Dragan, M., Leith, S. J., Mir, Z. M., Leong, H. S., Pampillo, M., . . . Bhattacharya, M. (2013). KISS1R induces invasiveness of estrogen receptor-negative human mammary epithelial and breast cancer cells. *Endocrinology, 154*(6), 1999-2014. doi: 10.1210/en.2012-2164
135. Kovacs, J. J., Hara, M. R., Davenport, C. L., Kim, J., & Lefkowitz, R. J. (2009). Arrestin development: emerging roles for beta-arrestins in developmental signaling pathways. *Dev Cell, 17*(4), 443-458. doi: 10.1016/j.devcel.2009.09.011

136. Shenoy, S. K., Han, S., Zhao, Y. L., Hara, M. R., Oliver, T., Cao, Y., & Dewhirst, M. W. (2012). beta-arrestin1 mediates metastatic growth of breast cancer cells by facilitating HIF-1-dependent VEGF expression. *Oncogene, 31*(3), 282-292. doi: 10.1038/onc.2011.238
137. Mythreye, K., & Blobe, G. C. (2009). The type III TGF-beta receptor regulates epithelial and cancer cell migration through beta-arrestin2-mediated activation of Cdc42. *Proc Natl Acad Sci U S A, 106*(20), 8221-8226. doi: 10.1073/pnas.0812879106
138. Ma, L., & Pei, G. (2007). Beta-arrestin signaling and regulation of transcription. *J Cell Sci, 120*(Pt 2), 213-218. doi: 10.1242/jcs.03338
139. Matsubayashi, J., Takanashi, M., Oikawa, K., Fujita, K., Tanaka, M., Xu, M., . . . Mukai, K. (2008). Expression of G protein-coupled receptor kinase 4 is associated with breast cancer tumourigenesis. *J Pathol, 216*(3), 317-327. doi: 10.1002/path.2414
140. Lundgren, K., Tobin, N. P., Lehn, S., Stal, O., Ryden, L., Jirstrom, K., & Landberg, G. (2011). Stromal expression of beta-arrestin-1 predicts clinical outcome and tamoxifen response in breast cancer. *J Mol Diagn, 13*(3), 340-351. doi: 10.1016/j.jmoldx.2011.01.009
141. Li, T. T., Alemayehu, M., Aziziyeh, A. I., Pape, C., Pampillo, M., Postovit, L. M., . . . Bhattacharya, M. (2009). Beta-arrestin/Ral signaling regulates lysophosphatidic acid-mediated migration and invasion of human breast tumor cells. *Mol Cancer Res, 7*(7), 1064-1077. doi: 10.1158/1541-7786.mcr-08-0578
142. Alemayehu, M., Dragan, M., Pape, C., Siddiqui, I., Sacks, D. B., Di Guglielmo, G. M., . . . Bhattacharya, M. (2013). beta-Arrestin2 regulates lysophosphatidic acid-induced human breast tumor cell migration and invasion via Rap1 and IQGAP1. *PLoS One, 8*(2), e56174. doi: 10.1371/journal.pone.0056174

Chapter 2 – Methods

2.1 cBioPortal Data Mining

cBioPortal for Cancer Genomics (www.cbioportal.org), a publicly available online data portal to search and analyze cancer genomics data sets was used to investigate the genes of interest, *EFEMP1* and *KISS1*. The Breast Invasive Carcinoma (TCGA, Provisional) data set (n=962) was used for all data mining [1,2].

2.2 Blood Collection and Fibulin-3 ELISA

The study was approved by the Western's Research Ethics Boards, and all participants provided informed consent. Blood (5 mL) was collected in BD Vacutainer K2 EDTA tubes (VWR International; Radnor, PA, USA) from healthy patients, non-metastatic TNBC patients or metastatic TNBC patients presenting at The Breast Care Program of St. Joseph's Health Care London. Blood was centrifuged at 3000 rpm for 10 min, and the plasma was collected and frozen immediately in liquid nitrogen. Samples were stored at -80 ℃ and subsequently thawed to quantify plasma fibulin-3 concentrations using an enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science Inc.; Wuhan, Hubei, China) according to manufacturer's instructions [3]. Absorbance was measured at 450 nm using a Victor 3V Multi-Detection Microplate Reader (PerkinElmer; Waltham, MA, USA).

2.3 Cell Culture

Human breast cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and were maintained at 37 °C with 5% CO_2 (**Table 2.2**). The cell lines differ in invasiveness and ERα expression. MCF10A cells were cultured in Mammary Epithelial Basal Medium supplemented with a MEGM Single Quots kit (bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, gentamicin/amphotericin)

(Clonetics-Cambrex; Walkersville, MD, USA) and cholera toxin (100 ng/mL). MCF7 cells were grown in DMEM (Invitrogen; Carlsbad, CA, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Sigma-Aldrich; St. Louis, MO, USA) and 0.3% insulin. SKBR3, Hs578T and MDA-MB-231 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen; Carlsbad, CA, USA) supplemented with 10% (vol/vol) FBS.

2.4 Stable Transfections and Gene Knockdown

The weakly invasive SKBR3 breast cancer cells express very low levels of KISS1R [4], and therefore a gain-of-function model was generated by stably expressing FLAG-KISS1R or pFLAG (vector control) constructs, as previously described [4]. FLAG-KISS1R or pFLAG (vector control) constructs were also stably expressed in the mammary epithelial cell line MCF10A, as previously described [4]. Cells (1×10^6) were transfected by microporation (1700 V, 10 pulse width, 3#) using the Neon™ Transfection System (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Heterogeneous populations of stable transfectants were selected using medium containing Geneticin (1.5 µg/mL). Overexpression of KISS1R was verified by Western blot analysis.

Gene knockdown of β-arrestin1 and β-arrestin2 in MDA-MB-231 cells has been previously described [5,6]. Cells (5×10^6) were transfected with gene specific small hairpin ribonucleic acid (shRNA) (OriGene Technologies; Rockville, MD, USA) by electroporation (Gene Pulser Xcell, Bio-Rad; Hercules, CA, USA) according to the instructions of the manufacturer (250 V, 950 µF). Gene knockdown of fibulin-3 was achieved using lentivirus-delivered pGFP-C-shLenti shRNA Cloning Plasmid (Origene Technologies; Rockville, MD, USA). Heterogeneous populations of stable transfectants were selected using medium containing puromycin (1.5 µg/mL). Cells expressing scrambled controls were also

generated. Knockdown of fibulin-3, β-arrestin1 and β-arrestin2 was verified by Western blot analysis.

Table 2.2. Human cell models used in the study.

Human Mammary Epithelial Cells	Characteristics
MCF10A: non-malignant mammary epithelial	Non-invasive [7] ERα negative [8]
• **FLAG-KISS1R**	Highly invasive [4]
Human Breast Cancer Cells	
MCF7	Non- invasive [9] ERα positive [10]
SKBR3	Weakly invasive [11] ERα negative [11]
• **FLAG-KISS1R**	Highly invasive [4]
Hs578T	Invasive [12] ERα negative [12]
• **Fibulin-3 shRNA #1** • **Fibulin-3 shRNA #2**	
MDA-MB-231	Highly invasive [13] ERα negative [13]
• **β-arrestin1 shRNA** • **β-arrestin2 shRNA**	Reduced invasion [5] Reduced invasion [5]
• **Fibulin-3 shRNA #1** • **Fibulin-3 shRNA #2**	

Note: all generated cell lines are stable cell lines.

2.5 Immunoblot Assays

Cells were treated with KP-10 (100 nM) (Phoenix Pharmaceuticals; Burlingame, CA, USA) as determined by previous dose response studies [4,14], in FBS supplemented media for 72 hours, where applicable. Cells were washed with ice-cold phosphate-buffered saline (PBS), lysed with RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 8, 5 mM EDTA, 1% NP40, 0.1% SDS, 10 mM NaF, 0.5% deoxycholate, 10 mM sodium pyrophosphate, protease inhibitors: 1 µL/mL aprotinin, 1 µL/mL leupeptin and 10 µL/mL AEBSF) and centrifuged at 14,000 rpm at 4 °C for 20 minutes. Protein (50 µg) was separated by SDS-PAGE and transferred to nitrocellulose, followed by blocking in 10% skim milk and incubation with primary antibody in 3% skim milk overnight at 4 °C. Protein expression was studied using the following primary antibodies: mouse anti-β-arrestin1 (1:300; BD Transduction Laboratories; San Jose, CA, USA), mouse anti-β-arrestin2 (1:1000; Abcam; Cambridge, UK), rabbit anti-fibulin-3 (1:1000; Abcam; Cambridge, UK), rabbit anti-KISS1R (1:2000, Abcam, Cambridge, UK), rabbit anti-MMP-9 (1:500; Abcam; Cambridge, UK), rabbit anti-ERK1/2 (1:1000; Cell Signaling; Beverly, MA, USA), rabbit anti-phoshpo-ERK1/2 (1:2000; Cell Signaling; Beverly, MA, USA), rabbit anti-AKT (1:1000; Cell Signaling; Beverly, MA, USA), rabbit anti-phospho-AKT (1:1000; Cell Signaling; Beverly, MA, USA). β-Actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as a loading control and was examined with a rabbit anti-Actin (1:2000; Sigma-Aldrich; St. Louis, MO, USA), mouse anti-Actin (1:2000; GeneTex Inc.; Irvine, CA, USA) or rabbit anti-GAPDH (1:5000; GeneTex Inc.; Irvine, CA, USA). After 1 hour incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, rabbit (1:2500, GE Healthcare; Buckinghamshire, UK) or mouse (1:2500, GE Healthcare; Buckinghamshire, UK) the proteins were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo

Scientific; Waltham, MA, USA) and a VersaDoc Imaging System (Bio-Rad; Hercules, CA, USA).

2.6 Immunofluorescence Microscopy

Immunostaining was performed as previously described [4,6,14]. Breast cells were washed with Hank's Balanced Salt Solution (HBSS), fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton-X (room temperature, 20 min), followed by blocking in 3% bovine serum albumin in HBSS for 30 minutes. The primary antibody fibulin-3 (rabbit 1:50; Abcam; Cambridge, UK) was incubated for 1 hour. After washing in HBSS, cells were incubated with the secondary antibody anti-rabbit AlexaFluor 488. Nuclei were stained with 0.01% Hoechst 33258 (Invitrogen; Carlsbad, CA, USA) for 5 minutes, and then washed 5 times with cold HBSS. Two replicates were conducted for each condition and images were acquired by confocal microscopy using an LSM-510 META laser-scanning microscope (Zeiss; Jena, Germany).

2.7 Immunoblot Assays for Fibulin-3 Secretion

MDA-MB-231 and Hs578T cells (3×10^6) expressing fibulin-3 shRNA or scrambled controls were plated in a 10 cm culture dish, serum starved for 24 hours and then the conditioned media was collected. SKBR3 pFLAG and FLAG-KISS1R cells (3×10^6) were also plated in 10 cm culture dishes, serum starved for 24 hours and then stimulated with 100 nM KP-10 for 24 hours, following stimulation, conditioned media were collected. Conditioned media were concentrated using Microsep™ 10K Advance Centrifugal Filters (Pall® Life Sciences; Port Washington, NY, USA) and centrifuged at 5000 rcf at 4 °C for 30 minutes. Protein (75 µl) was separated by SDS-PAGE and transferred to nitrocellulose,

followed by blocking in 10% skim milk and incubation with rabbit anti-fibulin-3 (1:1000; Abcam; Cambridge, UK) primary antibody in 3% skim milk overnight at 4 °C. After 1 hour incubation with HRP-conjugated secondary antibody, the proteins were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific; Waltham, MA, USA) and a VersaDoc Imaging System (Bio-Rad; Hercules, CA, USA).

2.8 Scratch Assays for Motility

MDA-MB-231 and Hs578T cells expressing fibulin-3 shRNA or scrambled control were plated in duplicate and grown to 100% confluence in a 12-well plate, serum starved for 24 hours and subsequently scratched with a sterile pipette tip, as described previously [4,14]. Cells in FBS supplemented media were allowed to migrate into the scratch for 18 hours and were imaged every 15 minutes using an automated Olympus IX-81 microscope (Olympus; Shinjuku, Tokyo, Japan). Distance travelled was measured and analyzed using InVivo Analyzer Suite (Media Cybernetics; Rockville, MD, USA). For each image taken (per time point), the width of the scratch (μm) was measured at seven points along the scratch. The distance migrated was calculated by subtracting the width of the scratch at each time point from the width of the scratch at time zero. The distances migrated at each of the seven points along the scratch were averaged to determine the distance migrated for each well.

2.9 Cell Migration and Invasion Assays

Transwell chamber migration and Matrigel invasion assays were conducted as previously stated [4,14], with MDA-MB-231 and Hs578T cells depleted of fibulin-3 or scrambled control. Transwell filters (8 μm pores) were placed into a 24-well plate containing either serum-free RPMI 1640 media or media supplemented with 10% FBS. Cells (4×10^4)

were plated in the upper chamber in either serum-free media or serum-free media supplemented with 100 nM KP-10 (Phoenix Pharmaceuticals; Burlingame, CA, USA) as determined by previous studies [4,14] and incubated for 20 hours. For invasion assays, cells were layered on top of a 1 in 10 dilution of Matrigel (8.0 mg/mL; BD Biosciences; Franklin Lakes, NJ, USA) dissolved in serum-free media and incubated for 20 hours. Cells were then fixed with a 20% acetone: 80% methanol solution and nuclei stained with 0.1% Hoechst 33258 (Invitrogen; Carlsbad, CA, USA). Two replicates were conducted for each condition and 12 fields (migration) or 24 fields (invasion) in each replicate were imaged and counted using an Olympus IX-71 inverted microscope (Olympus; Shinjuku, Tokyo, Japan), using InVivo Analyzer Suite (Media Cybernetics; Rockville, MD, USA). 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% FBS-supplemented medium).

2.10 Three-Dimensional (3D) Invasion Assays

3D invasion assays were conducted as described previously [4,14]. MDA-MB-231 and Hs578T cells depleted of fibulin-3 or scrambled control (2.5×10^4) were seeded in a 1:1 dilution of phenol red-free Matrigel (8.0 mg/mL; BD Biosciences; Franklin Lakes, NJ, USA) and culture medium on Matrigel-coated 35 mm glass-bottomed culture dishes (MatTek; Ashland, MA, USA). Cultures were maintained and imaged for 5 days. Images were taken with an Olympus IX-81 microscope (Olympus; Shinjuku, Tokyo, Japan), using InVivo Analyzer Suite (Media Cybernetics; Rockville, MD, USA). Cell colonies were scored blindly as being either stellate or spheroidal. A colony is deemed to be stellate if one or more projections from the central sphere of a colony of cells are observed.

2.11 Zymography

Zymographic analysis was performed as described previously [15]. MDA-MB-231 cells expressing fibulin-3 shRNA or scrambled control and SKBR3 cells expressing pFLAG and FLAG-KISS1R vectors (1×10^6) were plated in a 6-well plate. Subsequently, cells were serum starved for 24 hours and the media were collected. Cells (2.5×10^5) were serum starved for 24 hours and then stimulated with 100 nM KP-10 for 24 hours. Separately, cells (2.5×10^5) were serum starved for 24 hours and then stimulated with 500 nM EGFR inhibitor AG1478 (Millipore; Billerica, MA, USA) for 24 hours. Additionally, cells (2.5×10^5) serum starved for 24 hours were pre-treated for 30 minutes with the following drugs prior to stimulation with 100 nM KP-10 for 24 hours: 20 μM active ERK1/2 inhibitor UO126 (Millipore; Billerica, MA, USA), 20 μM inactive analog UO124 (Millipore; Billerica, MA, USA) [16], or vehicle dimethyl sulfoxide (DMSO). After the 24 hour stimulation period, media were collected. Samples were centrifuged at 350 rcf for 5 minutes to remove cellular debris. Samples were combined 1:1 with sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, glycerol, 1% bromophenol blue) and 40 μL of sample was loaded onto a 10% SDS-PAGE separating gel copolymerized with 0.1% gelatin. The gels were then washed in 2.5% Triton X-100 to remove the SDS and renature the MMP proteins. The gels were then incubated for 48 hours at 37 °C with gentle agitation in developing buffer (1 M Tris, 5M NaCl, 1M CaCl2 (anhydrous), 66.67 μl of 30% Brij-35, pH buffered to 7.4 with 6 N HCl) to allow enzymatic digestion of the gelatin. Following incubation, the gels were stained with Coomassie blue, destained, and imaged using the VersaDoc imaging system (Bio-Rad; Hercules, CA, USA).

2.12 EGFR Immunoprecipitation

Immunoprecipitation experiments were performed as previously described [4,14]. MDA-MB-231 and Hs578T cells expressing either fibulin-3 shRNA or scrambled control were cultured to 60% confluency, serum starved for 24 hours and treated with 20 ng/mL of EGF for 15 minutes where indicated. Cells were washed with ice-cold PBS and lysed in glycerol lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 2 mM Na3VO4, 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 (450 µg of total protein) were used for immunoprecipitation studies. EGFR was immunoprecipitated from total lysates using anti-EGFR antibody (4 µg, Millipore; Billerica, MA, USA), incubated at 4 °C overnight. Immunoprecipitated proteins were then pulled-down with protein G Dynabeads (Life Technologies; Carlsbad, CA, USA) and resolved by Western blot analysis. Phosphorylated EGFR was determined using an anti-mouse antiphosphotyrosine antibody (PY-20, Santa Cruz; Dallas, TX, USA) at 1:1000. Total EGFR was determined using an anti-EGFR antibody (1:4000, Millipore; Billerica, MA, USA). After 1 hour incubation with HRP-conjugated secondary antibody, the proteins were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific; Waltham, MA, USA) and a VersaDoc Imaging System (Bio-Rad; Hercules, CA, USA).

2.13 MTT Cell Viability Assays

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays were conducted as per manufacturer's instructions using a cell proliferation kit I (MTT) (Roche; Basel, Switzerland) to determine if depletion of endogenous fibulin-3 had any effect on cell viability. MDA-MB-231 and Hs578T cells expressing fibulin-3 shRNA or

scrambled control were plated in triplicates (5×10^4) on a 96-well plate. MTT labeling reagent (10 µl) was added to each well (final concentration 0.5 mg/ml) and cells were incubated for 4 hours at 37°C and 5% CO_2. Subsequently, solubilization solution (10% SDS in 0.01 M HCl, 100 µl) was added to each well and incubated overnight at 37°C and 5% CO_2. Absorbance was then measured at 595 nm using a Victor 3V Multi-Detection Microplate Reader (PerkinElmer) with a background subtraction at 750 nm.

2.14 Cell Growth Assays

To determine if shRNA expression had any impact on cell growth rate, MDA-MB-231 or Hs578T cells (4×10^5) expressing either fibulin-3 shRNA or scrambled control were plated in 60 mm culture plates. Cells were trypsinized and counted using a haemocytometer at 24 hour intervals (24, 48 and 72 h).

2.15 Statistical Analysis

Student's t-test, one-way analysis of variance (ANOVA) or two-way ANOVA with a Dunnett's or Bonferroni's post-hoc test were performed using GraphPad Prism 5 (GraphPad Software, Inc.; La Jolla, CA, USA). Differences were considered statistically significant at $P<0.05$.

