The Role of Osteopontin in Claudin-low Mammary Tumorigenesis

by

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A Thesis
Presented to
The Faculty of Graduate Studies
of
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Biomedical Science

Guelph, Ontario, Canada

ABSTRACT

THE ROLE OF OSTEOPONTIN IN CLAUDIN-LOW MAMMARY TUMORIGENESIS

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Osteopontin (OPN) is a cell-secreted phospho-glycoprotein that is implicated in both physiological and pathological processes in the body. Tumors of several cancer types, including breast cancer, exhibit high levels of OPN expression. Elevated expression of OPN in breast cancer is associated with increased tumor invasiveness and poor prognosis. Claudin-low breast cancer is the last molecular subtype of triple negative breast cancers to be identified; it has a prevalence of 7-14% and is associated with poor prognosis. Our laboratory screened for OPN expression in a murine model of claudin-low mammary tumors and found a 15-fold increase of OPN expression in claudin-low mammary tumors compared to normal mammary tissues. In this study, OPN downregulation via RNA interference (RNAi) reduced claudin-low tumor cell proliferation and survival. Additionally, short-term OPN knockdown impaired cellular migration and invasion in vitro. Furthermore, this study is the first to demonstrate OPN inhibition increases AKT and ERK1/2 kinase activity in claudin-low tumor cells. These data suggest that OPN may be a promising therapeutic target for triple negative breast cancers.

ACKNOWLEDGMENTS

I would like to take this opportunity to thank all those who helped me and provided me with guidance and support throughout my research journey.

To my advisor, Dr. Roger Moorehead, words cannot express my gratitude and gratefulness for you giving me the opportunity to join your research team and allowing me to pursue my dream. You have been a great mentor and I'm forever thankful for all your patience, guidance and support. Your research expertise and mentorship contributed greatly to my research success and influenced my future research goals.

I would also like to thank the other members of my advisory committee, Dr. Jim Petrik and Dr. Jonathan LaMarre. Thank you for all your support and contributions to my research experience; it was an honor to have you both on my committee.

I would also like to acknowledge all members of the Moorehead lab. First and foremost, Katrina Waston for being a great teacher, your mentorship and enthusiasm added a great flavour to my research experience. Other members, Robert Jones, Sarah Elizabeth Franks, Anthony Bruce, Leanne Stalker, Thomas Moran and Ritesh Briah, thank you all for being a wonderful team, it was a great pleasure to share my fantastic research experience with all of you.

Special thanks to members of the RHB lab, especially Monica Antenos and Ed Reyes, thank you for all the work you do to keep a safe and healthy lab environment. I am also grateful to have had the opportunity of meeting and working with the other graduate students in the Biomedical Science department.

Lastly, I would like to thank all my family, the ones across the ocean and the ones here and those who became family to me. Thank you all for being there for me, for helping stand every time I fall, for bringing me joy on the toughest days. I'm truly thankful to having you, family and friends, as the greatest treasure in my life, I couldn't have become who I'm today, without all of you.

DECLARATION OF WORK PERFORMED

I declare that all the work described in this thesis, with the exception of the items indicated below, was performed by me.

Devan Thompson established the most effective siRNA sequence out of the three OPN-targeting sequences tested. She also performed and analyzed the immunofluorescence staining of ki67 and cleaved caspase-3. The One Shot DH5-alpha competent bacteria cells used for DNA plasmid amplification was kindly donated by Dr. Jim Petrik.

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LIST OF ABBREVIATIONS

ABAM	Antibiotic-antimycotic
ANOVA	Analysis of variance
AKT	Protein kinase b
APS	Ammonium persulfate
Bcl-2	B-cell lymphoma gene-2
BSA	Bovine serum albumin
BRCA1/2	Breast Cancer Susceptibility gene family
BSP	Bone sialoprotein
BSPI	Sailoprotein I
BSPII	Sailoprotein II
BCT	Breast-conserving therapies
CCL5	Chemokine ligand 5
CD44	Cluster of differentiation 44
CT	Computed tomography scan
COX2	Cyclooxygenase 2
CBE	Clinical breast examination
DC	Ductal Carcinoma
Dc	Dendtritic cells
DMEM	Dulbecco's modified eagles medium
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal regulated kinases
EMT	Epithelial mesenchymal transition
ECM	Extracellular matrix
ErbB-2	Receptor tyrosine-protein kinase
ETOH	Ethanol
FAK	Focal adhesion kinase
FasL	Ligand of the fas death receptor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GC	guanine cystosine
HA	Hydroxyapatite
HCl	Hydrocloric acid
HIF	Hypoxia inducible factor
HepG2	Hepatocellular carcinoma cell line
HGF	Hepatocyte growth factor
HER2	Human epidermal growth factor receptor 2
iNOS	Inducible nitric oxide
IKK	IkB kinase
IL-12	Interleukin 12
IL-10	Interleukin 10
IDC	Invasive ductal carcinoma

ILC	Invasive lobular carcinoma
JAK2	Janus kinase 2
kDa	Kilo daltons
Ki-67	Antigen KI-67
LLC	Lewis lung carcinoma
LC	Lobular Carcinoma
LCIS	Lobular carcinoma in situ
LR	Local recurrence
MAPK	Mitogen activated protein kinase
MEK	MAPK/Erk Kinase
MSC	mesenchymal stromal cells
MMP	Matrix metalloproteinase
MMTV-LTR	Mammary tumor virus long terminal repeat
M	Distant metastasis
MRI	Magnetic resonance imaging
NaF	Sodium fluoride
NFkB	Nuclear factor kappa light chain enhancers of activated B cells
NK	Natural kill cells
NaV	Sodium orthovanadate
N	Lymph node involvement
OPN	Osteopontin
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor

PKC	Protein Kinase-C
PI3 Kinase	Phosphatidyl inositol kinase
PG2	Dependant prostaglandin E2
PMSF	Phenylmethanesulfony fluoride
rAAV	Recombinant adeno-associated virus
RANKL	Receptor activator of NF-kB ligand
RIPA	Radioimmunoprecipitation assay
RGD	Arginine glycine aspartate rich sequence
SDS	Sodium dodecyl sulfate
Src	Sarcoma
STS	Staurosporine
STAT-3	Signal Transducer and activator of transcription
TAF	Tumor associated fibroblasts
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TEMED	Tetramethylethylenediamine
Th1	T helper-type 1
TP53	Tumor Protein 53
TF	Transcription Factor
T cell	Lymphocytes
TGF	Transformin growth factor
T	Primary tumor size
V	Volts

VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
WAP	Whey acidic protein promoter

INTRODUCTION

Osteopontin (OPN) is a phospho-glycoprotein that is secreted by a number of cells in the body including specialized epithelial cells lining the mammary glands (Rangaswami et al. 2006). OPN plays a key role in a number of physiological and pathological processes and is heavily implicated in tumorigenesis (Rangaswami et al. 2006, Rittling and Chambers, 2004). Tumors of several breast cancer subtypes exhibit elevated levels of OPN and this has been linked to increased tumor malignancy and invasiveness (Brown et al. 1994, Rudland et al. 2002, Wang et al. 2003). Furthermore, high OPN serum levels in breast cancer patients is considered a prognostic marker of tumor invasiveness, therapy outcomes and overall survival (Tuck et al. 1998, Rodrigues et al. 2007).