2.16 References

1. Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., . . . Schultz, N. (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov, 2*(5), 401-404.
2. Gao, J., Aksoy, B. A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S. O., . . . Schultz, N. (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal, 6*(269), pl1.
3. Pass, H. I., Levin, S. M., Harbut, M. R., Melamed, J., Chiriboga, L., Donington, J., . . . Goparaju, C. (2012). Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. *N Engl J Med, 367*(15), 1417-1427.
4. Cvetkovic, D., Dragan, M., Leith, S. J., Mir, Z. M., Leong, H. S., Pampillo, M., . . . Bhattacharya, M. (2013). KISS1R induces invasiveness of estrogen receptor-negative human mammary epithelial and breast cancer cells. *Endocrinology, 154*(6), 1999-2014.
5. Li, T. T., Alemayehu, M., Aziziyeh, A. I., Pape, C., Pampillo, M., Postovit, L. M., . . . Bhattacharya, M. (2009). Beta-arrestin/Ral signaling regulates lysophosphatidic acid-mediated migration and invasion of human breast tumor cells. *Mol Cancer Res, 7*(7), 1064-1077.
6. Alemayehu, M., Dragan, M., Pape, C., Siddiqui, I., Sacks, D. B., Di Guglielmo, G. M., . . . Bhattacharya, M. (2013). beta-Arrestin2 regulates lysophosphatidic acid-induced human breast tumor cell migration and invasion via Rap1 and IQGAP1. *PLoS One, 8*(2), e56174.
7. Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., . . . Brooks, S. C. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res, 50*(18), 6075-6086.
8. Petersen, O. W., & Polyak, K. (2010). Stem cells in the human breast. *Cold Spring Harb Perspect Biol, 2*(5), a003160.
9. Soule, H. D., Vazguez, J., Long, A., Albert, S., & Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst, 51*(5), 1409-1416.
10. Brooks, S. C., Locke, E. R., & Soule, H. D. (1973). Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. *J Biol Chem, 248*(17), 6251-6253.
11. Trempe, G. L. (1976). Human breast cancer in culture. *Recent Results Cancer Res*(57), 33-41.
12. Hughes, Linda, Malone, Catherine, Chumsri, Saranya, Burger, AngelikaM, & McDonnell, Susan. (2008). Characterisation of breast cancer cell lines and establishment of a novel isogenic subclone to study migration, invasion and tumourigenicity. *Clinical & Experimental Metastasis, 25*(5), 549-557.
13. Cailleau, R., Young, R., Olive, M., & Reeves, W. J., Jr. (1974). Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst, 53*(3), 661-674.
14. Zajac, M., Law, J., Cvetkovic, D. D., Pampillo, M., McColl, L., Pape, C., . . . Bhattacharya, M. (2011). GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness. *PLoS One, 6*(6), e21599.

15. Walsh, L. A., Cepeda, M. A., & Damjanovski, S. (2012). Analysis of the MMP-dependent and independent functions of tissue inhibitor of metalloproteinase-2 on the invasiveness of breast cancer cells. *J Cell Commun Signal*, *6*(2), 87-95.
16. Li, D., Yallowitz, A., Ozog, L., & Marchenko, N. (2014). A gain-of-function mutant p53-HSF1 feed forward circuit governs adaptation of cancer cells to proteotoxic stress. *Cell Death Dis, 5,* e1194.

Chapter 3 – Results

3.1 Plasma fibulin-3 levels increase in metastatic TNBC patients

To date, fibulin-3 has been proposed as a plasma and effusion biomarker in malignant mesothelioma [1], however whether plasma fibulin-3 concentrations increase in breast cancer is unknown. Thus, we investigated whether plasma fibulin-3 concentrations are increased in metastatic TNBC patients compared to non-metastatic TNBC patients and healthy controls using a fibulin-3 ELISA. The metastatic TNBC cohort (n=7) displayed significantly higher plasma fibulin-3 concentrations than those of the healthy control (n=6) group (**Figure 3.1**). This study is ongoing as we acquire more samples from patients presenting at The Breast Care Program of St. Joseph's Health Care London, however our data suggests that plasma fibulin-3 levels are increased in metastatic TNBC patients.

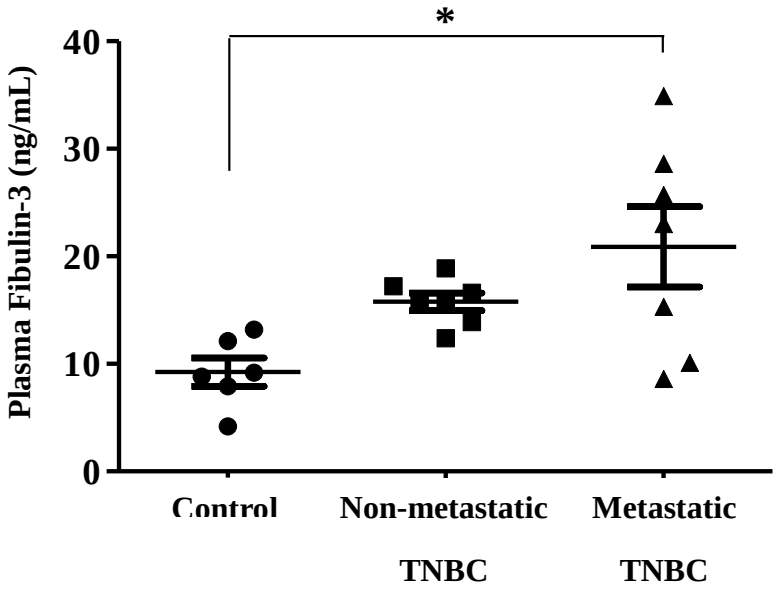

Figure 3.1. Plasma fibulin-3 levels in TNBC patients. Fresh blood samples were taken from healthy patients (n=6) patients with early triple negative breast cancer (n=8) and metastatic triple negative breast cancer patients (n=7). Plasma fibulin-3 levels (ng/mL) were measured by ELISA. One-way ANOVA followed by Bonferroni's multiple comparison test: *, $P<0.05$.

3.2 Altered *EFEMP1* and *KISS1* expression in invasive breast carcinoma

Genetic alterations have been shown to influence tumor development and progression, and therefore identification of these alterations is important for further understanding the malignant process and for the development of targeted therapies [2]. To investigate if the genes encoding our proteins of interest were altered in cancer patients, specifically in invasive breast cancer patients, we used cBioPortal, an online data portal to search and analyze cancer genomic data sets. Explorative analysis was performed on the fibulin-3 gene (*EFEMP1*), and the gene encoding KPs (*KISS1*) to determine if there was amplification, mRNA upregulation, and mutation of the genes. Pathology reports for invasive breast carcinoma patients were also examined, however due to incompleteness, the hormone receptor status of the majority of these patients remains unknown.

Cross-cancer gene alteration summaries for *KISS1* and *EFEMP1* were generated by cBioPortal. In breast invasive carcinoma, *KISS1* is amplified in 27.6% (8/29 cases; BCCRC Patient Xenograft British Columbia, Nature 2014) [3] and 13.6% (131/962 cases; TCGA, Provisional) (**Figure 3.2A**). Invasive breast cancer displayed the greatest alteration frequency of *EFEMP1* compared to other cancers (**Figure 3.2B**). In breast invasive carcinoma, *EFEMP1* is amplified 13.8% (4/29 cases; BCCRC Patient Xenograft British Columbia, Nature 2014) [3].

Additionally, the Breast Invasive Carcinoma (TCGA, Provisional) dataset was used to generate a Kaplan–Meier overall survival curve (n=849) for both genes (**Figure 3.3**). Patients with amplified *EFEMP1* (1.5%; 14/962 cases) showed no significant difference in survival compared to patients with unaltered *EFEMP1*. Patients with amplified *KISS1* (13.6%; 131/962 cases) displayed a drastic drop (approaching statistical significance) in

overall survival compared to those patients without amplification of the gene. Therefore, amplification of *KISS1* in invasive breast carcinoma appears to influence patient survival.

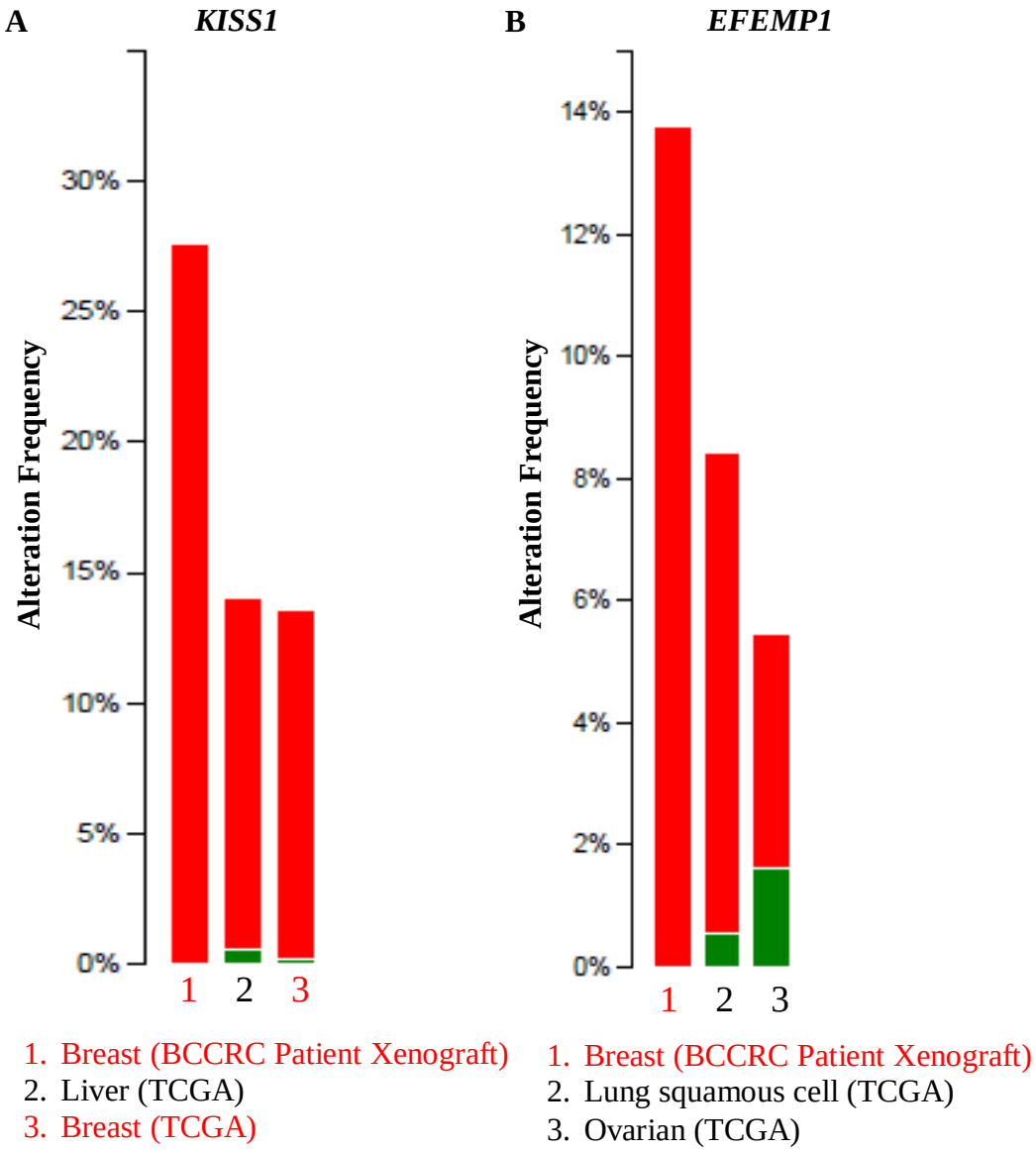

Figure 3.2. Cross-cancer gene alteration summaries for *KISS1* and *EFEMP1*. (**A**) Cross-cancer gene alteration summary for *KISS1*; only top three cancers are shown. In breast invasive carcinoma, *KISS1* is amplified in 27.6% (8/29 cases; BCCRC Patient Xenograft British Columbia, Nature 2014) and 13.6% (131/962 cases; TCGA, Provisional) based on Z-score threshold of 2.0. (**B**) Cross-cancer gene alteration summary for *EFEMP1;* only top three cancers are shown. In breast invasive carcinoma, *EFEMP1* is amplified in 13.8% (4/29 cases; BCCRC Patient Xenograft British Columbia, Nature 2014) based on Z-score threshold of 2.0. Red: gene amplification, green: gene mutation.

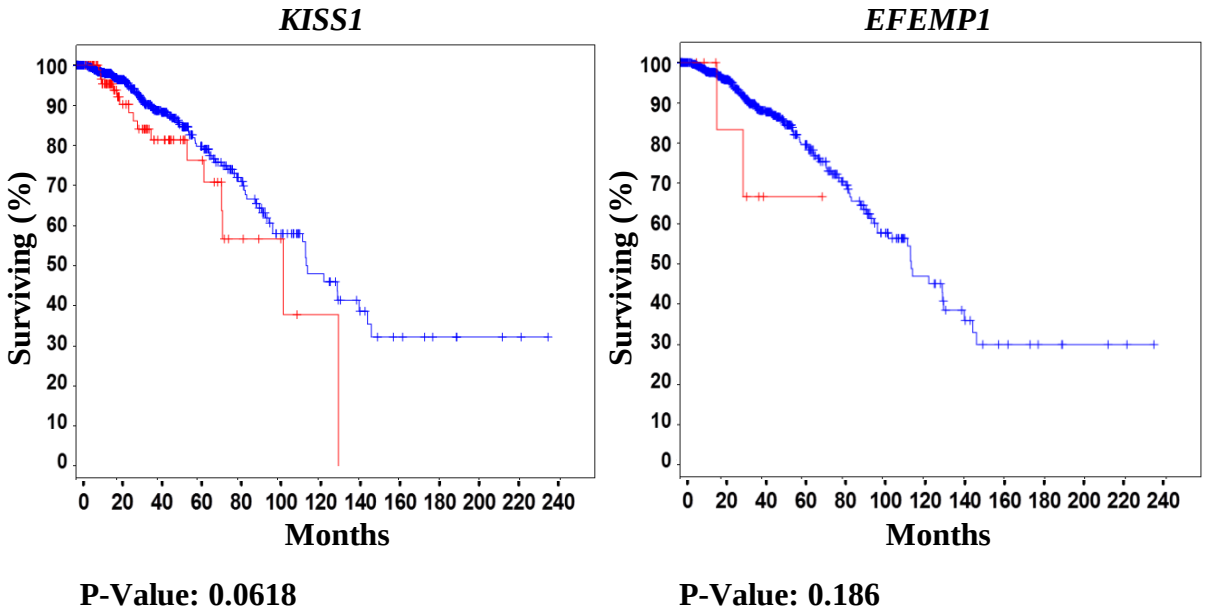

Figure 3.3. Kaplan–Meier overall survival curves comparing cases of gene alteration to cases without gene alteration for *KISS1* and *EFEMP1*. cBioPortal explorative data analysis was performed on both genes using the Breast Invasive Carcinoma (TCGA, Provisional) dataset and Kaplan–Meier overall survival curves (n=849) were generated. P-values were calculated by the log-rank test.

3.3 Fibulin-3 and KISS1R expression in breast cell lines

Although fibulin-3 mRNA was found to be elevated in breast cancer effusions [4], a role for fibulin-3 in regulating breast cancer invasion is unknown. We first examined fibulin-3 protein expression in mammary epithelial cells and breast cancer cell lines by western blotting. We found that the expression of endogenous fibulin-3 was higher in the highly metastatic TNBC MDA-MB-231 cells compared to less invasive SKBR3 pFLAG control cells, non-invasive cells (ERα+) MCF7 cells or non-malignant MCF10A cells (**Figure 3.4**). The ERα-negative non-malignant MCF10A mammary epithelial cells are non-motile and non-invasive [5,6]. We have previously shown that KP-10 treatment or overexpression of KISS1R in the MCF10A cells stimulates EMT and increases invasiveness [7]. Additionally, expression of KISS1R in the ERα-negative SKBR3 cells resulted in EMT, increased cell invasiveness and increased extravasation *in vivo* [7]. Our results demonstrated that expression of KISS1R in the non-malignant MCF10A and the ERα-negative SKBR3 breast cancer cells increased the expression of endogenous fibulin-3. This pattern of fibulin-3 expression was also observed by immunofluorescence microscopy (**Figure 3.5**). As previously reported [7], KISS1R protein expression was high in the highly invasive breast cells (SKBR3 FLAG-KISS1R, MDA-MB-231) compared with the less invasive and non-invasive cell lines. Hence, our results indicate that fibulin-3 may play a role in breast cancer cell invasion, and that KISS1R may regulate the expression of fibulin-3.

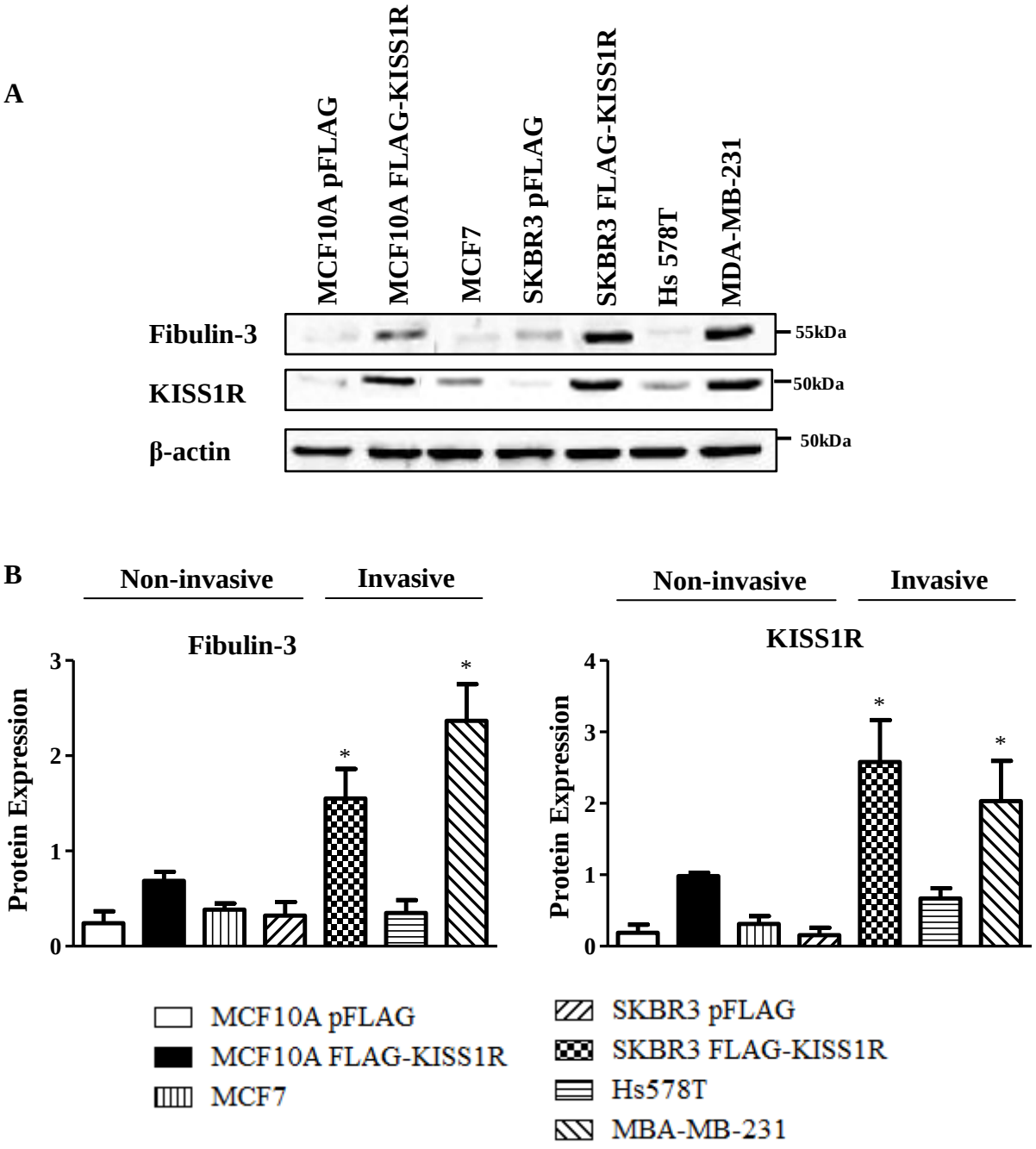

Figure 3.4. Expression of fibulin-3 and KISS1R in breast cell lines. (**A**) Representative western blots and (**B**) densitometric analysis of endogenous fibulin-3 (n=3) and KISS1R (n=4) in various breast cell lines. One-way ANOVA followed by Dunnett's post hoc test: *, P<0.05 compared to MCF10A pFLAG. β-actin, *loading control*. Columns represent protein expression ± standard error of the mean (SEM).

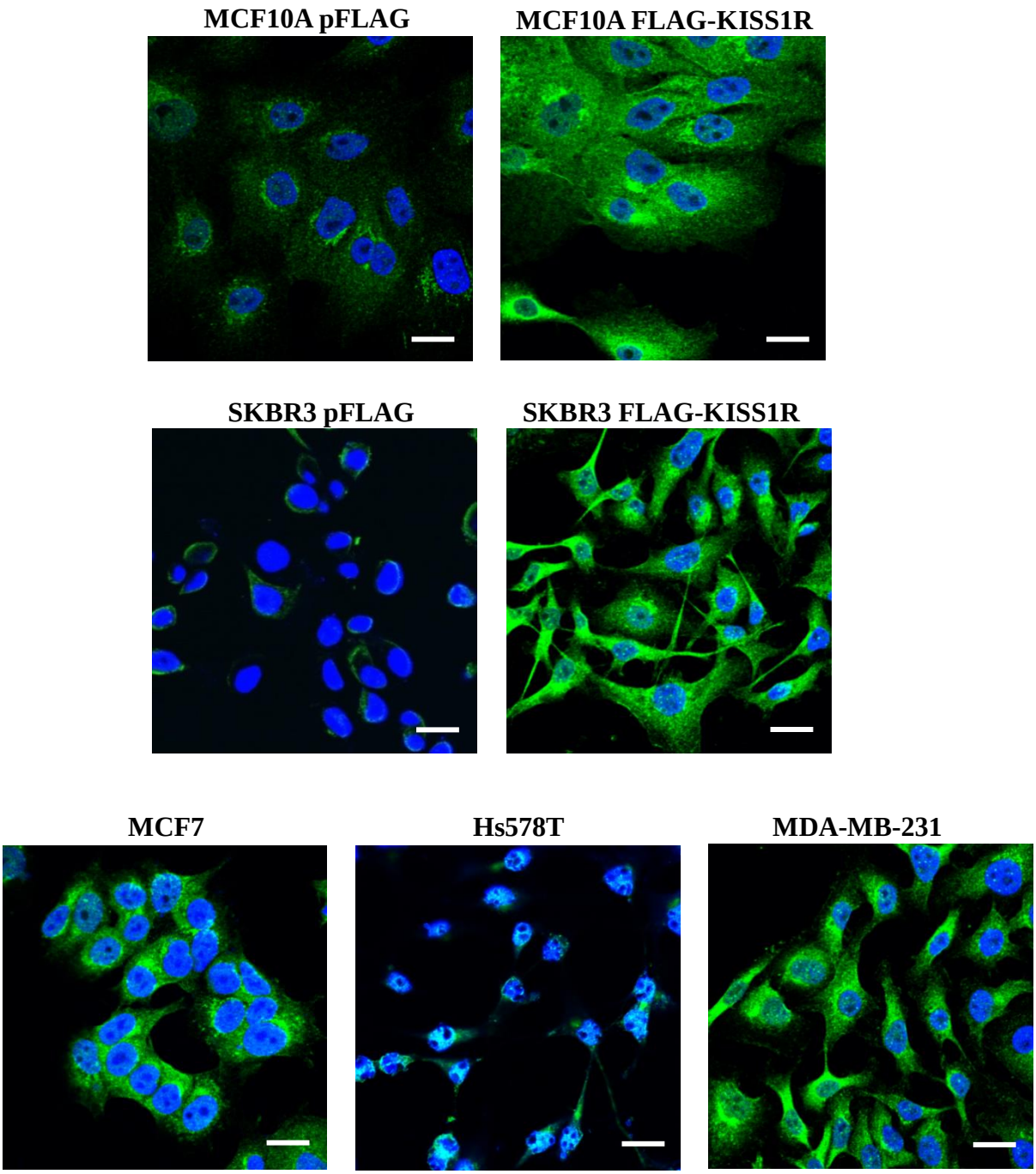

Figure 3.5. Endogenous fibulin-3 expression in breast cells. Representative confocal micrographs of various breast cell lines (n=4) immunostained with anti-fibulin-3 (rabbit) followed by AlexaFluor 488 (green). Nuclei stained with Hoechst 33258. Scale bar = 20 μm.

3.4 Kisspeptin-10 signaling stimulates fibulin-3 expression and secretion in ERα-negative breast cancer cells

Next, to investigate if activation of KISS1R promotes fibulin-3 expression we treated ERα-negative breast cancer cells with 100 nM kisspeptin-10, as determined by previous dose response studies [7,8], and examined fibulin-3 protein expression in cell lysates, as well as concentrated conditioned media by western blotting. Treatment of breast cancer cells with KP-10 (100 nM, 72 hr), significantly increased fibulin-3 protein levels compared to unstimulated cells, as determined by western blot analysis (**Figure 3.6**). Additionally, since fibulin-3 is a secreted protein, we also examined the ability of KP-10 to stimulate fibulin-3 secretion in ERα-negative breast cancer cells. KP-10 treatment also increased fibulin-3 protein levels in conditioned media (**Figure 3.7**). Therefore, KISS1R signaling regulates fibulin-3 expression and secretion in ERα-negative breast cancer cells.

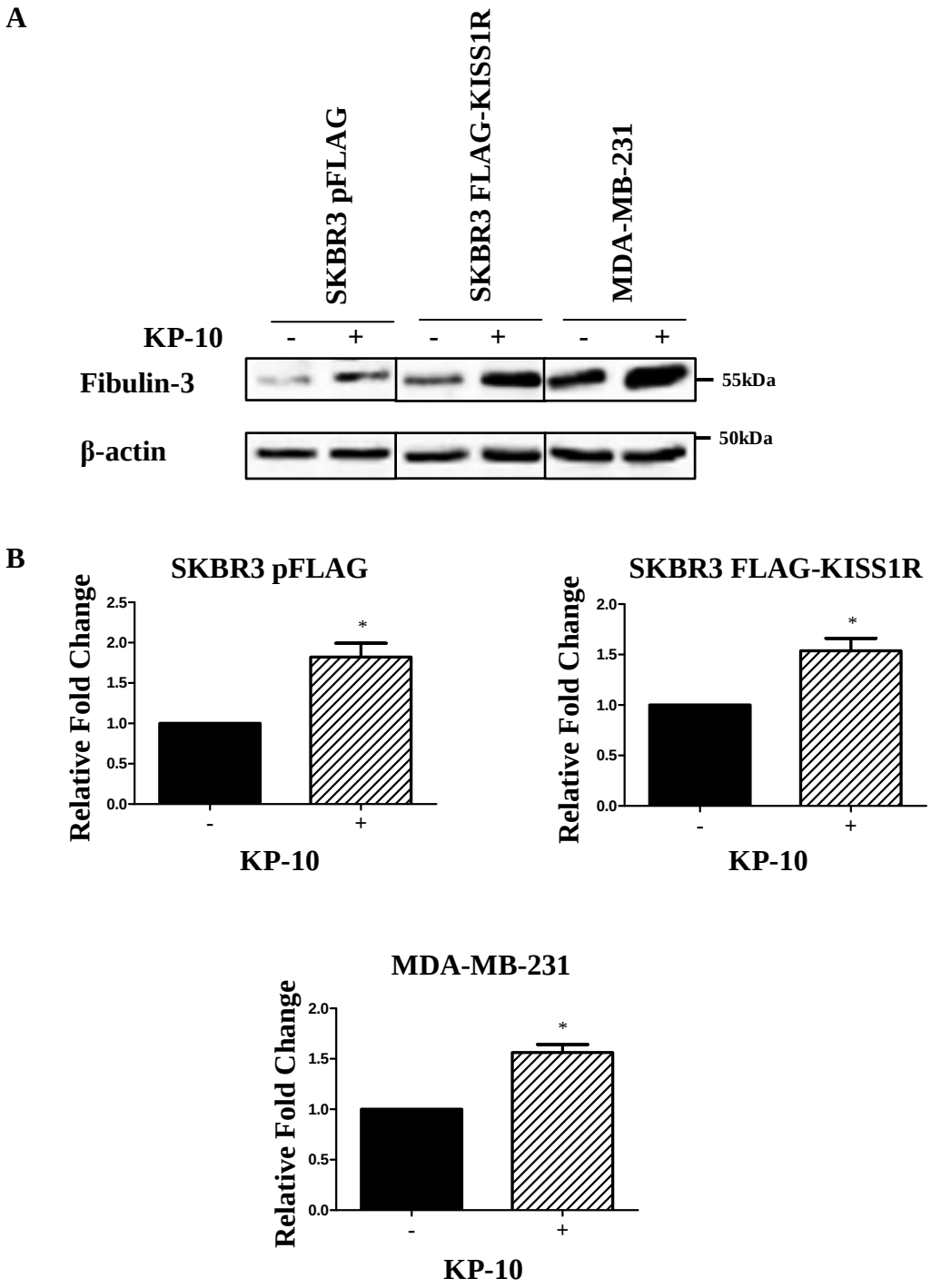

Figure 3.6. KP-10 stimulates the expression of fibulin-3 in ERα-negative breast cancer cells. (**A**) Representative western blot showing expression levels of fibulin-3 in ER-α negative breast cells unstimulated or after 100 nM kisspeptin-10 in complete media treatment for 72 hours. (**B**) Densitometric analysis of fibulin-3 (n=3) expression levels following kisspeptin-10 stimulation; β-actin, *loading control*. Student's t-test: *, P<0.05. Columns represent protein expression ± SEM.

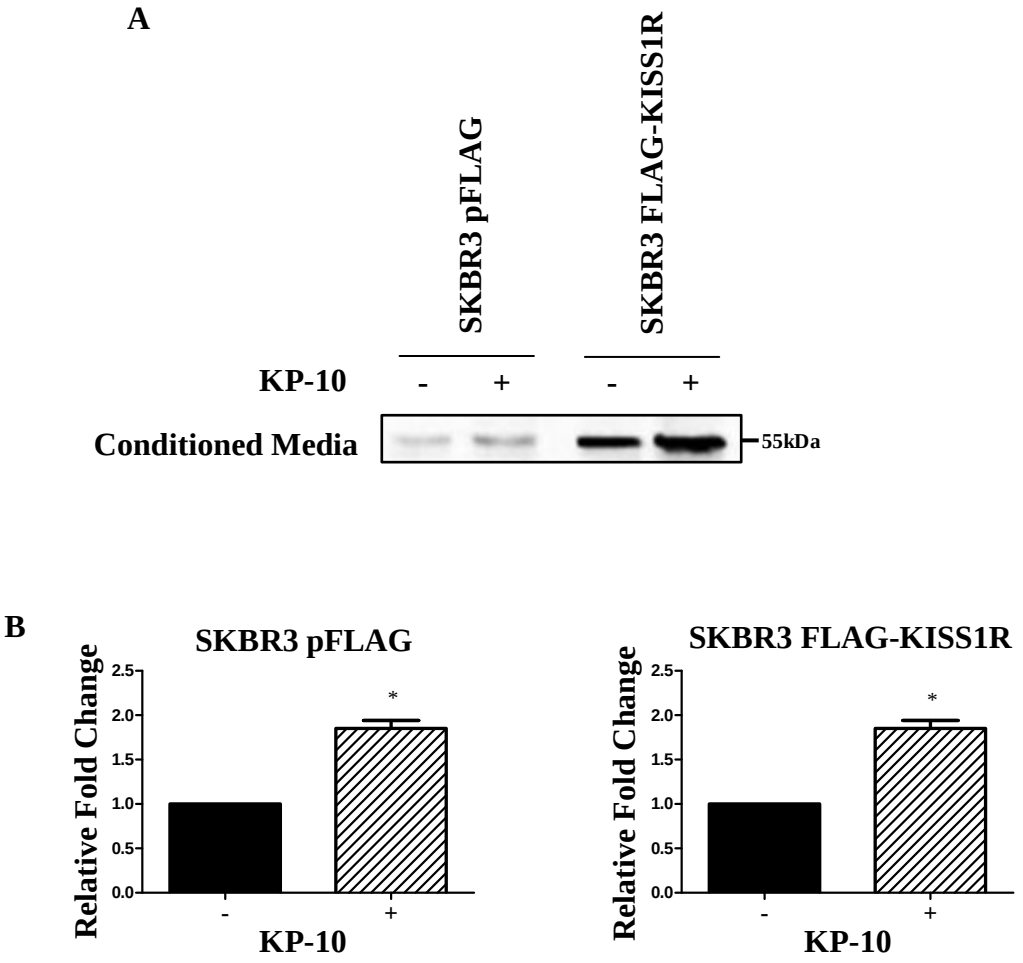

Figure 3.7. KP-10 stimulates the secretion of fibulin-3 in ERα-negative breast cancer cells. (**A**) Representative western blot showing fibulin-3 in conditioned media from ER-α negative breast cells unstimulated or after 100 nM kisspeptin-10 in serum free media treatment for 24 hours. (**B**) Densitometric analysis of fibulin-3 (n=3) levels following kisspeptin-10 stimulation. Student's t-test: *, $P<0.05$. Columns represent protein expression ± SEM.

3.5 β-arrestins regulate the expression of fibulin-3 protein in TNBC cells

We have previously shown that β-arrestin2 regulates KP-10 induced EGFR activation and cell invasion in TNBC cells [8]. Thus, we determined whether changes in β-arrestin protein levels altered fibulin-3 expression levels by western blotting. We observed that depletion of endogenous β-arrestin1 or β-arrestin2 in MDA-MB-231 cells decreased the expression of fibulin-3 protein levels, compared to cells expressing scrambled controls (**Figure 3.8**). This data suggests that β-arrestins also regulate the expression of fibulin-3 in TNBC cells.

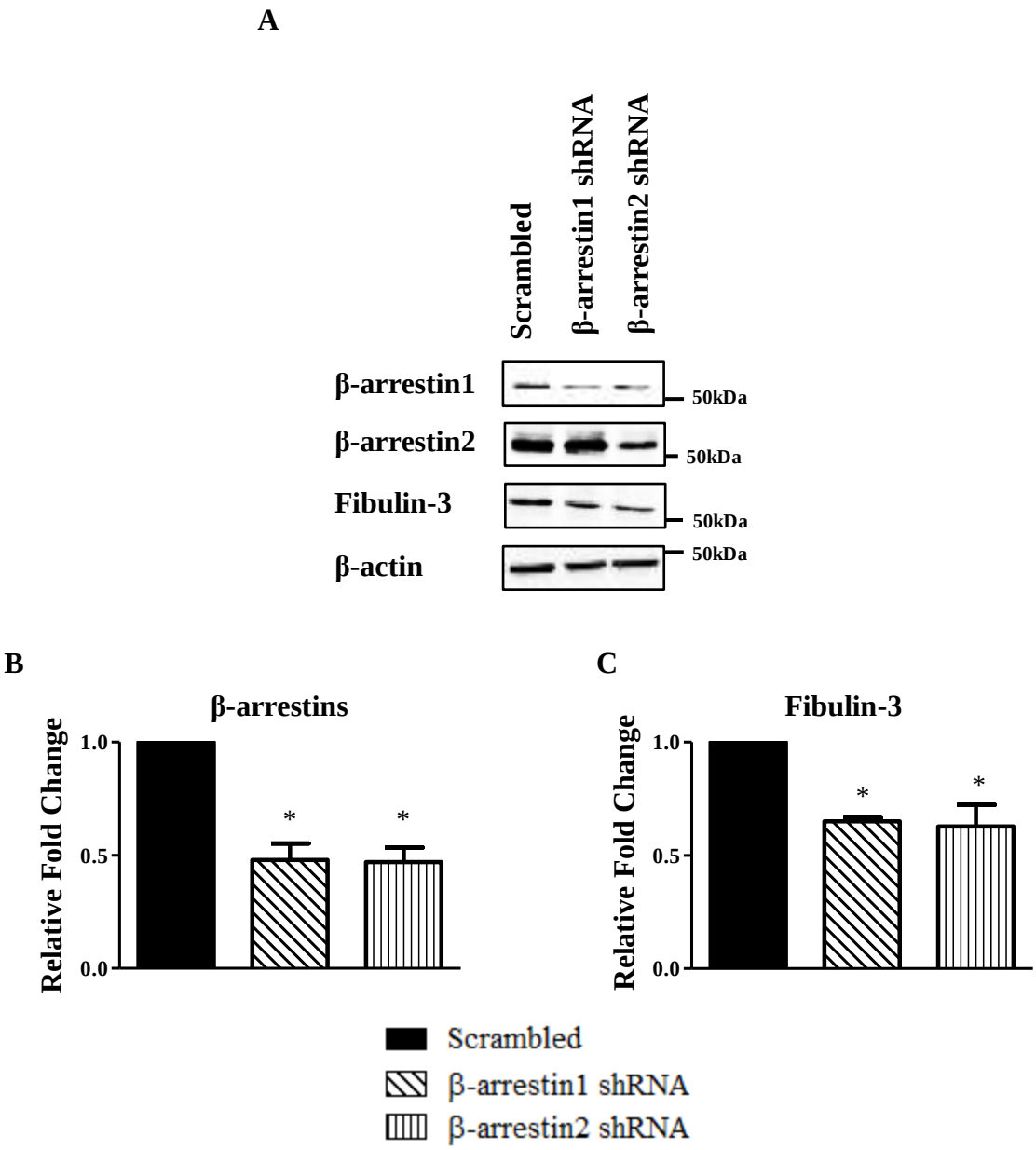

Figure 3.8. β-arrestins regulate the expression of fibulin-3 protein in TNBC cells. (**A**) Representative western blot and densitometric analysis of (**B**) β-arrestin 1 (n=8), and β-arrestin 2 (n=8) and (**C**) fibulin-3 (n=3) in MDA-MB-231 cells expressing β-arrestin1 or β-arrestin2 shRNA or scrambled control; β-actin, *loading control*. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$ compared to MDA-MB-231 scrambled. Columns represent protein expression ± SEM.

3.6 Depletion of fibulin-3 diminishes TNBC cell migration

Metastasis is dependent on the ability of cancer cells to invade the surounding tissue and the cell's ability to migrate away from the primary tumor [9,10]. To examine a potential mechanism by which fibulin-3 regulates TNBC cell migration, endogenous fibulin-3 was depleted in the invasive TNBC MDA-MB-231 and Hs578T cell lines using lentivirus-delivered shRNA and knockdown was verified by western blot (**Figure 3.9A, B**). Depletion of fibulin-3 had no effect on cell viability (**Figure 3.9C**). Additionally, we examined if secretion of fibulin-3 was also diminished in MDA-MB-231 and Hs578T cells stably expressing fibulin-3 shRNA, and observed that it was significantly depleted (**Figure 3.10**). To assess a role of fibulin-3 in TNBC cell migration, we first used scratch motility assays, as previously conducted [7,8,11]. Data showed a significant decrease in the distance migrated by cells depleted of endogenous fibulin-3 compared to the scrambled controls (**Figure 3.11**). Cell migration was also measured using transwell chamber migration assays, as previously described [7,8,11,12]. MDA-MB-231 cells depleted of fibulin-3 or scrambled control were seeded in serum-free media or serum-free media containing 100 nM KP-10 and allowed to migrate across the transwell migration membrane towards serum-free or FBS-supplemented media for 20 hours. The depletion of fibulin-3 significantly decreased the ability of cells to migrate across the membrane under basal and KP-10 stimulated conditions (**Figure 3.12**). Additionally, cell growth assays demonstrated that there were no differences in cell proliferation, indicating that the observed differences in scratch closure and transwell migration assays were a result of changes in cell migration and not differences in cell proliferation (**Figure 3.13**). Therefore, these results indicate that fibulin-3 regulates TNBC cell migration.