Several in vitro and in vivo studies have addressed the effect of OPN expression on breast cancer cell behavior and have demonstrated that increased OPN is strongly associated with tumor progression. OPN has been shown to mediate breast cancer cell migration and induces cell invasion. These effects are seen in various in vitro breast cancer models with native and induced OPN overexpression (Liaw et al. 1995, Seftor et al. 1999, Rathinam and Alahari 2010). Increased OPN expression has also been shown to promote breast cancer cell survival and proliferation in vitro and tumor growth and metastasis in vivo (Allan et al. 2006, Zhang et al. 2014). For example, inhibiting OPN expression in MDA-MB-435 invasive breast cancer cell line significantly reduced tumor growth and metastasis in vivo (Dia et al. 2010). In addition to its role in promoting tumor progression, OPN has the capacity to render non-malignant mammary epithelial cells, malignant (Oates et al. 1996).

Breast cancer is an inclusive term for several molecularly distinct breast tumor subtypes that differ in their gene expression pattern and tumorigenic behavior. Of the five classified subtypes of breast cancer, claudin-low is the most recently identified; it has a prevalence of 7-14% and is associated with poor prognosis. Claudin-low tumors are enriched with tumor initiating cells and mesenchymal features (Prat et al. 2010, Sabatier et al. 2014). Based on accumulated evidence supporting a role for OPN in breast cancer, our laboratory screened for OPN expression in a murine model of claudin-low mammary tumors. DNA microarray analysis comparing recurrent claudin-low mammary tumors to normal mammary tissues revealed a remarkable increase in the expression of OPN, which was15-fold higher in the recurrent claudin-low tumors compared to normal mammary tissues. The differential OPN expression in recurrent-claudin-low tumors raised a fundamental question as to whether increased OPN expression has a role in claudin-low tumor progression.

Based on the literature and previously mentioned findings in our laboratory, we hypothesized that increased expression of OPN in claudin-low mammary tumors leads to increased tumor cell migration, invasion, proliferation and survival. To test this hypothesis, we examined the effect of OPN downregulation on the migration, invasion, proliferation and survival of a murine claudin-low tumor cell line (RJ348) in vitro. Transient knockdown of OPN via RNAi, significantly decreased RJ348 migration and impaired cell invasion in vitro. Moreover, OPN downregulation significantly reduced cellular proliferation and survival of RJ348 cells in vitro.

Furthermore, we sought to explore the long-term effects of OPN knockdown on claudin-low tumor growth and metastasis using the RJ348 cell line. Although cells were

successfully transfected with OPN-targeting plasmids, OPN expression levels were not downregulated in RJ348 cells. In conclusion, this study demonstrated that transient knockdown of OPN significantly decreased claudin-low tumor cell migration, invasion, proliferation and survival in vitro. Although we were unable to explore the effects of long-term knockdown of OPN, in vitro results of this study suggests that OPN is a promising molecular target for triple negative subtypes of breast cancer. Additionally, our claudin-low mammary cell line provides a valuable model to examine the effect of OPN knockdown on claudin-low tumor progression in immunocompetent mice. Considering the substantial implication of the immune system in claudin-low breast cancer progression, this model is particularly valuable as it provides a more clinically relevant pre-clinical model.

REVIEW OF THE LITREATURE

Breast Cancer

Statistics

Breast cancer is a major health burden that affects women worldwide. Based on The International Agency For Research on Cancer (IARC) latest release of the GLOBOCAN project, global tumor burden increased to 14.1 million new cases in the year of 2012. They also reported a drastic increase in breast cancer incidence rates making up ~25% of all cancer cases (GLOBOCAN, 2012). Breast cancer incidence rates vary across the globe with a slightly higher rate in less developed regions (883,000 cases), than more developed regions (794,000 cases). However, breast cancer remains the most frequently diagnosed cancer among women worldwide (Bray et al. 2004, GLOBOCAN, 2012, Jemal et al. 2011).

In Canada, breast cancer incidence rate hit its peak in the early to mid-1980's as a result of intensified mammography screening. Incidence rates then leveled off to ~0.6% increase per year in the late 1980's. On the other hand, breast cancer mortality rates have declined since the 1980's and continue to decline as a result of earlier detection and effective therapy. Nonetheless, breast cancer remains the second leading cause of cancer-related deaths among Canadian women (Canadian Cancer Society, 2014). On a global scale, breast cancer ranks as the second most frequent cause of cancer related-death among women and the fifth cause of death from cancer overall. However, breast cancer mortality rates vary across the globe with a slightly higher rate in less developed regions (14.3%) than more developed regions (15.4%). (GLOBOCAN, 2012).

Risk Factors

Sex

Gender is a critical factor for the risk of developing breast cancer. Females are at a greater risk of developing breast cancer than males. The estimated incidence rate of developing breast cancer among males is ~1 in a 100,000, making up less than 1% of all breast cancer cases. Females on the other hand, have a greater incidence rate (~99 in a 100,000) of developing breast cancer, making up more than 99% of all breast cancer cases (Bray et al. 2004, Candian Cancer Socity, 2014).

Age

The risk of developing breast cancer increases with age among women. Throughout an average woman's life, the risk of developing breast cancer is doubled every 10 years of age until she reaches menopause. Post menopause, the risk of developing breast cancer decreases drastically. Along with aging a number of other factors contribute to breast cancer risk including: the onset of menarche, age at first pregnancy and the onset of menopause (Ritte et al. 2013).