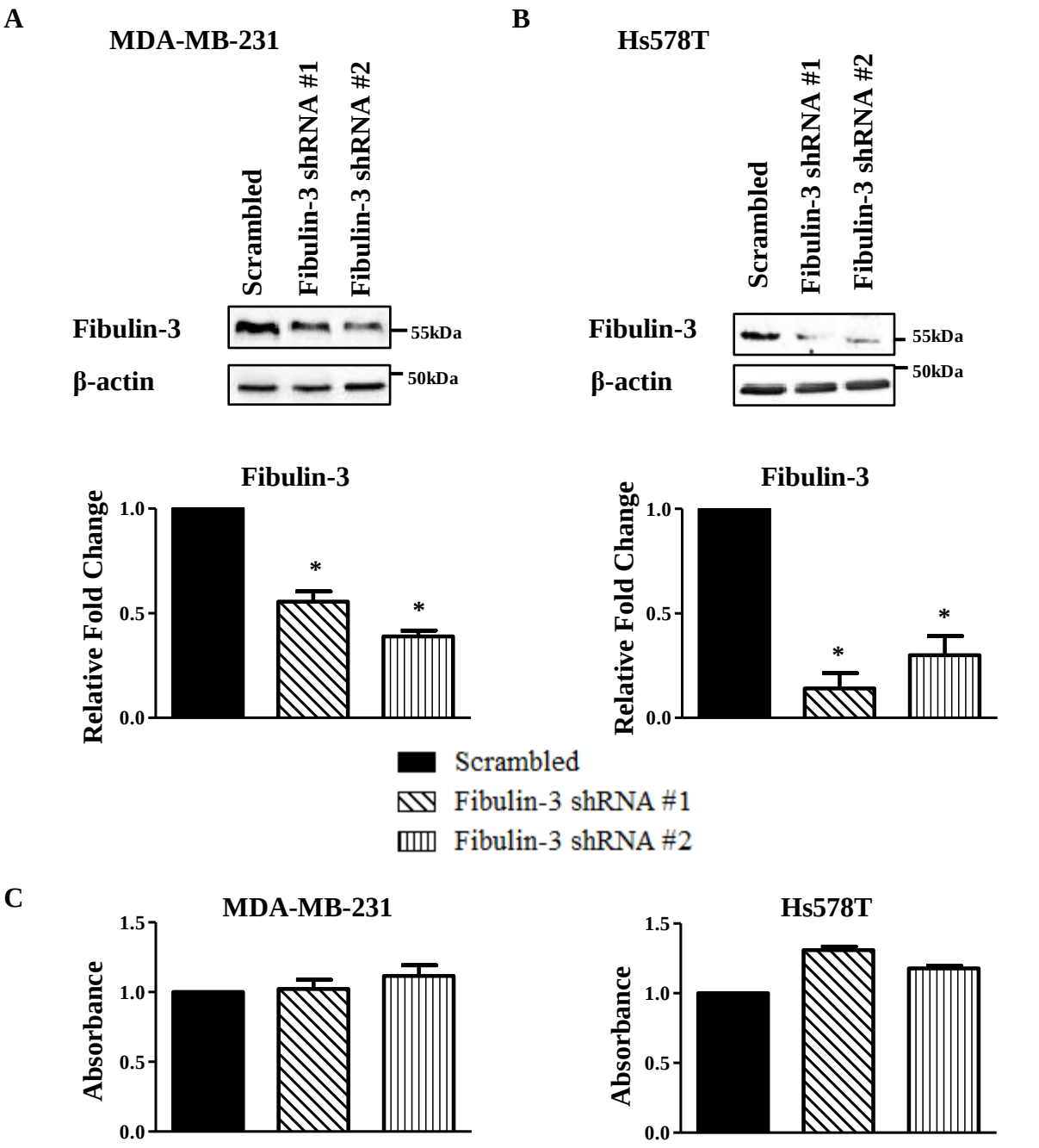

Figure 3.9. Fibulin-3 knockdown in TNBC cells using shRNA. Representative western blot and densitometric analysis of fibulin-3 (n=3) in (**A**) MDA-MB-231 cells stably expressing fibulin-3 shRNA or scrambled control and (**B**) Hs578T cells stably expressing fibulin-3 shRNA or scrambled control; β-actin, *loading control*. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$ compared to scrambled. Columns represent protein expression ± SEM. (**C**) MDA-MB-231 and Hs578T cells expressing fibulin-3 shRNA or scrambled control were cultured in 96-well plates and cell viability was determined by MTT assay (n=3). Columns represent absorbance measured at 550 nm minus background reading at 700 nm ± SEM.

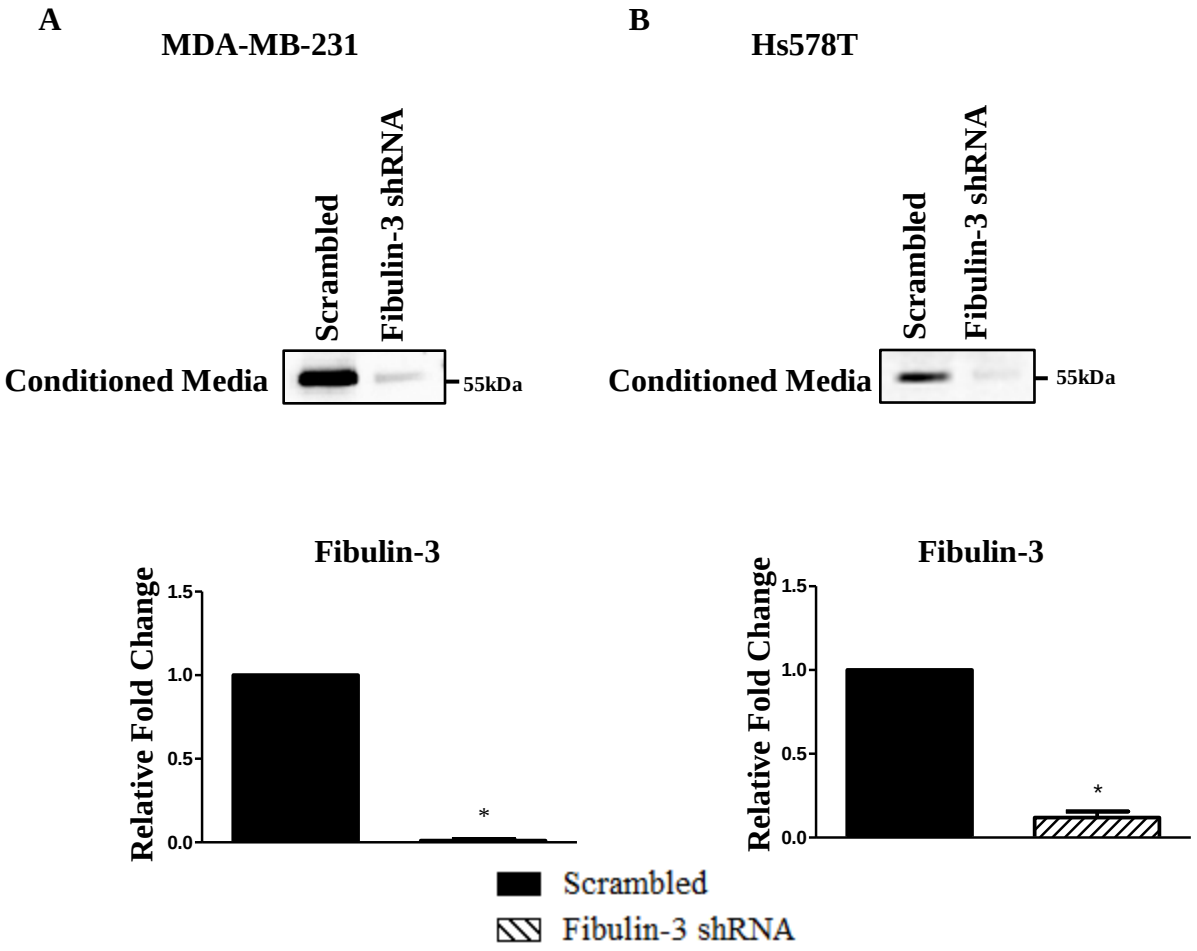

Figure 3.10. Fibulin-3 knockdown in TNBC cells decreases fibulin-3 secretion. Representative western blot and densitometric analysis of fibulin-3 (n=3) in conditioned media from (**A**) MDA-MB-231 cells stably expressing fibulin-3 shRNA or scrambled control and (**B**) Hs578T cells stably expressing fibulin-3 shRNA or scrambled control. Student's t-test: *, $P<0.05$. Columns represent protein expression ± SEM.

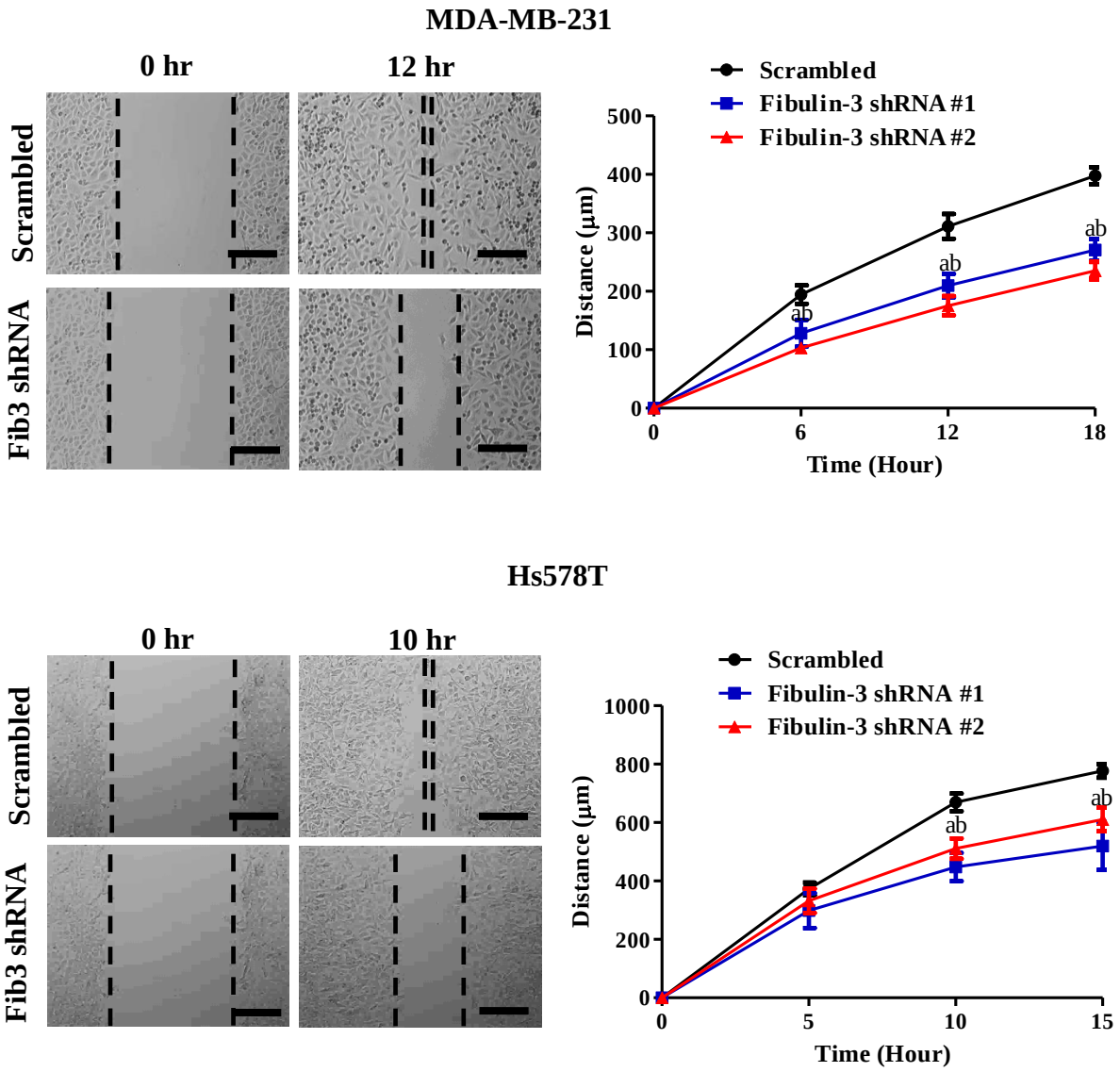

Figure 3.11. Depletion of fibulin-3 inhibits TNBC cell motility. Cell motility measured using the scratch assay (n=3) in both MDA-MB-231 and Hs578T cells depleted of endogenous fibulin-3 over an 18 hour and 15 hour period, respectively. Two-way ANOVA followed by Bonferroni's multiple comparison test: a, $P < 0.05$ for Fibulin-3 shRNA construct #1 vs. scrambled control; b, $P < 0.05$ Fibulin-3 shRNA construct #2 vs. scrambled control. Scale bar = 50 μm.

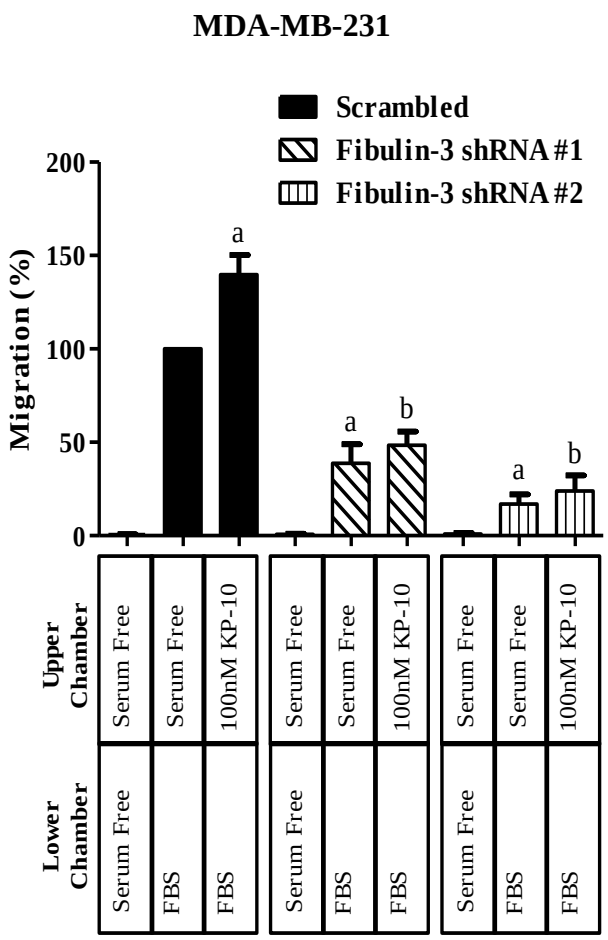

Figure 3.12. Depletion of fibulin-3 inhibits MDA-MB-231 cell migration in transwell chamber migration assay. MDA-MB-231 cells depleted of fibulin-3 display decreased migration towards 10% fetal bovine serum (FBS) under basal and kisspeptin-10 stimulated conditions (n=3). One-way ANOVA followed by Bonferroni's multiple comparison test: a, $P<0.05$ for significance difference vs scrambled control non-stimulated; b, $P<0.05$ for significance difference vs scrambled control 100 nM kisspeptin-10. Columns represent cells migrated to lower chamber ± SEM.

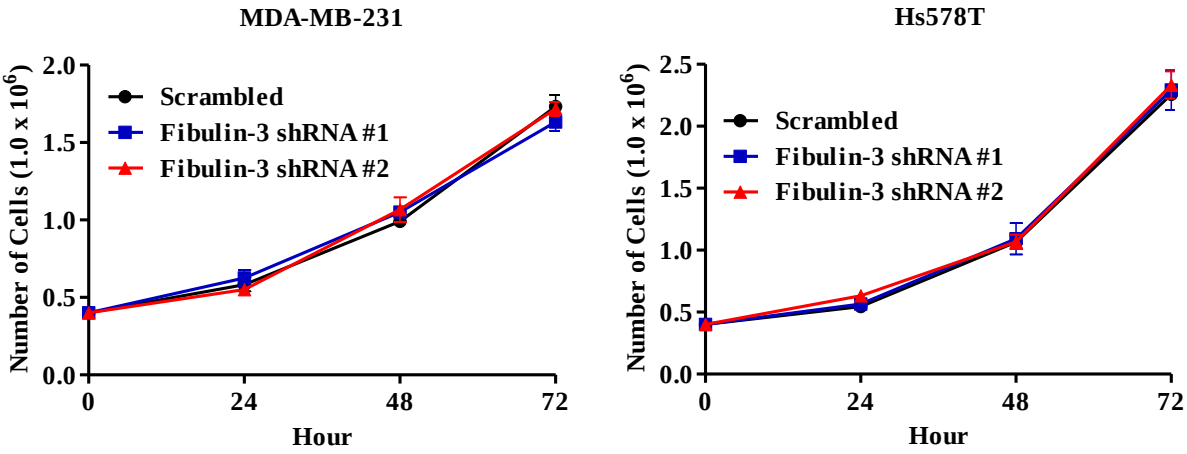

Figure 3.13. Depletion of endogenous fibulin-3 does not affect cell growth. MDA-MB-231 or Hs578T cells (4 x 10^5) expressing fibulin-3 shRNA or scrambled control were cultured for 72 hours and counted at 24 hour intervals (n=3).

3.7 Depletion of fibulin-3 inhibits TNBC cell invasion.

Matrigel is a reconstituted extracellular matrix that mimics the *in vivo* tumor microenvironment, enabling cells to grow in 3D [7,8,11,13]. To assess a role for fibulin-3 in TNBC cell invasion, a 3D Matrigel invasion assay was conducted, as previously described [7,8,11,12]. MDA-MB-231 and Hs578T cells depleted of fibulin-3 had a diminished ability to form invasive, or stellate, colonies when cultured in Matrigel (5 days) compared to scrambled control cells (**Figure 3.14, 3.15**). The viability of colonies was verified by examining cell nuclei integrity using Hoechst staining. We also assessed the role of fibulin-3 in cell invasion using transwell Matrigel invasion assays, as previously conducted [7,8,11,12]. Cells were layered on a 1 in 10 dilution of Matrigel in serum-free media or serum-free media containing 100 nM KP-10 and allowed to migrate across the transwell migration membrane towards serum-free or FBS-supplemented media for 20 hours. The depletion of fibulin-3 significantly decreased the ability of cells to invade across the membrane in unstimulated and KP-10 stimulated conditions (**Figure 3.16**). These findings demonstrate that fibulin-3 regulates the invasive ability of TNBC cells.

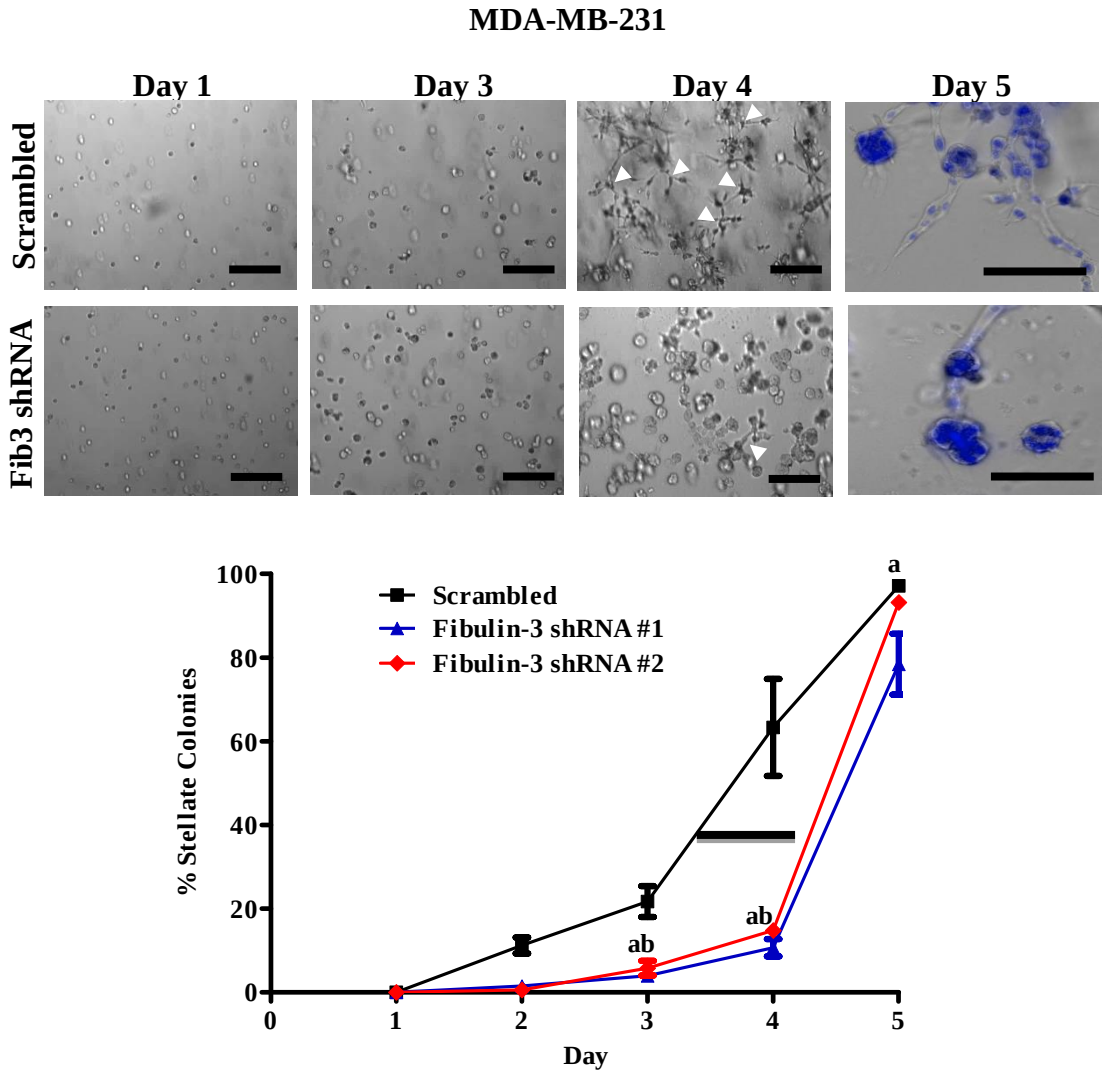

Figure 3.14. Fibulin-3 knockdown inhibits MDA-MB-231 TNBC cell invasion in three-dimensional Matrigel assay. MDA-MB-231 cells expressing fibulin-3 shRNA or scrambled controls were suspended in Matrigel for 5 days and the number of invasive stellate structures were counted (n=3). Cells were fixed and nuclei were stained with Hoechst 33258 on day 5. Representative images shown. White arrows: stellate invasive structures; scale bar = 50 µm. Two-way ANOVA followed by Bonferroni's multiple comparison test: a, $P<0.05$ scrambled control vs fibulin-3 shRNA construct #1; b, $P<0.05$ scrambled control vs fibulin-3 shRNA construct #2.

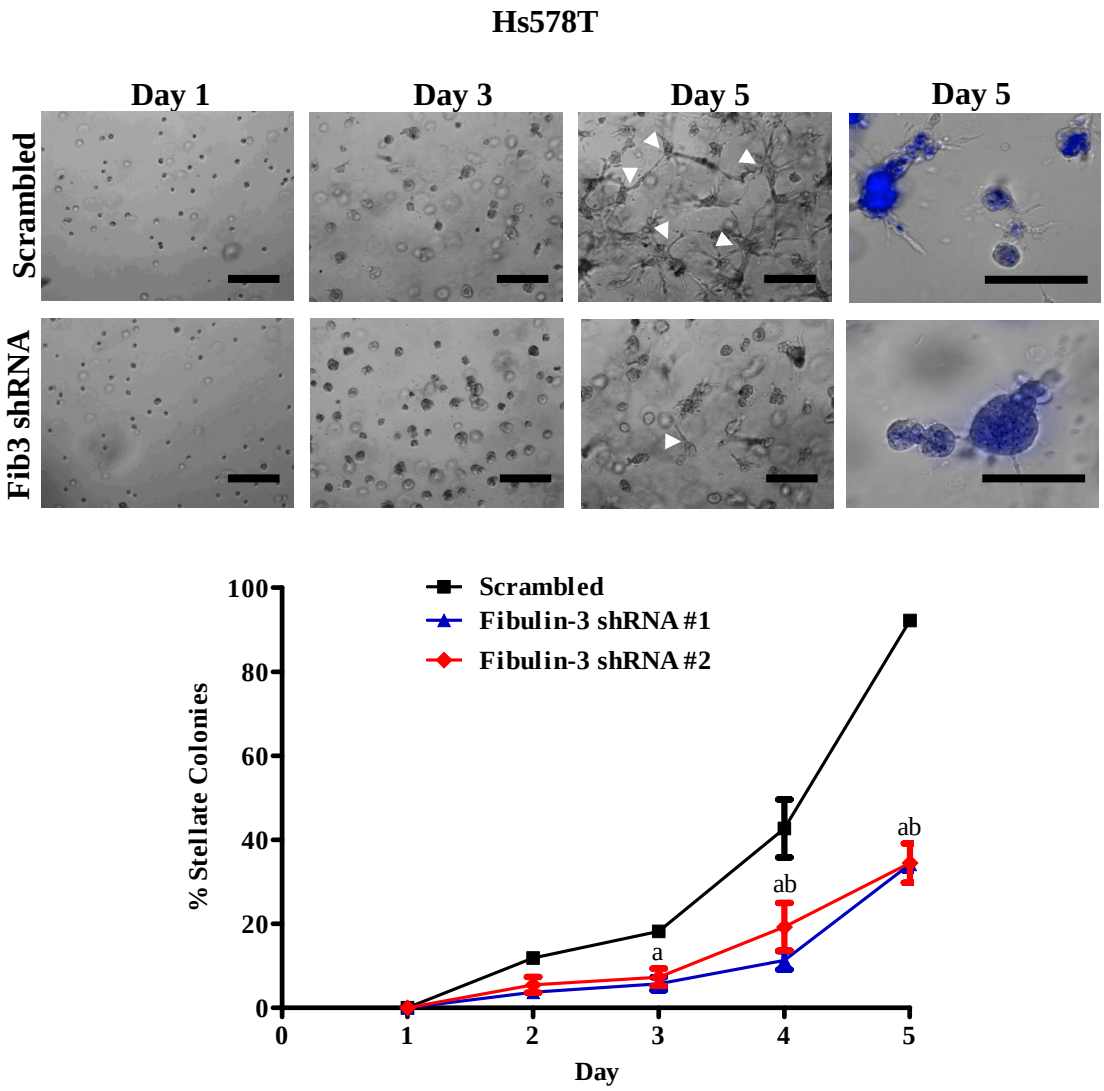

Figure 3.15. Fibulin-3 knockdown inhibits Hs578T TNBC cell invasion in three-dimensional Matrigel assay. Hs578T cells expressing fibulin-3 shRNA or scrambled controls were suspended in Matrigel for 5 days and the number of invasive stellate structures were counted (n=3). Cells were fixed and nuclei were stained with Hoechst 33258 on day 5. Representative images shown. White arrows: stellate invasive structures; scale bar = 50 µm. Two-way ANOVA followed by Bonferroni's multiple comparison test: a, $P<0.05$ scrambled control vs fibulin-3 shRNA construct #1; b, $P<0.05$ scrambled control vs fibulin-3 shRNA construct #2.

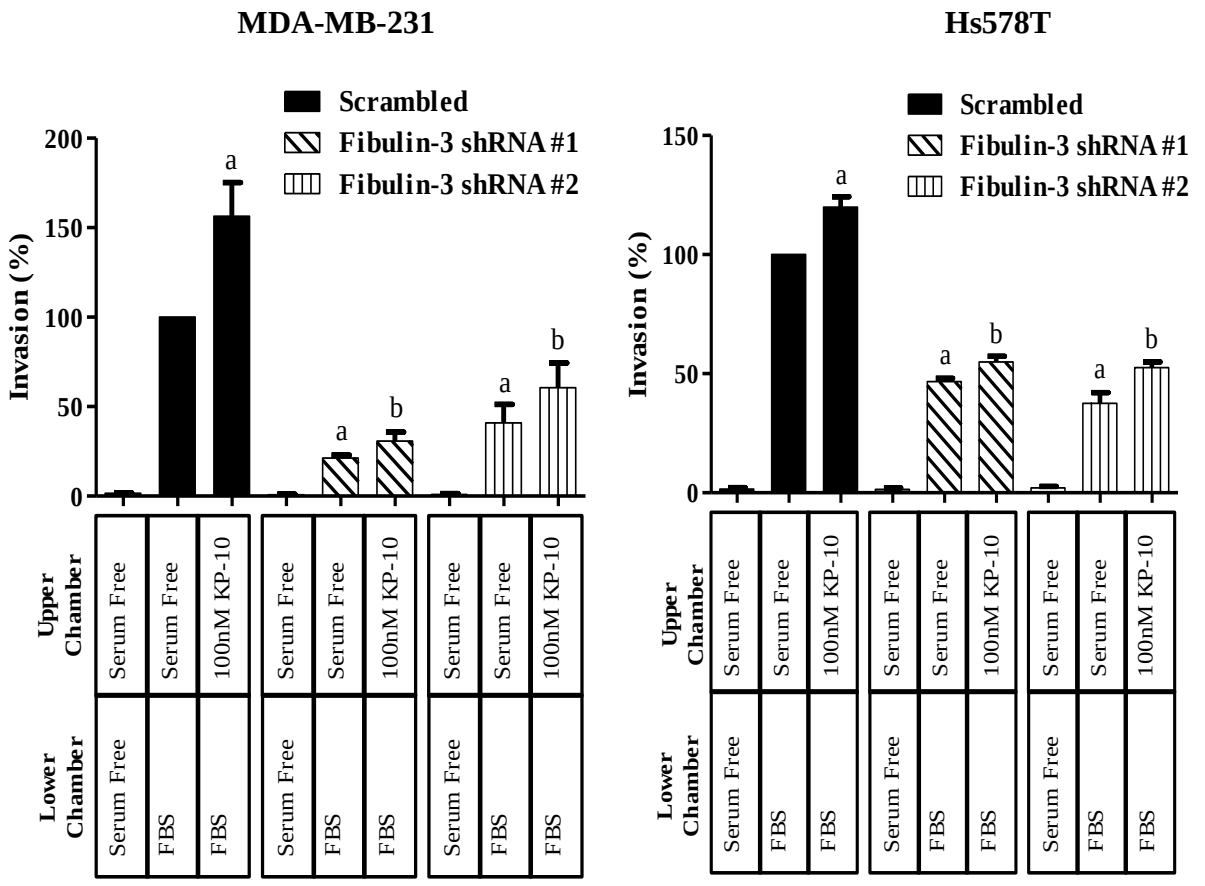

Figure 3.16. Depletion of fibulin-3 inhibits TNBC cell invasion in transwell chamber Matrigel invasion assay. MDA-MB-231 and Hs578T cells depleted of fibulin-3 display decreased invasion towards 10% fetal bovine serum (FBS) under basal conditions and inhibition of kisspeptin-10 induced cell invasion (n=3). One-way ANOVA followed by Bonferroni's multiple comparison test: a, $P<0.05$ for significance difference vs scrambled control non-stimulated; b, $P<0.05$ for significance difference vs scrambled control 100 nM kisspeptin-10. Columns represent cells invaded to lower chamber ± SEM.

3.8 Depletion of fibulin-3 decreases MAPK signaling and MMP-9 expression and activity

Fibulin-3 has been shown to regulate the phosphorylation of ERK and AKT downstream of EGFR in pancreatic cancer cells [14]. To initially examine potential mechanisms by which fibulin-3 regulates TNBC cell invasion, we investigated the phosphorylation status of ERK and AKT. Upon depletion of fibulin-3 in the TNBC cell lines, we observed decreased phosphorylation of ERK compared to scrambled controls (**Figure 3.17**). However, there was no significant change in AKT phosphorylation (**Figure 3.17**).

Fibulin-3 has also been shown to regulate MMP-9 expression and activity in lung cancer and malignant glioma [15,16]. Therefore to determine whether fibulin-3 regulates TNBC cell invasion *via* MMP-9, we examined the effect of fibulin-3 depletion on MMP-9 expression and activity by western blotting and zymography, respectively. MMP-9 expression significantly decreased upon depletion of fibulin-3 in both the MDA-MB-231 and Hs578T cell lines compared to scrambled controls (**Figure 3.18A**). Additionally, zymography demonstrated that MMP-9 activity was diminshed in the MDA-MB-231 cells depleted of fibulin-3 (**Figure 3.18B**). This data suggests that fibulin-3 may regulate TNBC cell invasion *via* an ERK and MMP-9 dependent pathway.

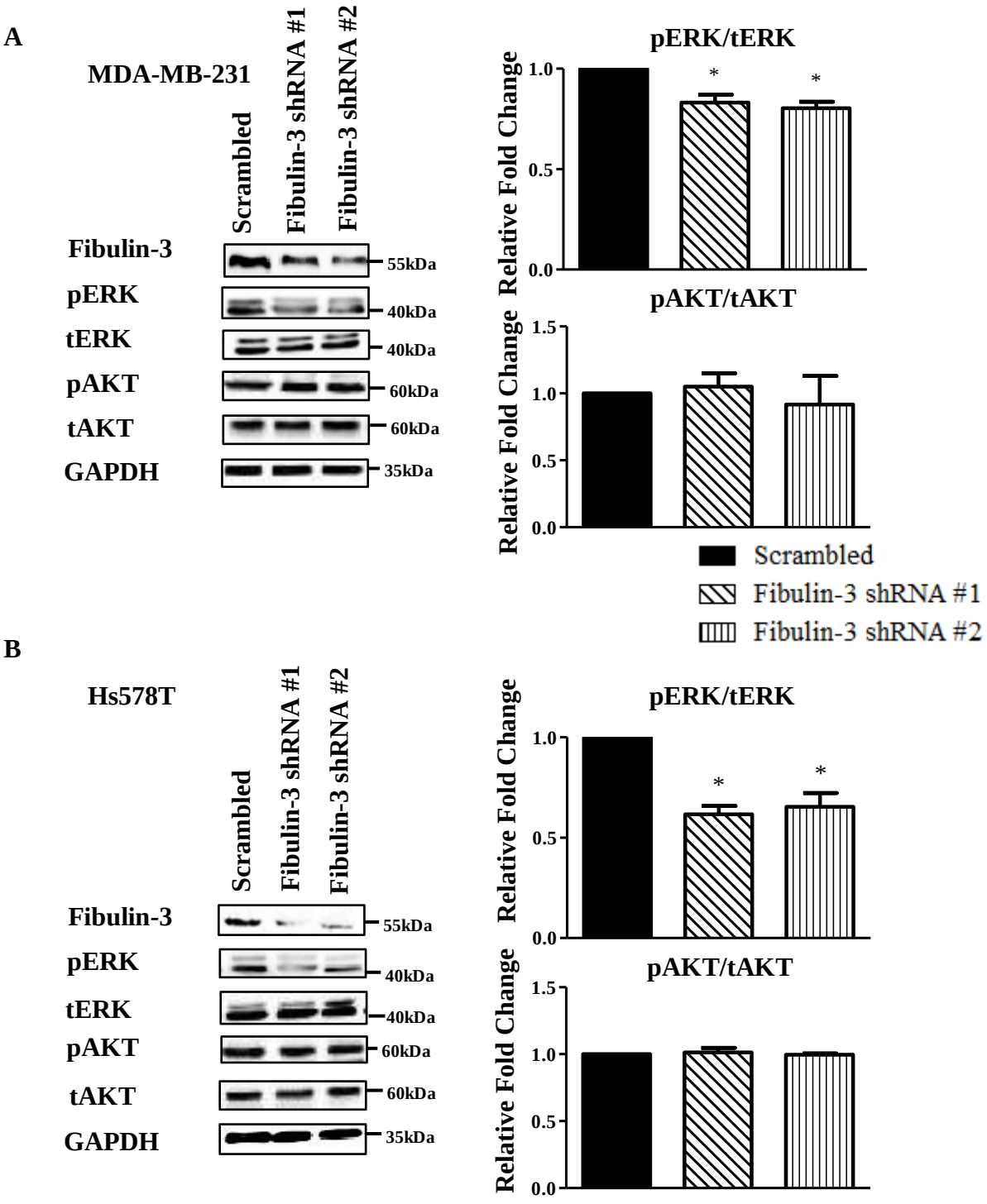

Figure 3.17. Depletion of fibulin-3 in TNBC cells reduces the phosphorylation of downstream signaling molecules. Representative western blot and densitometric analysis of pERK (n=3) and pAKT (n=3) in (**A**) MDA-MB-231 cells or (**B**) Hs578T cells expressing fibulin-3 shRNA or scrambled control; β-actin, *loading control*. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P<0.05$ compared to scrambled. Columns represent protein expression ± SEM.