Age at Menarche and Menopause

Cycling reproductive hormones are critical risk factors of developing breast cancer. Women who are exposed to cycling reproductive hormones for a longer period are at a higher risk of developing breast cancer than those with a shorter exposure period. For instance, women who start menstruating at a younger age and reach menopause at a later age

are at higher risk of developing breast cancer (Ritte et al. 2013). However, studies have shown that earlier onset of menarche contributes more to the increased breast cancer risk than the late onset of menopause (Amir et al. 2010). This may be attributed to the higher levels of circulating estrogen during early adolescence, a developmental stage when mammary epithelial cells are most receptive to the mitogenic effects of estrogen. Such exposure may result in errors/mutations thereby increasing the risk of developing breast cancer (Britt, 2012).

Age at First Pregnancy and Breast-Feeding

Late-age pregnancy and lack of breast-feeding have also been shown to influence breast cancer incidence. Women who have their first child after the age of 30 have double the risk of developing breast cancer compared to those who have their first child before the age of 20 (Li et al. 2006). However, women at highest risk of developing breast cancer are those who have the first child after the age of 35. First pregnancy at late-age is associated with the development of specific histological types of breast cancer. Several studies have revealed that first-child bearing at late age is more strongly associated with the development of lobular breast carcinoma than with ductal carcinoma (Ursin et al. 2005, Bernstein et al. 2005, Li et al. 2006, Reeves et al. 2009, Newcomb et al. 2011). The association of increased risk of developing breast cancer with late age-pregnancy may be attributed to the temporal development of the mammary glands. Mammary epithelial cell proliferation and differentiation is influenced by pregnancy. Prior to pregnancy the mammary ducts and lobules remain undifferentiated and highly mitogenic in response to high estrogen and progesterone exposure. During pregnancy, however, the lobules and ducts start to

differentiate and mature in preparation for lactation. This differentiation alters cells in a number of ways including a decrease in proliferation (Ursin et al. 2005, Hennighausen et al. 2001). Thereby, having the first pregnancy at a late age delays mammary gland differentiation and promotes the mitogenic effect of estrogen and progesterone, which may increase the risk of developing breast cancer.

The lack of breastfeeding as well as short-duration breastfeeding are also contributing factors to the risk of developing breast cancer. Studies have shown that women who never breastfed or only started nursing after the age of 35 are at higher risk of developing breast cancer in comparison to women who breastfed at an earlier age (Bray et al. 2004). The duration of breastfeeding is also important. A collaborative reanalysis of over 47 epidemiological studies have shown that the relative risk of developing breast cancer decreases by 4.3% for every 12 months of breastfeeding. This particular study also suggests that the incidence of breast cancer in developed countries would be reduced by 42%, solely by increasing the duration of breastfeeding to an average of 12 months per child (Moller et al. 2002).

Family History

While age remains the primary risk factor for developing breast cancer, family history ranks second on the scale. Studies have shown that individuals with breast cancer have a 20-30% chance of having at least one relative with the disease. That being said, genetically inherited breast cancer only accounts for 5-10% of all breast cancer cases. A number of factors are associated with hereditary breast cancer risk; however, germline mutations in the breast cancer susceptibility gene family, BRCA1 and BRCA2 (BRCA1/2)

are the best-characterized (Edlich et al. 2005, Amir et al. 2010). It is estimated that BRCA carriers have a 40-90% lifetime risk of developing breast cancer. In addition, BRCA1 and BRCA2 mutations account for approximately 29% and 25%, respectively, of all cases in families with high breast cancer prevalence (Fackenthal and Olopade 2007). Mutations of the BRCA gene family have also been implicated in an increased risk of developing bilateral breast cancer (Narod et al. 2014).

Life Style

Lifestyle-associated risk factors, also known as modifiable risk factors, account for ~21% of all breast cancer deaths worldwide. Those factors include alcohol consumption, obesity, and lack of physical activity. Alcohol consumption both at an earlier and later age has been shown to be associated with increased risk of developing breast cancer (Brooks et al. 2013). Studies have shown that women who consume as low as 5 to 9.5g of alcohol daily, averaging 3-6 drinks per week, have a higher risk of developing breast cancer than those that do not consume alcohol. An additional 10% increase to the reported estimated risk of developing breast cancer is added with every 10g per day of alcohol intake (Chen et al. 2011).

On the other hand, obesity, especially in post-menopausal women, has been shown to be associated with an increased risk of developing breast cancer. For instance, post-menopausal women who lack heart rate training are at a higher risk of developing breast cancer along with increased weight, body mass index and waist circumference in

comparison to women who use heart rate training (Lahmann et al. 2004). Correspondingly, consistent physical activity has been shown to significantly reduce the risk of developing breast cancer in a dose-dependent manner. Dose-response analyses suggest that moderate physical activity; equivalent to 25 hours/week of basic recreation and household activity reduces the risk of breast cancer by 2%. Meanwhile, vigorous physical activity ranging from 4hr/week of walking or 1h/week of running reduces the risk of developing breast cancer by 5% (Wu et al. 2013).

Development

Nearly all breast cancers are adenocarcinomas originating from the mammary glandular system. Traditionally, breast cancers are classified based on tumor histopathology and the location of origin. The two most common histological types of breast cancer are ductal carcinoma (DC) and lobular carcinoma (LC). Both types are further divided into invasive, and non- invasive carcinomas: invasive ductal carcinoma (IDC); invasive lobular carcinoma (ILC); ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) respectively. Statistically, DC is the most common type, it accounts for ~80% of all breast cancers, while LC accounts for ~10% of all cases (Malhotra et al. 2010).

DCIS are the most common type of non-invasive breast tumors, they account for ~15% of all newly diagnosed breast cancer cases. DCIS tumors are characterized by the hyperproliferation of epithelial cells lining the ducts of the mammary gland. Majority of these tumors remain confined to the luminal layer of the ducts, as they are non-malignant. On the other hand, LCIS breast tumors are less common than DCIS, and are associated with less risk of developing invasive breast cancer. LCIS tumors are characterized by the

uncontrolled proliferation of cells lining the terminal ducts and ductules of the breast and they are often multifocal and bilateral tumors (Keshtgar et al. 2010).

IDC is the most common type of invasive breast cancer and it accounts for ~80% of all invasive breast cancer cases. IDC cells invade through the ductal basement membrane and spread into the surrounding tissue. This type of breast cancer also has a great potential to metastasize to other parts of the body (Weigelt et al. 2008). A less common type of invasive breast cancer is ILC which accounts only for ~10% of all invasive breast cancer cases. It is often difficult to distinguish between IDC and ILC histologically, however, immunohistochemistry staining for E-cadherin is usually used to determine the difference, since ILC lacks the expression of E-cadherin (Keshtgar et al. 2010).