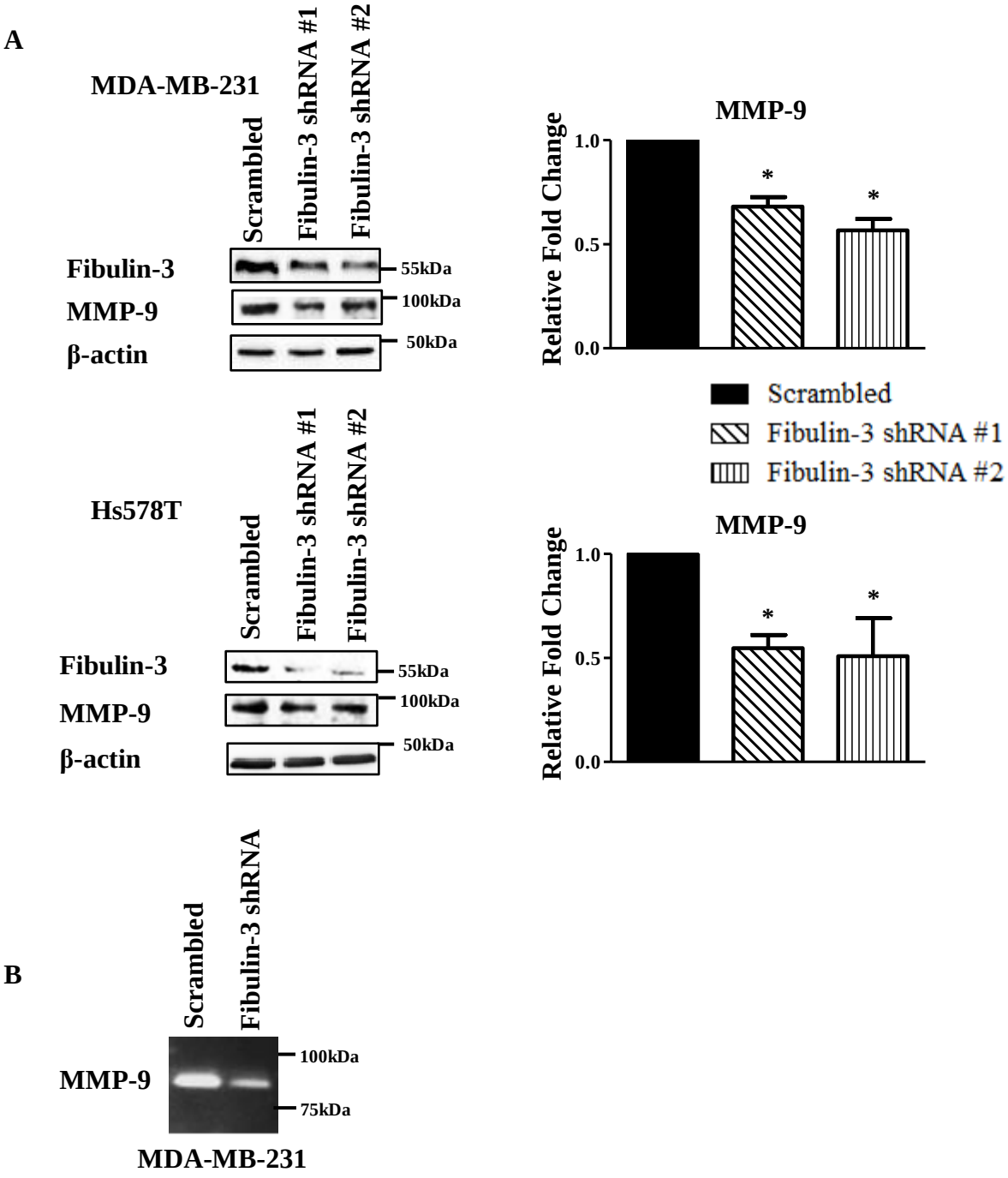

Figure 3.18. Depletion of fibulin-3 in TNBC cells decreases the expression and activity of MMP-9. (**A**) Representative western blot and densitometric analysis of MMP-9 (n=3) in MDA-MB-231 and Hs578T cells expressing fibulin-3 shRNA or scrambled contro; β-actin, *loading control*. One-way ANOVA followed by Dunnett's multiple comparison test: *, P<0.05 compared to scrambled. Columns represent protein expression ± SEM. (**B**) Depletion of fibulin-3 in MDA-MB-231 cells reduces MMP-9 activity (n=3) shown by zymography.

3.9 Depletion of fibulin-3 inhibits kisspeptin-10-induced MMP-9 secretion and activity

Our lab has previously shown that KP-10 stimulates MMP-9 secretion and activity in MDA-MB-231 cells [4]. Thus, we examined MMP-9 activity in the SKBR3 breast cancer cells overexpressing KISS1R, and observed that KISS1R overexpression also increased MMP-9 secretion and activity (**Figure 3.19A**). We also sought to determine if fibulin-3 regulates KP-10 induced MMP-9 activity by conducting zymography with the MDA-MB-231 cells depleted of fibulin-3. Zymography revealed that depletion of fibulin-3 inhibited the KP-10 induced secretion and activity of MMP-9 (**Figure 3.19B**). Therefore, KISS1R signaling stimulates MMP-9 secretion and activity *via* a fibulin-3 dependent mechanism.

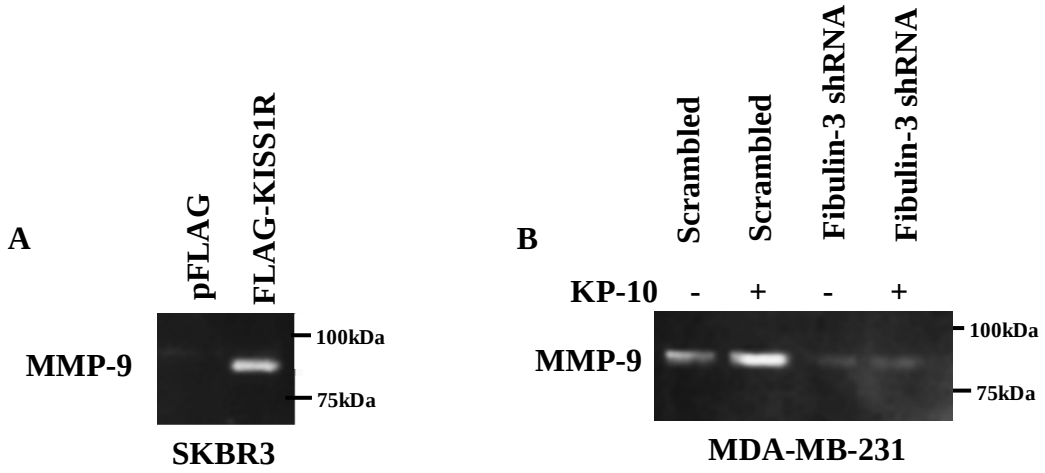

Figure 3.19. Depletion of fibulin-3 inhibits KP-10-induced MMP-9 secretion and actvity. (**A**) Overexpression of KISS1R in SKBR3 breast cancer cells increases MMP-9 secretion and activity (n=3), shown by zymography. (**B**) Depletion of fibulin-3 in MDA-MB-231 cells blocks basal and KP-10 stimulated MMP-9 secretion and activity (n=4).

3.10 Inhibition of ERK signaling further diminshes MMP-9 secretion and acitivty

MMP-9 has been shown to regulate invasion downstream of ERK signaling in MCF7 and MDA-MB-231 breast cancer cells [17]. Additionally, since the western blot results revealed that phosphorylation of ERK is diminished in fibulin-3 knockdown cells, we next examined the effect of inhibiting ERK signaling on MMP-9 secretion and activity. MDA-MB-231 cells stably expressing fibulin-3 shRNA or scrambled control displayed a decrease in basal and KP-10 induced MMP-9 secretion and activity upon pretreatment with ERK1/2 inhibitor, UO126 (**Figure 3.20**). In contrast, MMP-9 secretion and activity did not change in cells treated with the inactive analog, UO124, compared to vehicle control (**Figure 3.20**). This data demonstrates that KP-10 induced MMP-9 secretion and activity is dependent on ERK signaling, and this may occur downstream of fibulin-3.

EGFR signaling has been shown to regulate MMP-9 *via* an ERK-dependent pathway in SKBR3 breast cancer cells [18]. Thus, to determine if the involvement of fibulin-3 in MMP-9 secretion and activity is EGFR dependent, we repeated zymography with MDA-MB-231 cells stably expressing fibulin-3 shRNA or scrambled control stimulated with EGFR inhibitor, AG1478. We found that AG1478 treatment appeared to slightly decrease MMP-9 activity in the scrambled control, however there was no noticeable further decrease in the fibulin-3 knockdown cells (**Figure 3.21**). Therefore, this suggests that fibulin-3 regulates MMP-9 secretion and activity in TNBC cells, and that this may occur independently of EGFR activity.

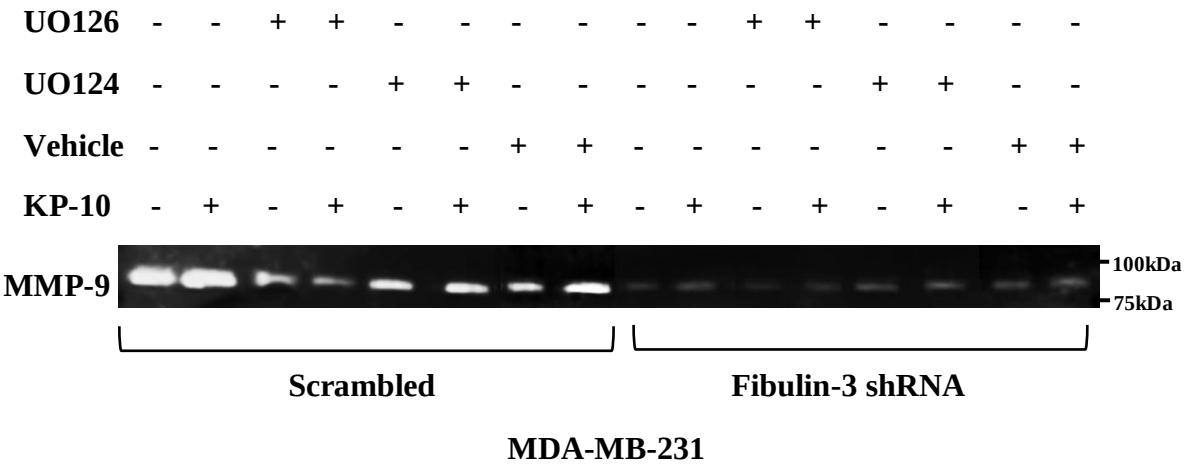

Figure 3.20. Inhibition of ERK signaling blocks basal and KP-10-induced MMP-9 secretion and actvity. MDA-MB-231 cells stably expressing fibulin-3 shRNA or scrambled control were pre-treated for 30 minutes with the following drugs prior to stimulation with 100 nM KP-10 for 24 hours: 20 μM active ERK1/2 inhibitor, UO126 or 20 μM inactive analog, UO124. Representative zymograph of three independent experiments is shown.

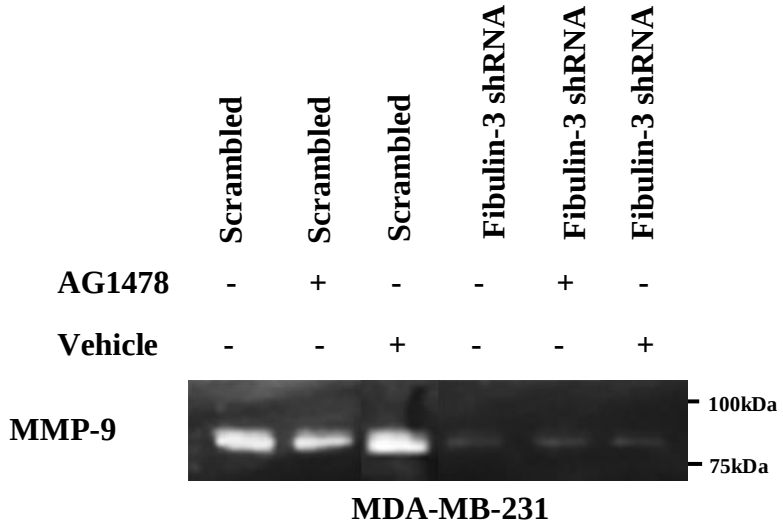

Figure 3.21. EGFR inhibition decreasesMMP-9 secretion and actvity in scrambled control but not in fibulin-3 depleted cells. MDA-MB-231 cells stably expressing fibulin-3 shRNA or scrambled control were treated with 500 nM EGFR inhibitor, AG1478. Representative zymograph of three independent experiments is shown.

3.11 Depletion of fibulin-3 decreases EGFR phosphorylation in TNBC cells

Fibulin-3 has been shown to compete with EGF for binding and activation of EGFR in pancreatic cancer cells [14], however whether fibulin-3 activates EGFR in breast cancer cells remains unknown. Therefore, to assess whether fibulin-3 activates EGFR in TNBC cells, lysates from unstimulated and EGF treated MDA-MB-231 and Hs578T cells stably expressing fibulin-3 shRNA or scrambled control were examined for phosphorylated endogenous EGFR by western blotting. We observed that both MDA-MB-231 and Hs578T cells depleted of fibulin-3 displayed significantly less phosphorylation of EGFR (**Figure 3.22**). These results indicate that fibulin-3 regulates EGFR phosphorylation in TNBC cells.

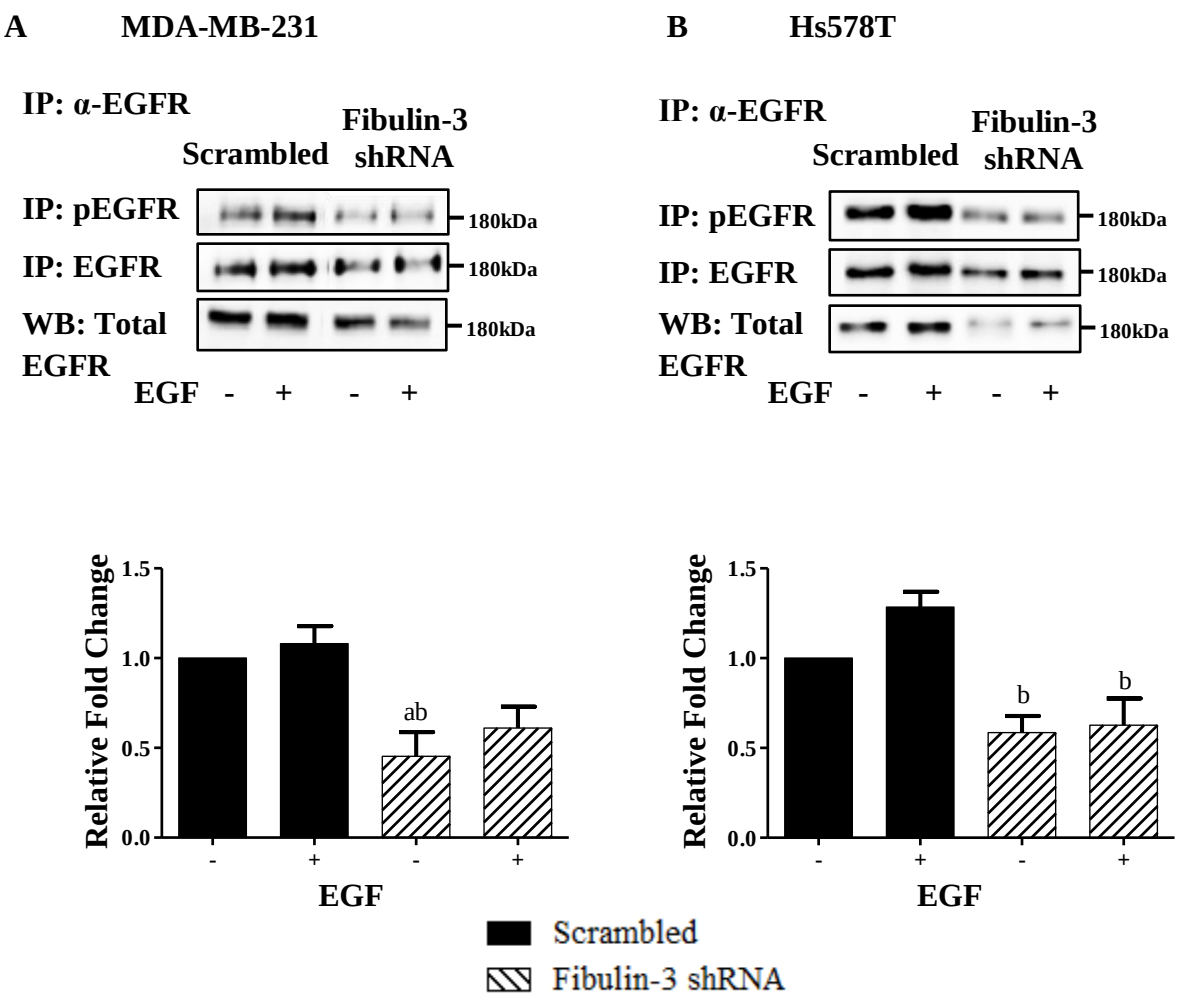

Figure 3.22. Depletion of fibulin-3 decreases EGFR phosphorylation in TNBC cells. (**A**) MDA-MB-231 (n=3) and (**B**) Hs578T (n=4) cells stably expressing fibulin-3 shRNA or scrambled control treated with 20 ng/mL EGF for 0 or 15 minutes. EGFR was imunopreciptated and blots were probed with phosphorylated and total EGFR. Quantification of phosphorylated EGFR expression normalized to EGFR pulled down. One-way ANOVA followed by Bonferroni's multiple comparison test: a, $P<0.05$ for significance difference vs scrambled control non-stimulated; b, $P<0.05$ for significance difference vs scrambled control 20 ng/mL EGF. Columns represent protein expression ± SEM.

3.12 References

1. Pass, H. I., Levin, S. M., Harbut, M. R., Melamed, J., Chiriboga, L., Donington, J., . . . Goparaju, C. (2012). Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. *N Engl J Med, 367*(15), 1417-1427. doi: 10.1056/NEJMoa1115050
2. Hyman, E., Kauraniemi, P., Hautaniemi, S., Wolf, M., Mousses, S., Rozenblum, E., . . . Kallioniemi, A. (2002). Impact of DNA amplification on gene expression patterns in breast cancer. *Cancer Res, 62*(21), 6240-6245.
3. Eirew, P., Steif, A., Khattra, J., Ha, G., Yap, D., Farahani, H., . . . Aparicio, S. (2015). Dynamics of genomic clones in breast cancer patient xenografts at single-cell resolution. *Nature, 518*(7539), 422-426. doi: 10.1038/nature13952
4. Davidson, B., Stavnes, H. T., Holth, A., Chen, X., Yang, Y., Shih, I.-M., & Wang, T.-L. (2011). Gene expression signatures differentiate ovarian/peritoneal serous carcinoma from breast carcinoma in effusions. *Journal of Cellular and Molecular Medicine, 15*(3), 535-544. doi: 10.1111/j.1582-4934.2010.01019.x
5. Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., . . . Brooks, S. C. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res, 50*(18), 6075-6086.
6. Petersen, O. W., & Polyak, K. (2010). Stem cells in the human breast. *Cold Spring Harb Perspect Biol, 2*(5), a003160. doi: 10.1101/cshperspect.a003160
7. Cvetkovic, D., Dragan, M., Leith, S. J., Mir, Z. M., Leong, H. S., Pampillo, M., . . . Bhattacharya, M. (2013). KISS1R induces invasiveness of estrogen receptor-negative human mammary epithelial and breast cancer cells. *Endocrinology, 154*(6), 1999-2014. doi: 10.1210/en.2012-2164
8. Zajac, M., Law, J., Cvetkovic, D. D., Pampillo, M., McColl, L., Pape, C., . . . Bhattacharya, M. (2011). GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness. *PLoS One, 6*(6), e21599. doi: 10.1371/journal.pone.0021599
9. Yilmaz, M., & Christofori, G. (2009). EMT, the cytoskeleton, and cancer cell invasion. *Cancer and Metastasis Reviews, 28*(1-2), 15-33.
10. Deryugina, E. I., & Quigley, J. P. (2006). Matrix metalloproteinases and tumor metastasis. *Cancer and Metastasis Reviews, 25*(1), 9-34.
11. Alemayehu, M., Dragan, M., Pape, C., Siddiqui, I., Sacks, D. B., Di Guglielmo, G. M., . . . Bhattacharya, M. (2013). beta-Arrestin2 regulates lysophosphatidic acid-induced human breast tumor cell migration and invasion via Rap1 and IQGAP1. *PLoS One, 8*(2), e56174. doi: 10.1371/journal.pone.0056174
12. Li, T. T., Alemayehu, M., Aziziyeh, A. I., Pape, C., Pampillo, M., Postovit, L. M., . . . Bhattacharya, M. (2009). Beta-arrestin/Ral signaling regulates lysophosphatidic acid-mediated migration and invasion of human breast tumor cells. *Mol Cancer Res, 7*(7), 1064-1077. doi: 10.1158/1541-7786.mcr-08-0578
13. Cvetkovic, D., Goertzen, C. G., & Bhattacharya, M. (2014). Quantification of breast cancer cell invasiveness using a three-dimensional (3D) model. *J Vis Exp*(88). doi: 10.3791/51341
14. Camaj, P., Seeliger, H., Ischenko, I., Krebs, S., Blum, H., De Toni, E. N., . . . Bruns, C. J. (2009). EFEMP1 binds the EGF receptor and activates MAPK and Akt pathways in pancreatic carcinoma cells. *Biol Chem, 390*(12), 1293-1302. doi: 10.1515/bc.2009.140

15. Xu, S., Yang, Y., Sun, Y. B., Wang, H. Y., Sun, C. B., & Zhang, X. (2014). Role of fibulin-3 in lung cancer: in vivo and in vitro analyses. *Oncol Rep, 31*(1), 79-86. doi: 10.3892/or.2013.2799
16. Hu, B., Thirtamara-Rajamani, K. K., Sim, H., & Viapiano, M. S. (2009). Fibulin-3 is uniquely upregulated in malignant gliomas and promotes tumor cell motility and invasion. *Mol Cancer Res, 7*(11), 1756-1770. doi: 10.1158/1541-7786.mcr-09-0207
17. Moulik, S., Pal, S., Biswas, J., & Chatterjee, A. (2014). Role of ERK in Modulating MMP 2 and MMP 9 with Respect to Tumour Invasiveness in Human Cancer Cell Line MCF-7 and MDA-MB-231. *Journal of Tumor, 2*(2).
18. Kim, S., Choi, J. H., Lim, H. I., Lee, S. K., Kim, W. W., Cho, S., . . . Yang, J. H. (2009). EGF-induced MMP-9 expression is mediated by the JAK3/ERK pathway, but not by the JAK3/STAT-3 pathway in a SKBR3 breast cancer cell line. *Cell Signal, 21*(6), 892-898.