There are other less common types of breast cancers including micropapillary carcinoma, mucinous carcinoma, tubular carcinoma, neuroendocrine carcinoma, metaplastic carcinoma, apocrine carcinoma, adenoid cystic carcinoma and medullary carcinoma. Majority of those carcinomas are invasive, however they have better prognosis that IDC and ILC. Together those types account for 5-7% of all invasive breast cancer cases (Weigelt et al. 2008)

Diagnosis

Breast cancer is often detected by clinical breast examination (CBE) and during routine mammograms. Less informative screening methods include self-breast examination. Physicians encourage women to perform self-breast examination monthly, mostly to get familiar with the normal anatomy of the breast and be aware of abnormal changes (Keshtgar

et al. 2010). However, self- breast examination is not an effective method to detect non-palpable masses. On the other hand, mammography is the first and most effective screening method for early, non-palpable breast tumors. A disadvantage of mammography, however, is that it doesn't distinguish between malignant and benign tumors, and has been shown to detect 35-45% of non-cancerous mammary calcifications (Smetherman and Dana, 2013). The high sensitivity of mammography to non-cancerous masses often results in tumor misclassification and false-positive diagnosis (Gotzsche and Jorgensen, 2013). Thus, the use of mammography is controversial, and often women are advised to weigh personal benefits and harms of mammography screening. The National Comprehensive Caner Network guidelines (2013) suggests annual screening for women at 40 years or older of average risk, and at 25 years for those at high risk. Diagnosis of a tumorous mass by clinical examination or mammography assessment is followed by core biopsies to confirm a tumor's malignancy.

Once malignancy is confirmed, sets of diagnostic assessments including Magnetic resonance imaging (MRI), ultrasound and computed tomography scan (CT) are carried out to examine the extent of tumor progression. Diagnostic assessments combining clinical examination, mammography and MRI has been shown to produce a more accurate tumor characterization than any other combination of tests (Berg et al. 2004, Rhodes et al. 2011). Patients are then clinically staged using the revised TNM breast cancer staging system. This system stages breast cancer from 0-IV based on primary tumor size (T), regional lymph node involvement (N) and distant metastasis (M). Stage 0 tumors are defined as being a pre-cancerous tumor, stage I refers to tumors that exhibit micro-invasion to surrounding tissue. Stage II tumors are classified based on the extent of tumor dissemination in lymph nodes.

Stage III, tumors are < 5cm in diameter with extensive lymph node involvement and stage IV refers to tumors with distant metastasis (Singletary et al. 2002).

Management

There are several therapeutic plans for breast cancer management including surgery, radiation therapy, chemotherapy and hormonal therapy. Treatment plans are assigned to patients based on tumor stage and type. Surgery is often used to eliminate tumors. For early stage breast cancers, breast-conserving therapies (BCT) such as lumpectomy and partial mastectomy are used. Studies have demonstrated that there is no difference in disease-free, metastasis-free, or overall survival between patients treated with total mastectomy or lumpectomy (Fisher et al. 2002A). As a result, breast-conserving therapies are recommended for early stage breast cancers (Fisher et al. 2002B). Having said that, total mastectomy is recommended for patients with high risk of recurrence including, patients previously diagnosed with breast cancer and carriers of the BRCA gene mutation (Shah et al. 2014).

In addition to surgery, radiation and adjuvant chemotherapy are also used for breast cancer management. Studies have demonstrated that applying radiation therapy post-surgical debulking reduces the risk of local recurrence (LR) by 50% in breast cancer patients. The Early Breast Cancer Trialists Collaborative Group has also demonstrated the importance of radiotherapy in multiple studies. In the first study, EBCTCG group analyzed data from 10 trials with 7300 cancer patients and found the risk of LR to be significantly reduced from 26% after lumpectomy alone to 7% after lumpectomy with radiation therapy; an absolute reduction of 19% (Clarke et al. 2005). They then further confirmed this association by

expanding their analysis to 17 randomized trials including 10801 breast cancer patients undergoing breast-conserving surgery with and without radiotherapy. Results demonstrated an absolute reduction of ~ 16% in LR in response to radiation therapy compared with those not receiving radiation (Darby et al. 2011). Similarly, administrating adjuvant chemotherapy post-surgery has been shown to decrease the annual relative risk of breast cancer relapse and mortality by 23% and 17% respectively (Early Breast Cancer Trialists' Collaborative Group, 2005). Commonly used conventional adjuvant chemotherapeutic agents include antimicrotubules, alkylating agents, antimetabolites and anthracyclines. There are also molecular-specific therapeutic agents that target specific hormone receptors and cancer-signature onco-proteins (Peppercorn et al. 2008). For example, tamoxifen is a hormone-receptor inhibitor; it is used for estrogen receptor positive tumors, which accounts for 70% of all breast cancers (Malhotra et al. 2010). Studies have demonstrated that treatment with tamoxifen for 5 years reduces the risk of recurrence by 41% and mortality by 34% (Early Breast Cancer Trialists' Collaborative Group, 2005). In contrast, aromatase inhibitors are used to inhibit estrogen production in postmenopausal women with early breast cancer. Anastrozole is a commonly used aromatase inhibitor and has been shown superior to tamoxifen when treating postmenopausal women in terms of disease-free survival, time for local recurrence and incidence of contra lateral cancer development (Cuzik et al 2010). Another molecular-target agent is trastuzumab, a monoclonal antibody targeting the extracellular domain of the Human Epidermal Growth Factor Receptor 2 (HER2) protein (Salmon et al. 2011). HER2 receptor is amplified in 15-25% of all breast cancers (Ravdin et al.1995). Studies have shown Trastuzumab to significantly improve the clinical outcomes of HER2 positive breast cancer therapy when used in combination with chemotherapy

compared with chemotherapy alone (Gianni et al. 2011). Neoadjuvant therapy is another form of breast cancer management that has been shown effective when used in combination with other treatment regimens. For example, Bevacizumab, an anti-angiogenic therapeutic molecule that targets Vascular Endothelial Growth Factor A, was recently shown effective as a neoadjuvant drug for breast cancer therapy (VEGF-A) (Valachis et al. 2010). The use of bevacizumab with docetaxel for HER-2 positive breast cancer prior to surgery resulted in a modest but significant increase in complete pathological response (Bear et al. 2012).