Chapter 4 – Discussion and Conclusions

4.1 Summary of Novel Findings and Conclusions

Objective #1: To determine if plasma fibulin-3 levels are increased in TNBC patients compared to healthy controls.

cBioPortal data analysis of the TCGA database revealed that *KISS1* gene had the highest amplification frequency in invasive breast carcinoma and patients with alteration displayed a drastic decrease in survival. However, *EFEMP1* gene was altered only 1.5% in invasive breast carcinoma patients. Despite the apparent lack of amplification, plasma fibulin-3 concentrations are elevated in metastatic TNBC patients compared to non-metastatic TNBC and healthy patients. These results indicate that fibulin-3 may be a marker of metastasis in TNBC patients.

Objective #2: To determine if fibulin-3 positively regulates KISS1R-induced TNBC cell invasion, and the underlying mechanism(s).

We show for the first time that depletion of fibulin-3 resulted in decreased basal and KP-10 stimulated TNBC cell migration and invasion, two important processes involved in cancer metastasis. We also found that fibulin-3 depletion resulted in decreased phosphorylation of ERK and reduced expression and activity of MMP-9. Additionally, KP-10-induced MMP-9 activity was further reduced by ERK inhibition. Thus, our results suggest that fibulin-3 promotes TNBC cell invasion downstream of KISS1R signaling by regulating ERK and MMP-9 secretion and activity (**Figure 4.1**).

Objective #3: To determine if fibulin-3 regulates EGFR phosphorylation in TNBC cells.

Depletion of fibulin-3 in TNBC cells resulted in decreased EGFR phosphorylation. These results demonstrate that fibulin-3 may regulate EGFR activation in TNBC cells.

4.2 Contributions of Research to Current State of Knowledge

GPCR signaling has been shown to be an important mediator of intracellular signaling in both physiological and pathological conditions [1,2]. GPCR signaling involves complex G-protein independent and G-protein dependent downstream signaling pathways. In addition, GPCRs have been shown to transactivate receptor tyrosine kinases (RTKs) by stimulating tyrosine phosphorylation through a variety of mechanisms [3]. GPCR transactivation of the RTK, EGFR, has been demonstrated in lung, ovarian, colon, prostate and breast cancer [4]. Overexpression of EGFR in many cancers has been correlated with increased cell survival, growth and invasion [5,6]. Specifically, TNBC, which currently lacks effective targeted therapies, overexpresses EGFR [7]. However, clinical therapies designed to inhibit EGFR signaling by targeting the extracellular domain or the tyrosine kinase domain have been ineffective due to acquired drug resistance [8]. Our lab has mechanistically shown that KISS1R regulates TNBC cell invasion *via* β-arrestin2 and IQGAP1, and that these proteins are required for KISS1R transactivation of EGFR [9,10]. Therefore, further elucidating the mechanism by which KISS1R regulates EGFR activity and cell invasion in TNBC may identify novel therapeutic targets for use in combination therapy.

The tumor microenvironment has been implicated in many of the processes associated with the progression of cancer, including initial carcinogenesis, metastasis and drug resistance [11]. Therefore, components of the tumor microenvironment are emerging as potential preventative and therapeutic targets in the treatment of cancer [12]. Specifically, proteins secreted by cancer cells into the tumor microenvironment may represent important targets, as their expression profiles in cancers have the potential to aid in earlier diagnosis and the assessment and prevention of cancer progression [13]. Additionally, ECM proteins

have an influential role in tumor progression and metastasis and targeted ECM inhibitors are worth considering as anti-metastatic therapies [14].

Fibulin-3 Expression in TNBC

Fibulin-3 expression has been examined in several cancers, including breast cancer, however whether fibulin-3 expression increases in TNBC remains unknown. Our results demonstrated that compared to *EFEMP1, KISS1* is amplified more in invasive breast cancer, compared to other cancers. Gene amplification is a common genetic alteration in cancer that is useful for prognostic and diagnostic purposes [15]. Additionally, invasive breast carcinoma patients with amplified *KISS1* displayed a drastic decrease in overall survival compared to patients without alterations in the gene. Whether plasma kisspeptin levels are increased in TNBC patients is being examined as part of another ongoing study in our lab. We found that plasma fibulin-3 concentrations in metastatic TNBC patients are elevated compared to healthy controls. We next examined fibulin-3 protein levels and found that fibulin-3 is higher in invasive cells compared to weakly invasive or non-malignant cells.

To date, one study indicates that fibulin-3 is overexpressed in breast cancer effusions [16] whereas another study suggests that fibulin-3 may be down-regulated in primary breast tumors [17]. These inconsistent reports could be the result of numerous factors, such as differences in the origin of the sample, breast cancer subtype and advancement of the disease. Additionally, a recent study reported that fibulin-3 mRNA is decreased in primary breast tumors compared to normal breast [16]. Using the Oncomine database, the authors demonstrated that fibulin-3 mRNA decreases with increasing staging of breast cancer and that low fibulin-3 expression correlated with poor patient survival [16]. They suggested that decreased fibulin-3 expression is a result of epigenetic silencing through promoter

methylation [16]. Moreover, using MCF10A and MDA-MB-231 cells, the study demonstrated that fibulin-3 mRNA decreased in isogenic lines that had greater invasive capabilities [16]. One possible reason for the difference between this study and our observations is that the correlation between mRNA and protein expression does not always go hand in hand [17]. Translation of mRNA in cancer has been shown to be dysregulated through changes in translational efficiency, availability of translational machinery and activation of translation through aberrantly activated signaling pathways [18]. Additionally, since proteins are the final product of transcription and translation and the main effectors of signaling cascades, they may better reflect gene function [17]. Thus, our novel data revealing increased fiublin-3 protein in the circulation of metastatic TNBC patients may accurately represent fibulin-3 expression in TNBC.

Fibulin-3 in TNBC Cell Migration and Invasion

KISS1R stimulates the secretion of the GnRH hormone [19,20], however we have shown for the first time, that KISS1R signaling is capable of regulating the expression and secretion of the ECM protein, fibulin-3. Our results demonstrated that kisspeptin-10 stimulation significantly increased the expression and secretion of fibulin-3 in the ERα-negative SKBR3 breast cancer overexpressing KISS1R and pFLAG controls. Thus, our results support that fibulin-3 may be a downstream effector of KISS1R signaling, and that KISS1R signaling may influence components of the tumor microenvironment.

ECM remodeling, migration and invasion are all integral to the process of cancer cell metastasis [21]. For our study, we used two human TNBC cell lines, MDA-MB-231 and Hs578T. Our lab has previously shown that kisspeptin-10 stimulation of these cells promotes migration and invasion [9,10]. Our study extended these findings by demonstrating that

fibulin-3 depletion reduced TNBC cell migration as assessed by scratch and transwell migration assays, thereby supporting a role for fibulin-3 in TNBC cell migration. Moreover, fibulin-3 depletion inhibited both basal and kisspeptin-10 stimulated migration assessed in the transwell chamber migration assay, therefore suggesting that fibulin-3 may positively regulate kisspeptin-10-induced TNBC cell migration.

Our findings also supported a role for fibulin-3 in TNBC cell invasion by demonstrating that depletion of fibulin-3 resulted in decreased invasion of MDA-MB-231 and Hs578T cells in the 3D Matrigel invasion assays and transwell Matrigel invasion assays. In addition, fibulin-3 depletion inhibited both basal and kisspeptin-10 stimulated invasion in the transwell Matrigel invasion assays. Tian and colleagues reported that overexpression of fibulin-3 inhibits TGF-β signaling, EMT, invasion and metastasis *in vivo* [16]. However, the TGF-β pathway has been reported to be both tumor suppressive and tumor promoting in breast cancer [22,23,24]. The effects of TGF-β are dependent on the cell type, differentiation and microenvironment [24]. Additionally, mouse models have demonstrated that mammary epithelial cell specific expression of activated TGF-β ligand or expression of a constitutively active type I TGF-β receptor results in increased lung metastases *in vivo* [25,26]. It has been proposed that TGF-β signaling could suppress primary tumor growth while promoting metastases through EMT in a subpopulation of breast cancer cells [24]. Interestingly, treatment of TNBC mouse models with TGF-β neutralizing antibodies or receptor kinase inhibitors prevents the development of lung and bone metastases [27]. Therefore, perhaps the effects of fibulin-3 on TGF-β signaling and metastasis differ in TNBC patients.

MMP-9 has been shown to mediate the pro-invasive role of fibulin-3 in malignant glioma [28]. Similarly, we observed decreased expression and activity of MMP-9 in TNBC cells depleted of fibulin-3. Our lab has previously demonstrated that kisspeptin-10 stimulates

MMP-9 secretion and activity in MDA-MB-231 cells [9]. Our current results also demonstrated that KISS1R overexpression in the SKBR3 breast cancer cells increases MMP-9 secretion and activity, thereby supporting the previous study. Additionally, our results demonstrated that kisspeptin-10 induced stimulation of MMP-9 is dependent on fibulin-3. Interestingly, ERK signaling has been reported to regulate MMP-9 mediated invasion in breast cancer cells [29]. We observed significant decreases in ERK phosphorylation in TNBC cells depleted of fibulin-3, and ERK inhibition decreased both scrambled and depleted fibulin-3 TNBC cell basal and kisspeptin-10-induced MMP-9 secretion and activity. Despite observing a significant decrease in ERK phosphorylation in the MDA-MB-231 cells depleted of fibulin-3, a high level of phosphorylation remained that can be explained by previous reports that MDA-MB-231 cells carry an activated kristen rat sarcoma viral oncogene homolog (K-ras) due to a mutation in the *KRAS* gene, resulting in high basal ERK phosphorylation [30,31]. Thus, our studies uncover a mechanism by which kisspeptin-10-induced MMP-9 activity and TNBC cell invasion are dependent on fibulin-3 and ERK signaling.

Fibulin-3 regulates EGFR Activation in TNBC

Fibulin-3 has been shown to compete with EGF for binding and activation of EGFR in pancreatic cancer cells [32]. Notably, EGFR is overexpressed in TNBC [7], and contributes to increased cell survival, proliferation and invasion [5,6]. Our results revealed that fibulin-3 depletion in the TNBC cells significantly reduced EGFR phosphorylation and therefore activation. Thus, our results are the first to demonstrate that fibulin-3 is required for EGFR phosphorylation in TNBC. Additionally, KISS1R signaling has been reported to lead to β-arrestin2 dependent ERK activation [33,34]. Our lab has previously shown that

kisspeptin-10 stimulation leads to β-arrestin2 dependent transactivation of EGFR, a well-known ERK activator [9,35]. Our current results demonstrated that depletion of endogenous β-arrestin1 or 2 significantly decreases fibulin-3 protein expression in the MDA-MB-231 cells. For that reason, KISS1R may transactivate EGFR in a β-arrestin2 and fibulin-3 dependent mechanism, however, this needs to be investigated further.

4.3 Limitations and Future Directions

Our research involving patient plasma has revealed that fibulin-3 concentrations are elevated in metastatic TNBC. The sample size for each group is currently being increased as we collect more patient plasma samples and evaluate the fibulin-3 concentration by ELISA. Currently we are only evaluating secreted fibulin-3 levels in the circulation and stability of fibulin-3 in the blood is unknown. Therefore, it may be of interest to evaluate fibulin-3 mRNA and protein expression (the latter by immunohistochemistry) in TNBC primary tumors compared to adjacent normal tissue and these studies are underway. Secondly, using an *in vivo* orthotopic xenograft mouse model we could examine the effects of fibulin-3 depletion on metastasis. The orthotopic xenograft mouse model has been shown to accurately resemble breast cancer gene expression profiles observed in patient primary breast tumors, thus providing an accurate model to assess the role of fibulin-3 in human TNBC metastasis [36]. Orthotopic xenograft mouse models could be established in immunodeficient mice by subcutaneous injection of MDA-MB-231 cells depleted of fibulin-3 and number and size of lung metastases could be evaluated. These studies will be conducted in the near future.

Our results demonstrated that KISS1R signaling regulates fibulin-3 expression, however the mechanism(s) by which this is occurring is currently unknown. To determine

whether this is occurring transcriptionally fibulin-3 mRNA can be measured in KP-10-induced cells by real-time PCR. Alternately, fibulin-3 protein stability may be increased and this can be assessed by examining the rate of protein degradation through differences in half-life [37]. We also demonstrated that β-arrestins regulate the expression of fibulin-3. β-arrestins function as substrates and adaptors for ubiquination [38]. Additionally, β-arrestins can escort E3 ubiquitin ligases to mediate ubiquitination of various proteins, including transmembrane receptors, ion channels, kinases and other regulatory proteins such as the tumor suppressor p53 [38]. Thus, β-arrestins could be regulating fibulin-3 protein stability downstream of KISS1R signaling and this requires further investigation.

Additionally, the data supporting a role for fibulin-3 in TNBC could be strengthened by the use of a gain of function model, by performing the migration and invasion assays in TNBC cells overexpressing fibulin-3. Our results also demonstrated that fibulin-3 positively regulates MMP-9 secretion and activity in TNBC, specifically MDA-MB-231 cells. One limitation of the use of zymography to establish the effect of EGFR inhibition in MDA-MB-231 cells depleted of fibulin-3 is that MMP-9 activity was already severely diminished in the knockdown cells, making it difficult to observe any further decrease and confidently conclude that fibulin-3 stimulation of MMP-9 is EGFR independent. Therefore, to improve our confidence regarding the effect of EGFR inhibition on fibulin-3, it would be greatly beneficial to repeat this experiment by treating cells overexpressing fibulin-3 with the EGFR inhibitor, AG1478, and examining MMP-9 activity by zymography. Fibulin-3 has also been shown to mediate invasion through MMP-2 expression and activity [28]. MDA-MB-231 cells have been shown to express MMP-9, but lack MMP-2, conversely Hs578T cells have high levels of MMP-2 and lack MMP-9 [39]. Therefore, to further our findings, the effect of fibulin-3 depletion in Hs578T cells on MMP-2 expression and activity could also be

examined by zymography. These studies would allow us to determine a role for MMP-2 in TNBC cell invasion, in addition to MMP-9.

Our results demonstrated that EGFR phosphorylation was diminished in TNBC cells depleted of fibulin-3. We could strengthen these results by also treating MDA-MB-231 cells with exogenous purified fibulin-3 and examining the phosphorylation of EGFR. The ability of KISS1R to transactivate EGFR *via* fibulin-3 remains unknown. Therefore transactivation experiments will be conducted as previously described [9,10], by stimulating TNBC cells depleted of fibulin-3 with kisspeptin-10 and examining phosphorylation of EGFR. Total EGFR expression also appeared to decrease in TNBC cells depleted of fibulin-3. Therefore, further knowledge regarding EGFR regulation could be obtained by examining total EGFR mRNA and protein expression in the TNBC cells depleted of fibulin-3. Depending on the results acquired, a potential transcriptional or translational mechanism could be further investigated to determine the mechanism by which fibulin-3 may be regulating EGFR in TNBC cells.

4.4 Conclusions

Our results demonstrate for the first time that KISS1R signaling stimulates fibulin-3 expression and secretion and that fibulin-3 positively regulates EGFR activation and KISS1R-induced cell invasion in TNBC (**Figure 4.1**). Additionally, this KISS1R-induced cell invasion occurs *via* fibulin-3 dependent stimulation of MMP-9 secretion and activity in an ERK-dependent manner. Patient data demonstrates that plasma fibulin-3 concentrations may be a useful marker of metastasis in TNBC. Thus, this study has identified fibulin-3 as a novel potential therapeutic target for prevention of human TNBC cell invasion and metastasis.

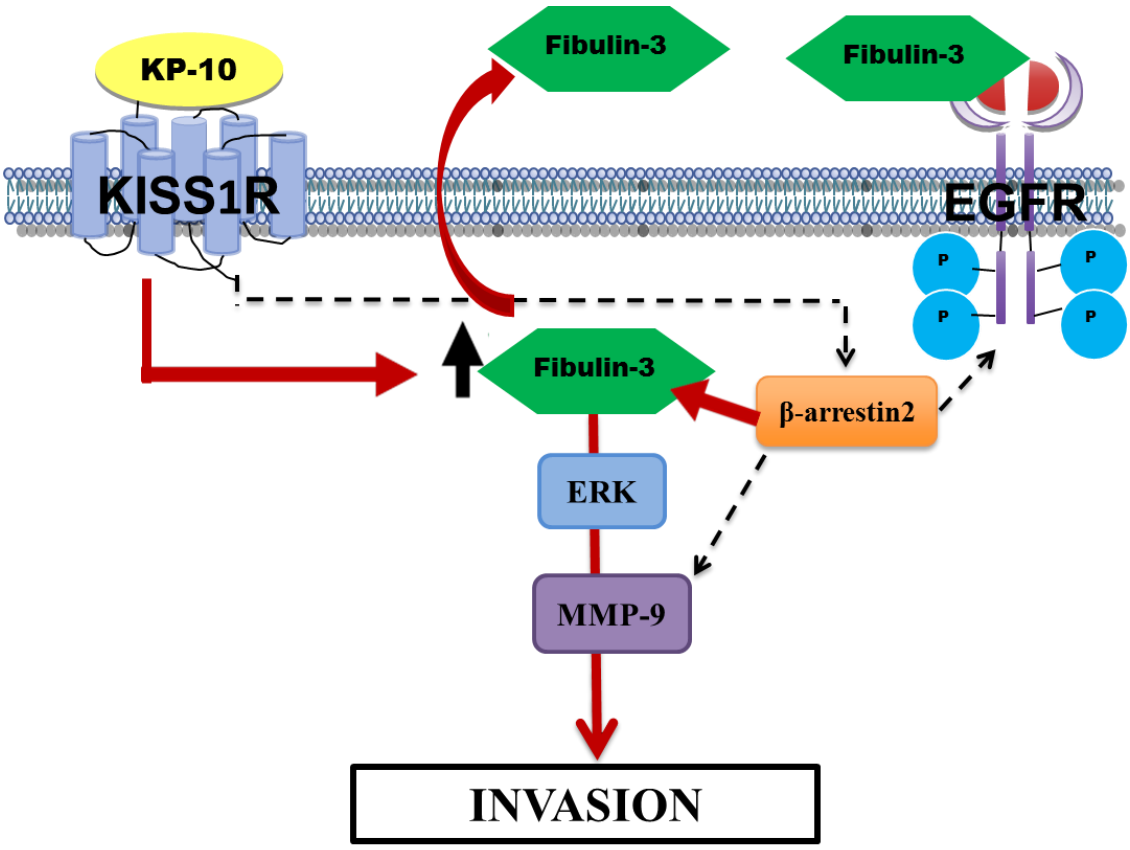

Figure 4.1. Our proposed model for fibulin-3 in regulating TNBC cell invasion. Previous studies in our lab have demonstrated that KISS1R activates EGFR *via* β-arrestin2 and that β-arrestin2 is required for KISS1R-induced MMP-9 activity and invasion in TNBC cells (black dashed arrows). Our current results (red arrows) demonstrate that KISS1R signaling stimulates fibulin-3 expression and secretion in TNBC cells. Additionally, we have found that β-arrestin2 also regulates fibulin-3 expression. We demonstrated that fibulin-3 regulates KISS1R-induced TNBC cell invasion *via* ERK and MMP-9 and regulates EGFR phosphorylation. Thus the ECM protein, fibulin-3 is a signaling partner for KISS1R in regulating TNBC cell invasion.