Subtypes of Breast Cancer

Earlier classification of breast cancers was mainly based on tumor histology and location of origin. However, with the ongoing research advancement, gene expression profiling became a critical technique to further categorize breast cancers into different molecular subtypes with distinct gene expression patterns that mirrors the diversity of clinical outcomes. At least five different molecular subtypes of breast cancer have been identified: luminal A; luminal B; HER2 enriched; basal-like and claudin-low type breast cancer. Luminal A and luminal B breast cancer subtypes make up the majority of all diagnosed breast cancer cases. Both subtypes are estrogen and progesterone-receptor positive which makes them response well to endocrine therapy. However, Luminal A and B tumors differ slightly in their gene expression pattern; luminal B tumors are often HER2-positive and express high levels of the proliferation marker Ki67. They are also frequently diagnosed with a higher grade than luminal A tumors (Malhotra et al. 2010)

In contrast, the HER2-enriched breast cancer subtype, as the name suggests, expresses high levels of HER2 and is typically estrogen and progesterone receptor negative.

HER2 enriched tumors make up about 12% - 20% of all breast cancer cases, they are high-grade tumors and are often associated with lymph node-metastasis. (Weigelt et al. 2008) In contrast, breast tumors that typically lack ER, PR and HER2 are referred to as triple negative breast cancer. Basal-like tumors, are a subtype of triple negative breast cancers and are characterized by the high expression of basal markers such as laminin, keratin-5 and keratin-17. They are high-grade tumors and are associated with poor prognosis (Peppercorn et al. 2008). Nevertheless, claudin-low subtype of breast cancer is the most recently identified triple negative subtype; it accounts for ~ 7-14% of all breast cancer cases. Claudin-low tumors are characterized by the low expression of luminal differentiation markers such as E-cadherin, claudin 3, 4 and 7 tight junction proteins and increased expression of epithelial-mesenchymal transition (EMT) and tumor initiating cell markers. Clinically, most of claudin-low tumors are ductal carcinoma that rarely response to endocrine therapy and are strongly associated with poor prognosis (Prat et al. 2010).

Modeling Breast Cancer

Cell Lines

The most commonly used modeling system of breast cancer is established murine and human breast cancer cell lines in vitro. The use of cell lines in vitro is advantageous because cell lines are relatively easy to handle, they provide an unlimited self-replicating supply and can be grown in infinite quantities. Additionally, established cell lines are relatively homogeneous, which makes their replacement feasible from frozen stocks in the case of cell loss. However, cell lines also have their disadvantages. For example, they are prone to phenotypic and genotypic drift during continual culturing. Such drift can be

attributed to cells' natural selection for clones with higher growth and survival rates in culture, which often results in phenotypic changes (Burdall et al. 2003). This could occur even in the most stable cell lines. Osborne and his colleagues were the first to demonstrate this phenomenon is the MCF-7 breast cancer cells obtained from different laboratories. Though cells were phenotypically identical; there was variation in cell growth rate, hormone receptor content and even karyotypes (Osborne et al. 1987). Another key disadvantage to the use of cell lines is limited clinical relevance, as isolated cell lines don't depict the nature of the primary tumors. The majority of breast cancer cell lines are isolated from tumor metastases via plural effusion or tumor aspiration. Thereby, isolated cells depict the nature of metastatic and extremely malignant tumors that likely differ from primary tumors. Studies conducted using those cell lines are often biased to more aggressive types of breast cancer and therefore less relevant to clinical cases. In addition, in vitro two-dimensional cell cultures lack the complexity of tumor environment and cells' interaction present in vivo (Holiday and Speirs, 2011).

Xenograft Models

Breast cancer xenograft models are derived from injecting human breast cancer cell lines into immune deficient mice. This type of breast cancer modeling was designed to overcome the limitation of in vitro models and understand the complex nature of tumor progression in vivo. Xenograft models allow researchers to explore crucial interactions between tumors and the host tissues including endocrinologic and tumor-stroma interactions (Clarke, 1996). Having said that, human xenograft models have a number of disadvantages. For example cells are injected into immune deficient mice, which decreases the model

relevance to clinical human breast cancers. Another limitation of this model is the difference between human and mouse stroma which has be shown to play a key role in tumor progression (Holiday and Sepirs, 2011). Apart from these pitfalls, breast cancer xenograft models often have a limited capacity to invade and metastasize. When they do metastasize, however, they mainly do so to the lungs, which is an uncommon site for human breast cancer metastases. To overcome this disadvantage, intravenous-site specific injections were developed to promote cells' colonization in specific organs. For example, intracarotid artery injections are used to study brain metastasis and left ventricle injection are used for metastasis to the bone. Cell lines that are commonly used in human xenograft models include the luminal A type MCF-7 and claudin-low type MDA-MB-231 cell lines. Other cell lines also used include BT474, luminal B type, MDA-MB-468, Basal-like type and MDA-MB-253 HER2 type breast cancer (Holiday and Sepire, 2011).

Transgenic Animal Models

Before transgenic mouse models were employed, there was a large gap in the literature on the basis and molecular determinants responsible for human breast cancer heterogeneity. However, the emergence of genetically engineered mice (GEM), helped identify and understand the role of genetic determinants on breast cancer initiation, progression and heterogeneity. The simplest forms of transgenic mouse models, GEM, are those with induction or amplification of a particular gene. Those models help address the role of a particular gene of interest in breast cancer pathogenesis (Huchinson and Muller, 2000). Several gain-of function transgenic models have been engineered to understand the role of proto-oncogenes including the c-myc, ErbB-2 and the cyclin D1 genes. In contrast,

knockout models are used to examine the role of key tumor suppressor genes including the p53 gene (Huchinson and Muller, 2000).

Gain of function transgenic models are established by using transgenic promoters that target transgene expression in the mammary glands. The two most commonly used transgenic promoters are the mouse mammary tumor virus long terminal repeat (MMTV-LTR) and the whey acidic protein promoter (WAP) (Behera et al. 2010). The MMTV-LTR is active throughout mammary development and its transcriptional activity increases during pregnancy (Muller et al. 1988, Pattengale et al.1989). Alternatively, the WAP promoter is only active during mid-pregnancy (Lipnik et al. 2005). This spatial difference in expression of MMTV-LTR and WAP promoters give raise to phenotypic and genotypic differences in each mouse model depending on the developmental stage of the individual mouse being examined. There are also other less commonly used promoters including the 5' flanking region of the C3 (1) component of the rat prostate steroid binding protein, beta-lactalbumin and metallothionein (Green et al. 2000, Palmiter et al. 1993).

Recent advancement in research has generated transgenic models with spatial and temporal control of gene expression. Those models have been generated to understand the role of a given gene at different stages of tumor progression. Transgene expression of a given gene can be modulated by the use of the bacteria-derived tetracycline-inducible system permitting the switching on or off (Tet-On/Tet-Off system) in a tissue- and time-specific manner. Similarly, ablation of a gene of interest in an inducible manner can be obtained by combining the Cre/loxP phage recombinase system with Tet-On/Off system (Fantozzi and Christofori, 2006).

Osteopontin

Osteopontin (OPN) is a secreted phospho-gylcoprotein that was first identified as a 60 kDa (Approx.) transformation-specific protein (Senger et al. 1979). During that time, OPN was also discovered in bone tissue along with another protein known as bone Sialoprotein. The two proteins were initially identified as bone extracellular matrix-associated proteins, and were named Sialoprotein I (BSPI), and Sialoprotein II (BSPII). The name Osteopontin was then given to the BSPI protein for its role as a bridge between cells and hydroxyapatite minerals through its RGD and polyaspartic motifs, that were discovered in the primary sequence of the protein (Prince et al. 1987, Zhang et al. 1990). OPN remains the most commonly used name when referring to this protein, in keeping with the nomenclature used for the human gene.

Thereafter, OPN was found present in several tissues in the body including bone, kidneys, vascular tissue, dentin hypertrophic cartilage, immune cells, and in the specialized epithelial cells of the mammary glands. It is also secreted in body fluids such as milk, urine, seminal fluid and plasma. Correspondingly, OPN was implicated in various physiological and pathological processes. However, soon after it was discovered, OPN gained the greatest emphasis on its implication in the process of tumorigenesis (rangaswami et al 2006).

Osteopontin Structure and Expression

The human Osteopontin gene is a single copy gene that maps to the long arm of chromosome 4 (4q13). It contains 7 exons and spans approximately 11.1kb in length (Hijiya et al. 1994). In contrast, the mouse Osteopontin gene spans ~4.8kb and maps to chromosome 5, on the locus of the Rickettsia resistance gene (Miyazaki et al. 1989). The mammalian

Osteopontin gene is well conserved among species. In humans, alternative splicing of the OPN mRNA give rise to three different splice variants of OPN. Those are OPN-a, the full length OPN, OPN-b lacking exon 5 and OPN-c lacking exon 4. Each variant is translated into a protein, with a predicted molecular weight of approximately 35kDa (Chae et al. 2009).

Translated OPN is composed of approximately 314 amino acid residues, and is subjected to various posttranslational modifications. Depending on the type of posttranslational modification, OPN protein molecular weight ranges from 34-75 kDa. The peptide's backbone is composed of multiple functional domains that are recognized by members of two main surface-receptor families, namely, integrin protein family and CD44 splice variants. OPN's N terminal fragment contains an arginine-glycine-aspartate rich sequence (RGD), a (SVVYGLR) sequence, a thrombin cleavage site and an aspartic acid rich sequence. In contrast, the C terminus of OPN contains a calcium binding site and a CD44 binding site (Bellahcene and Castronovo 1995). Although, the C terminus domain of OPN is able to bind CD44 surface receptor directly, the N terminal domain activity is thrombin cleavage-dependent; it requires cleavage and phosphorylation to activate the RGD motif and promote protein binding to members of the integrin family. OPN also acts as substrates for liver-transglutaminase and studies have shown a role for cross-linked OPN in mediating the process of extracellular matrix (ECM) remodeling (Prince et al. 1991).

Regulation of Osteopontin Gene Expression

Several transcription factors, growth factors and hormones regulate OPN gene expression. Transcription factors (TF) mediate OPN expression by activating specific response elements in the OPN promoter. OPN gene promoter contains a number of TF

response-elements including the TATA-like and CCAAT- like sequences; GATA-1; BRCA1; Runx2; vitamin D responsive motif as well as several AP, Ets and PEA recognition sites (Weber, 2001). For example, AP-1, Ets and Runx2 transcription factors have been shown to directly bind specific sites in the OPN promoter and induce OPN gene expression (Dsilva et al. 2012). Activated Ras has also been shown to activate OPN gene expression by binding to Ras-response element in the gene promoter and another independent element known as activated Ras enhancer site (Chambers et al. 1991, Guo et al. 1995). Thereby, the presence of a Ras enhancer element may contribute to the increased OPN expression in activated-Ras tumors (Denhardt et al. 2003).

Growth factors regulating OPN gene expression include transforming growth factor-beta (TGF-β), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). TGF- β promotes OPN gene expression via a multistep signaling system. The signaling cascade starts with TGF- β ligation to its type1 receptor, this induces the formation and translocation of Smad2-Smad3-Smad4 complex into the nucleus, where Smad3 binds to the OPN promoter (bases-179 to -229). Smad3 ligation is then followed by Smad4 binding to the transcription repressor Hoxa-9, a competitive ligand for OPN promoter; this blocks Hoxa-9- OPN interaction and induces OPN gene transcription (Shi et al. 2001). In contrast, epidermal growth factor (EGF) induces OPN expression via multiple pathways, including PI3K kinase and protein kinase-C (PKC). For example, in renal proximal tubule cells, induced expression of a dominant-negative PKC reduced the OPN expression levels in response to high glucose exposure. Meanwhile, renal proximal tubule cells expressing wild-type PKC showed a drastic increase in OPN expression in response to high glucose exposure (Hiseh et al.2006). Similarly, PI3K inhibitor-treated liver cells had a drastically lower OPN

expression in response to EGF treatment, which was shown to induce OPN overexpression in hepG2 liver cells (Zhang et al.2004). Vascular endothelial growth factors (VEGFs) also regulate OPN gene expression via its VEGFR1 receptor, which is also known as flt-1. For example, treating fibroblast cells with VEGF increased OPN expression in a dose dependent manner. In contrast, inhibiting the flt-1 receptor in those cells resulted in a significant decrease in VEGF and OPN expression (Li et al. 2012). However, these signaling pathways may be cell-type specific, and may not be involved in breast tumors. However, these findings serve as a platform for potential signaling pathways that may be involved in regulating OPN expression in breast tumors. Further studies are therefore needed to explore the implication of these pathways in OPN gene regulation in breast tumors.

Osteopontin Signaling

OPN has been shown to mediate multiple critical signaling pathways that are involved in cellular growth, survival and migration. As previously mentioned, OPN mediates its action by binding members of two cell surface-receptor families; integrin and CD44 family. For instance, OPN-integrin alphav-beta3 interaction mediates the activation of FAK, ERK1/2 and NF-kB signaling pathways, which induces cellular migration (Fong et al. 2009, Liu et al. 2014, Zou et al. 2013). OPN-integrin alphav-beta3 interaction is also implicated in modulating cellular proliferation and apoptosis via the activation of the STAT-3 signaling pathway. Signal Transducer and Activator of Transcription (STAT)-3 signaling pathways is a key regulator of cell cycle progression, cellular proliferation and apoptosis (Behera et al. 2010). OPN has also been shown to enhance VEGF expression via integrin alphav-beta3 and mediate VEGF-induced angiogenesis (Dias et al. 2009).