4.5 References

1. Katritch, V., Cherezov, V., & Stevens, R. C. (2013). Structure-function of the G protein-coupled receptor superfamily. *Annu Rev Pharmacol Toxicol, 53*, 531-556. doi: 10.1146/annurev-pharmtox-032112-135923
2. Premont, R. T., & Gainetdinov, R. R. (2007). Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol, 69*, 511-534. doi: 10.1146/annurev.physiol.69.022405.154731
3. Cattaneo, F., Guerra, G., Parisi, M., De Marinis, M., Tafuri, D., Cinelli, M., & Ammendola, R. (2014). Cell-surface receptors transactivation mediated by g protein-coupled receptors. *Int J Mol Sci, 15*(11), 19700-19728. doi: 10.3390/ijms151119700
4. Bhola, N. E., & Grandis, J. R. (2008). Crosstalk between G-protein-coupled receptors and epidermal growth factor receptor in cancer. *Front Biosci, 13*, 1857-1865.
5. Rodland, K. D., Bollinger, N., Ippolito, D., Opresko, L. K., Coffey, R. J., Zangar, R., & Wiley, H. S. (2008). Multiple mechanisms are responsible for transactivation of the epidermal growth factor receptor in mammary epithelial cells. *J Biol Chem, 283*(46), 31477-31487. doi: 10.1074/jbc.M800456200
6. Thomas, S. M., Bhola, N. E., Zhang, Q., Contrucci, S. C., Wentzel, A. L., Freilino, M. L., . . . Grandis, J. R. (2006). Cross-talk between G protein-coupled receptor and epidermal growth factor receptor signaling pathways contributes to growth and invasion of head and neck squamous cell carcinoma. *Cancer Res, 66*(24), 11831-11839. doi: 10.1158/0008-5472.can-06-2876
7. Siziopikou, K. P., Ariga, R., Proussaloglou, K. E., Gattuso, P., & Cobleigh, M. (2006). The challenging estrogen receptor-negative/ progesterone receptor-negative/HER-2-negative patient: a promising candidate for epidermal growth factor receptor-targeted therapy? *Breast J, 12*(4), 360-362. doi: 10.1111/j.1075-122X.2006.00276.x
8. Engelman, J. A., & Janne, P. A. (2008). Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res, 14*(10), 2895-2899. doi: 10.1158/1078-0432.ccr-07-2248
9. Zajac, M., Law, J., Cvetkovic, D. D., Pampillo, M., McColl, L., Pape, C., . . . Bhattacharya, M. (2011). GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness. *PLoS One, 6*(6), e21599. doi: 10.1371/journal.pone.0021599
10. Cvetkovic, D., Dragan, M., Leith, S. J., Mir, Z. M., Leong, H. S., Pampillo, M., . . . Bhattacharya, M. (2013). KISS1R induces invasiveness of estrogen receptor-negative human mammary epithelial and breast cancer cells. *Endocrinology, 154*(6), 1999-2014. doi: 10.1210/en.2012-2164
11. Sounni, N. E., & Noel, A. (2013). Targeting the tumor microenvironment for cancer therapy. *Clin Chem, 59*(1), 85-93. doi: 10.1373/clinchem.2012.185363
12. Albini, A., & Sporn, M. B. (2007). The tumour microenvironment as a target for chemoprevention. *Nat Rev Cancer, 7*(2), 139-147. doi: 10.1038/nrc2067
13. Mbeunkui, F., & Johann, D. J., Jr. (2009). Cancer and the tumor microenvironment: a review of an essential relationship. *Cancer Chemother Pharmacol, 63*(4), 571-582. doi: 10.1007/s00280-008-0881-9
14. Harisi, R., & Jeney, A. (2015). Extracellular matrix as target for antitumor therapy. *Onco Targets Ther, 8*, 1387-1398. doi: 10.2147/ott.s48883

15. Matsui, A., Ihara, T., Suda, H., Mikami, H., & Semba, K. (2013). Gene amplification: mechanisms and involvement in cancer. *Biomol Concepts, 4*(6), 567-582. doi: 10.1515/bmc-2013-0026
16. Tian, H., Liu, J., Chen, J., Gatza, M. L., & Blobe, G. C. (2015). Fibulin-3 is a novel TGF-beta pathway inhibitor in the breast cancer microenvironment. *Oncogene*. doi: 10.1038/onc.2015.13
17. Maier, T., Guell, M., & Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. *FEBS Lett, 583*(24), 3966-3973. doi: 10.1016/j.febslet.2009.10.036
18. Meric, F., & Hunt, K. K. (2002). Translation initiation in cancer: a novel target for therapy. *Mol Cancer Ther, 1*(11), 971-979.
19. Seminara, S. B., Messager, S., Chatzidaki, E. E., Thresher, R. R., Acierno, J. S., Jr., Shagoury, J. K., . . . Colledge, W. H. (2003). The GPR54 gene as a regulator of puberty. *N Engl J Med, 349*(17), 1614-1627. doi: 10.1056/NEJMoa035322
20. de Roux, N., Genin, E., Carel, J. C., Matsuda, F., Chaussain, J. L., & Milgrom, E. (2003). Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A, 100*(19), 10972-10976. doi: 10.1073/pnas.1834399100
21. Hunter, K. W., Crawford, N. P., & Alsarraj, J. (2008). Mechanisms of metastasis. *Breast Cancer Res, 10 Suppl 1*, S2. doi: 10.1186/bcr1988
22. Blobe, G. C., Schiemann, W. P., & Lodish, H. F. (2000). Role of transforming growth factor beta in human disease. *N Engl J Med, 342*(18), 1350-1358. doi: 10.1056/nejm200005043421807
23. Muraoka-Cook, R. S., Dumont, N., & Arteaga, C. L. (2005). Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res, 11*(2 Pt 2), 937s-943s.
24. Moses, H., & Barcellos-Hoff, M. H. (2011). TGF-beta biology in mammary development and breast cancer. *Cold Spring Harb Perspect Biol, 3*(1), a003277. doi: 10.1101/cshperspect.a003277
25. Muraoka-Cook, R. S., Kurokawa, H., Koh, Y., Forbes, J. T., Roebuck, L. R., Barcellos-Hoff, M. H., . . . Arteaga, C. L. (2004). Conditional overexpression of active transforming growth factor beta1 in vivo accelerates metastases of transgenic mammary tumors. *Cancer Res, 64*(24), 9002-9011. doi: 10.1158/0008-5472.can-04-2111
26. Siegel, P. M., Shu, W., Cardiff, R. D., Muller, W. J., & Massague, J. (2003). Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci U S A, 100*(14), 8430-8435. doi: 10.1073/pnas.0932636100
27. Tan, A. R., Alexe, G., & Reiss, M. (2009). Transforming growth factor-beta signaling: emerging stem cell target in metastatic breast cancer? *Breast Cancer Res Treat, 115*(3), 453-495. doi: 10.1007/s10549-008-0184-1
28. Hu, B., Thirtamara-Rajamani, K. K., Sim, H., & Viapiano, M. S. (2009). Fibulin-3 is uniquely upregulated in malignant gliomas and promotes tumor cell motility and invasion. *Mol Cancer Res, 7*(11), 1756-1770. doi: 10.1158/1541-7786.mcr-09-0207
29. Moulik, S., Pal, S., Biswas, J., & Chatterjee, A. (2014). Role of ERK in Modulating MMP 2 and MMP 9 with Respect to Tumour Invasiveness in Human Cancer Cell Line MCF-7 and MDA-MB-231. *Journal of Tumor, 2*(2).

30. Lonne, G. K., Masoumi, K. C., Lennartsson, J., & Larsson, C. (2009). Protein kinase Cdelta supports survival of MDA-MB-231 breast cancer cells by suppressing the ERK1/2 pathway. *J Biol Chem, 284*(48), 33456-33465. doi: 10.1074/jbc.M109.036186
31. Kozma, S. C., Bogaard, M. E., Buser, K., Saurer, S. M., Bos, J. L., Groner, B., & Hynes, N. E. (1987). The human c-Kirsten ras gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231. *Nucleic Acids Res, 15*(15), 5963-5971.
32. Camaj, P., Seeliger, H., Ischenko, I., Krebs, S., Blum, H., De Toni, E. N., . . . Bruns, C. J. (2009). EFEMP1 binds the EGF receptor and activates MAPK and Akt pathways in pancreatic carcinoma cells. *Biol Chem, 390*(12), 1293-1302. doi: 10.1515/bc.2009.140
33. Pampillo, M., Camuso, N., Taylor, J. E., Szereszewski, J. M., Ahow, M. R., Zajac, M., . . . Babwah, A. V. (2009). Regulation of GPR54 signaling by GRK2 and {beta}-arrestin. *Mol Endocrinol, 23*(12), 2060-2074. doi: 10.1210/me.2009-0013
34. Szereszewski, J. M., Pampillo, M., Ahow, M. R., Offermanns, S., Bhattacharya, M., & Babwah, A. V. (2010). GPR54 regulates ERK1/2 activity and hypothalamic gene expression in a Galpha(q/11) and beta-arrestin-dependent manner. *PLoS One, 5*(9), e12964. doi: 10.1371/journal.pone.0012964
35. Gan, Y., Shi, C., Inge, L., Hibner, M., Balducci, J., & Huang, Y. (2010). Differential roles of ERK and Akt pathways in regulation of EGFR-mediated signaling and motility in prostate cancer cells. *Oncogene, 29*(35), 4947-4958. doi: 10.1038/onc.2010.240
36. Petrillo, L. A., Wolf, D. M., Kapoun, A. M., Wang, N. J., Barczak, A., Xiao, Y., . . . Esserman, L. J. (2012). Xenografts faithfully recapitulate breast cancer-specific gene expression patterns of parent primary breast tumors. *Breast Cancer Res Treat, 135*(3), 913-922. doi: 10.1007/s10549-012-2226-y
37. Zhou, P. (2004). Determining protein half-lives. *Methods Mol Biol, 284*, 67-77. doi: 10.1385/1-59259-816-1:067
38. Kommaddi, R. P., & Shenoy, S. K. (2013). Arrestins and protein ubiquitination. *Prog Mol Biol Transl Sci, 118*, 175-204. doi: 10.1016/b978-0-12-394440-5.00007-3
39. Nawrocki Raby, B., Polette, M., Gilles, C., Clavel, C., Strumane, K., Matos, M., . . . Birembaut, P. (2001). Quantitative cell dispersion analysis: new test to measure tumor cell aggressiveness. *Int J Cancer, 93*(5), 644-652.

Appendices

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB 2014 Continuing Ethics Approval Notice

Date: April 24, 2015
Principal Investigator: Dr. Moshmi Bhattacharya
Department & Institution: Schulich School of Medicine and Dentistry\Physiology & Pharmacology,Western University

HSREB File Number: 104761
Study Title: The Role of Kisspeptin in Breast Cancer Progression and Metastasis
Sponsor: Canadian Institutes of Health Research

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2015/12/31
Expiry Date -2016/01/07

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Signature has been removed for thesis purposes

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer to Contact for Further Information

✓ Erika Basile	Grace Kelly	Mina Mekhail	Vikki Tran

This is an official document. Please retain the original in your files.

THESIS COPYRIGHT PERMISSION FORM

Title(s) of the Image(s): Terese Winslow LLC owns the copyright to the following image(s):

"Breast Cancer Stage IA" © 2012 Terese Winslow LLC, U.S. Govt. has certain rights

"Breast Cancer Stage IIA" © 2012 Terese Winslow LLC, U.S. Govt. has certain rights

"Breast Cancer Stage IIIA" © 2012 Terese Winslow LLC, U.S. Govt. has certain rights
"Breast Cancer Stage IV" © 2012 Terese Winslow LLC, U.S. Govt. has certain rights

Description of the Work: Terese Winslow LLC hereby grants permission to reproduce the above image(s) for use in the work specified:

Thesis title: Fibulin-3 Promotes Triple Negative Breast Cancer Cell Invasion

University: The University of Western Ontario

License Granted: Terese Winslow LLC hereby grants limited, non-exclusive worldwide print and electronic rights only for use in the Work specified. In addition Terese Winslow LLC grants Library and Archives Canada and ProQuest/UMI a non-exclusive license to reproduce, loan, distribute, or sell single copies of the Work by any means and in any form or format. **Images may not be used in any form or format outside the Work.** Terese Winslow LLC grants such rights "AS IS" without representation or warranty of any kind and shall have no liability in connection with such license.

Restrictions: Reproduction for use in any other work, derivative works, or by any third party by manual or electronic methods is prohibited, however, Ownership of original artwork, copyright, and all rights not specifically transferred herein remain the exclusive property of Terese Winslow LLC. Additional license(s) are required for ancillary usage(s).

Credit must be placed adjacent to the image(s) as follows:

For the National Cancer Institute © 2012 Terese Winslow LLC, U.S. Govt. has certain rights

Permission granted to:

Author name: Michelle Noonan

Author Signature (sign above line) Date

Terese Winslow, CMI, Member Date

Terese Winslow LLC Medical Illustration

ELSEVIER LICENSE
TERMS AND CONDITIONS
Jul 08, 2015

This is a License Agreement between Michelle Noonan ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited
Registered Company Number	1982084
Customer name	Michelle Noonan
Customer address	
License number	3664450869292
License date	Jul 08, 2015
Licensed content publisher	Elsevier
Licensed content publication	Cell
Licensed content title	Tumor Metastasis: Molecular Insights and Evolving Paradigms
Licensed content author	Scott Valastyan,Robert A. Weinberg
Licensed content date	14 October 2011
Licensed content volume number	147
Licensed content issue number	2
Number of pages	18
Start Page	275
End Page	292
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Figure 1
Title of your thesis/dissertation	The role of fibulin-3 in triple negative breast cancer

Expected completion date	Aug 2015
Estimated size (number of pages)	100
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 CAD
VAT/Local Sales Tax	0.00 CAD / 0.00 GBP
Total	0.00 CAD

Springer

PERMISSION LETTER

July 9, 2015

Springer reference

Cellular and Molecular Life Sciences

June 2009, Volume 66, Issue 11-12, pp 1890-1902

Date: 05 Feb 2009

Fibulins: Multiple roles in matrix structures and tissue functions

S. de Vega, T. Iwamoto, Y. Yamada

DOI 10.1007/s00018-009-8632-6

Print ISSN 1420-682X

Online ISSN 1420-9071

Journal number: 00018

Material to be used: Figure 1

Your project Requestor:	Michelle Noonan
University:	University of Western Ontario
Purpose:	Dissertation/Thesis

ELSEVIER LICENSE
TERMS AND CONDITIONS
Jul 08, 2015

This is a License Agreement between Michelle Noonan ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited
Registered Company Number	1982084
Customer name	Michelle Noonan
Customer address	
License number	3664460174219
License date	Jul 08, 2015
Licensed content publisher	Elsevier
Licensed content publication	Biochimica et Biophysica Acta (BBA) - Reviews on Cancer
Licensed content title	New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: Outside-in signaling and relationship to tumor progression
Licensed content author	Brigitte Bauvois
Licensed content date	January 2012
Licensed content volume number	1825
Licensed content issue number	1
Number of pages	8
Start Page	29
End Page	36
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this	No

Elsevier article?	
Will you be translating?	No
Original figure numbers	Figure 3
Title of your thesis/dissertation	The role of fibulin-3 in triple negative breast cancer
Expected completion date	Aug 2015
Estimated size (number of pages)	100
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 CAD
VAT/Local Sales Tax	0.00 CAD / 0.00 GBP
Total	0.00 CAD

Curriculum Vitae

MICHELLE NOONAN

EDUCATION:

University of Western Ontario, MSc 2015, Department of Physiology & Pharmacology
Supervisor: Dr. Moshmi Bhattacharya (Entrance Average 89%)

University of Western Ontario, BMSc 2013, Honors Specialization in Medical Sciences with distinction

PUBLICATIONS:

Calder M, Chan YM, Raj R, Pampillo M, Elbert A, **Noonan M**, Gillio-Meina C, Caligioni C, Berube NG, Bhattacharya M, Watson AJ, Seminara SB, Babwah AV (2014). Implantation Failure in Female Kiss1-/- Mice is Independent of their Hypogonadic State and can be partially rescued by Leukemia Inhibitory Factor. *Endocrinology, 155*(8), 3065-3078.

RESEARCH PRESENTATIONS:

June 2015 Department of Oncology Research and Education Day: **Noonan M,** Dragan M, Brackstone M, Babwah AV, Bhattacharya M. Role of fibulin-3 in triple negative breast cancer. – Poster Presentation

April 2015 London Health Research Day: **Noonan M,** Dragan M, Babwah AV, Bhattacharya M. Role of fibulin-3 in breast cancer. – Poster Presentation

Nov 2014 Windsor Cancer Research Group Biennial Conference: **Noonan M,** Dragan M, Babwah AV, Bhattacharya M. Role of fibulin-3 in breast cancer. – Poster Presentation

Nov 2014 Gowdey Lecture and Research Day: **Noonan M,** Dragan M, Babwah AV, Bhattacharya M. Role of fibulin-3 in breast cancer. – Poster Presentation

June 2014 Department of Oncology Research and Education Day: **Noonan M,** Dragan M, Bhattacharya M. Role of β-arrestin signaling in breast cancer cell survival and invasion. – Poster Presentation

March 2014 London Health Research Day: **Noonan M,** Dragan M, Bhattacharya M. Role of β-arrestin signaling in breast cancer cell survival and invasion. – Poster Presentation

AWARDS AND SCHOLARSHIPS:

Graduate

2014-2015 CIHR Cancer Research and Technology Transfer (CaRTT) Studentship
2014-2015 Translational Breast Cancer Research Studentship
2014 Lawson Internal Research Fund Studentship

2013- 2015 Western Graduate Research Scholarship

Undergraduate

2010-2013 Dean's Honor List
2009-2013 Received A.O Smith Scholarship
2010 Certificate of Academic Engagement
2009-2010 Received The Royal Canadian Legion Bursary
2009 Western Scholarship of Excellence

EMPLOYMENT:

2013-2015 Teaching Assistant, Department of Physiology and Pharmacology, Western
2012-2013 Weld Associate**,** Jefferson Elora Corporation, Elora
2010-2011 Concession Stand Supervisor, Elora Gorge Conservation Area, Elora
2007-2009 Concession Stand Operator, Elora Gorge Conservation Area, Elora

VOLUNTEER EXPERIENCE:

2014-2015 Department of Physiology and Pharmacology, UWO, SOGS Councilor
2014-2015 Bust a Move for Breast Health, London
2003-2015 Ladies Auxiliary, Royal Canadian Legion Br.229, Elora

CERTIFICATIONS AND TRAINING:

2014 Laser Safety Training
2014 Accessibility at Western (AODA) - Accessibility in Service
2013 St. John's Ambulance: Standard First Aid with CPR/AED Level C
2013 General Laboratory Safety and Hazardous Waste Management Training
2013 Mental Health Interactive Learning Module
2013 Safe Campus Community
2012 Advanced Rat Training
2012 Biosafety Training
2012 WHMIS Training