Role of Osteopontin in Physiological Processes

Bone Formation and Remodeling

OPN plays a key role in the homeostasis of bone resorption and remodeling. It is abundantly expressed in the bone tissue by osteoblasts, osteoclasts, and in the bone-matrix as a soluble-OPN. It mediates multiple processes in the bone tissue all of which play an important role in the homeostasis of bone resorption.

Part of OPN function in the bone tissue is mediating osteoclastogenesis; osteoclast differentiation; osteoclasts-bone tissue ligation and maintaining healthy bone matrix. Soluble-OPN in the bone matrix acts as a ligand for osteoclast binding to the bone tissue. Studies have shown that OPN mediates osteoclast attachment to mineralized bone tissue via integrin alphav-beta3 and CD44 surface receptors (Chellaiah and Hruska 2003). OPN also contributes to osteoclast generation and studies have demonstrated its importance for osteoclastogenesis in pathological states. For example, studies using organ culture systems have shown that bone culture lacking OPN have a significantly lower number of osteoclasts in comparison to normal bone culture. Additionally, knockdown of OPN in bone culture reduces osteoclastogenesis mediated by the receptor activator of NF-κB ligand (RANKL) signaling pathway (Ihara et al. 2001).

The role of OPN-RANKL signaling pathway in osteoclastogenesis was further supported by several studies. For instance, in vitro neutralization of OPN in arthritic joint cells suppresses the process of osteoclastogenesis, while the addition of recombinant OPN to

OPN-deficient cells enhanced the RANK-RANKL induced osteoclastogenesis (Ishii et al. 2004). In addition, OPN deficiency in OPN knockout mice prevent ovariectomy-induced osteoclastogenesis and result in impaired bone resorption (Yoshitake et al. 1999). Similar failure of osteoclastogenesis and bone resorption is observed in OPN-deficient mice post mechanical stress suspension, which is known to result in bone-mass and strength reduction (Ishijima et al. 2002).

Inflammation and Immunity

In the immune system OPN is expressed by several cell types including macrophages, neutrophils, natural kill cells (NK), lymphocytes (T cells) and dendritic cells (Dc). In accordance with its abundant expression by immune cells, OPN plays a key role in the immune response and mediates both innate and adaptive immunity. As a part of the innate immune response, OPN mediates mucosal defense against viral and bacterial pathogens. In innate immunity, OPN mechanism of defense stems from its differential regulation of macrophages IL-12 and IL-10 cytokine expression. This was demonstrated in multiple murine studies; in one study examining the effect of OPN on mucosal defense against viral pathogens, OPN- deficient mice suffered a prolong disease compared to wild-type mice, which showed elevated levels of OPN expression in the intestinal epithelial cells in response to rotavirus and had a better prognosis (Rollo et al. 2005). Similarly, Ashkar et al reported that OPN-deficient mice have an impaired T helper-type I (Th1) immunity and lack the ability to clear bacterial infections including Listeria monoctyogenes bacteria (Ashkar et al. 2000).

In addition to its regulation of macrophage-cytokines expression, OPN also mediates macrophage migration and survival at acute and chronic inflammation sites. OPN mediates migration by binding to integrin alpha4 and alpha9 via its SLAYGLR binding site. OPN-SLAYGLR interaction with integrin alpha4 also protects macrophages from apoptosis (Lund et al. 2013). Similar to its effect on macrophages, OPN also mediates neutrophil migration and recruitment by acting as a chemotactic cytokine. OPN binds neutrophil-associated integrin alpha9-beta1 and recruits neutrophil cells to an injury sites (Nishimichi et al. 2009). Thereby, OPN-deficient mice exhibit an impaired peritoneal recruitment of neutrophils in response to injury. However, the addition of exogenous OPN rescues the effect of OPN knockdown and resulted in a significant increase in neutrophils infiltration at an injury site (Koh et al. 2007, Atai et al. 2011).

In adaptive immunity, OPN mediates dendritic cells (Dc) differentiation and survival. As a result lack of OPN expression in Immature Dc cells inhibits their maturation. Additionally, neutralization of OPN in immature Dc cells inhibits the expression of key differentiation molecules and induces cell apoptosis (Kawamura et al. 2005).

In contrast to its pro-inflammatory role, OPN has also been shown to play a role in anti-inflammatory processes. It acts as a trans-repressor of the inducible nitric oxide synthase (iNOS) expression in macrophages. It does so by increasing proteasome depredation and ubiquitination of STAT1, which inhibits STAT1-induced iNOS gene transcription and protein expression (Guo et al.2007).

Mammary Gland Development

OPN is highly expressed in mammary epithelial cells and mammary gland-associated macrophages during pregnancy and lactation. OPN's expression in epithelial cells during pregnancy has been shown to play a key role in mammary gland maturation and alveolar development. For example, transgenic mice with knocked down OPN expression in the mammary epithelia had an impaired mammary gland-alveolar development. The lack of proper alveolar development further impaired the process of milk production. This is in part attributed to OPN's role in the synthesis of whey acidic milk protein (Nemir et al.2000). In fact OPN serves as a key component in the human secreted milk and is normally found in abundant amounts. OPN differential expression in human milk suggests an important role for OPN in an infants' physiological development. Additionally, it has also been hypothesized by a number of studies that OPN may play a key role in the infant's immunological defense development (Nagatomo et al. 2004, Maningat et al. 2009, Bai et al. 2012). The importance of OPN for infants' immunity may be attributed to its ability to activate Th1immunity, which is crucial for the innate immune defense in response to bacterial and viral infection (Ashkar et al. 2000). In addition, OPN may also be involved in infants' initial bone development.

Osteopontin and Tumorigenesis

In the past three decades and a half since its first description, OPN has been identified as tumor marker and is thought to play a key role in tumor progression and malignancy. OPN was first identified by Senger and coworkers (1979) as a phosphoprotein secreted in culture by transformed epithelial cells. Subsequent studies revealed high levels of OPN expression in tumor tissues of multiple origins including mammary tumors.

Bellahcène and Castronovo were the first to report high levels of OPN expression in malignant breast tumors. Results of their study showed OPN levels to be significantly higher in both in situ and invasive breast cancer lesions in comparison to normal breast tissues (Bellahcene and Castronovo 1995). After Bellahcene and Castronovo's report, researchers were interested in exploring the source of OPN in breast tumor tissues. Tuck et al (1998) evaluated OPN expression in 154 lymph-node negative breast tumors and found OPN mRNA and protein present in both infiltrating inflammatory and mammary tumor cells. They found that only 26% of tumor cells stained positive for OPN, meanwhile 70% of infiltrating inflammatory cells were positive for OPN (Tuck et al. 1998). This is not surprising, as different types of immune cells express OPN. However, the presence of multiple sources of OPN imposes an important question to whether OPN from different sources differentially regulate its signaling and function.

Increased OPN expression in mammary tumors is associated with increased invasiveness, poor prognosis and low survival. Tuck et al (1997) were the first to address this association in a study where they evaluated one patient's bilateral breast tumors. Extracted tumors from the two different breasts had similar histological characteristics; however, unlike the left-sided tumors, the right breast tumor had spread to the lymph nodes and was followed by local recurrence and then metastatic dissemination. Characterization of the primary tumors showed that only the right-sided primary tumors expressed high levels of OPN. Therefore, Tuck et al proposed that increased OPN expression in primary tumors may be associated with increased tumor invasiveness and metastasis (Tuck et al, 1997). However, this association was not conclusive due to the limited sample size of the study. Moving forward to drawing a more conclusive association, Chambers and her group used a

semi-quantitative scoring system to assess tumor cells IHC staining intensity for OPN in primary tumors of 154 lymph-node negative breast cancer patients. Univariate analysis revealed a strong association between OPN positivity in tumor cells and decreased disease-free survival and overall survival (Tuck et al. 1998). This inversed association was further supported by subsequent studies including Wang et al. (2003) and Rudland et al. (2002), who demonstrated that malignant tumors expressed significantly higher levels of OPN compared to benign and normal breast tissues. Rudland et al (2002) also demonstrated a negative association between tumor-OPN immunopositivity and patients' survival. These studies are further supported by other data that suggests a direct association between OPN expression and tumorigenesis. As a result, one may hypothesize that OPN is a key onco-protein that contributes to breast cancer invasiveness and metastasis.

Osteopontin Role in Tumor Growth and Survival

OPN is involved in promoting cell survival and tumor growth. As mentioned previously, OPN's N-terminal fragment contains an RGD motif that binds specifically to members of the integrin family. OPN's ligation to integrins triggers a variety of downstream effectors that mediate cell survival and tumor growth. However, the effect of OPN on STAT-3 signaling pathways is the best characterized. OPN binding to integrin alphav-beta3 activates the Signal Transducer and Activator of Transcription (STAT)-3 signaling pathway via the phosphorylation of Janus kinase2 (JAK2) signaling molecule. STAT-3 is a well-known onco-protein that regulates cell cycle progression, cellular proliferation and apoptosis and is upregulated in ~60% of all breast tumors. Neutralization of integrin alphav-beta3 receptor in breast cancer cells inhibits OPN-induced JAK2 phosphorylation and JAK2-

dependent nuclear-translocation and DNA binding of STAT-3. Results also demonstrated a significant decrease in STAT-3 activation and the expression its downstream targets, Cyclin D1 and Bcl2, which are key modulators of cell cycle progression. Additionally, OPN-induced breast tumor growth was significantly reduced in human xenograft models expressing mutant STAT-3 in comparison to tumors expressing wildtype STAT-3. In contrast, treating cells with OPN rescued breast tumor cells from staurosporine (STS)-induced apoptosis via the activation of JAK2-STAT-3 signaling pathway. The OPN-rescue effect seen was completely reversed in response to JAK2 inhibitors and mutant STAT-3 expression (Behera et al. 2010).

Moreover, OPN has also been shown to inhibit breast tumor cell apoptosis via the MAPK signaling pathway. In ME-C breast cancer cells, which are highly resistance to starvation- and Doxorubicin-induced apoptosis, OPN was the most differentially expressed anti-apoptotic protein in ME-C media compared to the media of the apoptotic-sensitive ME-A cells. Correspondingly, the addition of ME-C conditioned media to ME-A breast cancer cells significantly reduced their propensity to undergo apoptosis. However, co-treating ME-A cells with ME-C media concentrate and ERK1/2 inhibitor simultaneously restored ME-A apoptotic rate in response to serum deprivation and Doxorubicin treatment (Graessmann et al. 2006). This suggests a key role for OPN-ERK1/2 signaling pathway in promoting tumor cell survival and protects cells from apoptosis.

Osteopontin Role in Angiogenesis

Angiogenesis is the process of new vasculature formation that is crucial for sustained tumor growth and metastasis. It is a complex process that requires the interaction

of several pro-angiogenic factors, their receptors, extracellular proteins and adhesion molecules (Carmeliet and Jain 2011). OPN has been implicated in angiogenesis as a consequence of its ability to interact with integrin alphav-beta3, which is a known marker of angiogenesis (Liaw et al. 1995). Brooks and coworkers have shown that integrin alphav-beta3 expression is upregulated during angiogenesis and antagonist of this integrin inhibits the process of angiogenesis. Moreover, the introduction of integrin alphav-beta3 antagonist during angiogenesis induced apoptosis of the proliferative angiogenic vascular cells and impaired the formation of new vasculature (Brooks, Clark et al. 1994, Brooks, Montgomery et al. 1994). OPN is a known activator of integrin alphav-beta3 and has been shown to induce vascular regeneration post-injury by promoting endothelial cell migration and proliferation in vivo. Additionally, OPN and integrin alphav-beta3 mRNA levels correlated spatially and temporally with endothelial cell proliferation and migration (Liaw et al. 1995). OPN-integrin alphav-beta3 interaction has also been shown to enhance the process of angiogenesis via the activation of PI3K/Akt and ERK1/2 pathways. Treating endothelial cells with OPN also enhanced VEGF expression. OPN-induced VEGF is thought to signal a positive feedback loop where it activates Akt and ERK1/2 pathways and enhance the process of angiogenesis. Having said that, treating cells locally with anti-OPN antibody resulted in a greater negative effect on OPN-induced neovessel formation than anti-VEGF antibody in vivo. This suggests that VEGF partially participates in the process of OPN-induced angiogenesis (Dai et al. 2009).

Osteopontin Role in Migration and Invasion

OPN plays a critical role in two key processes that mediate tumor metastasis, namely, cell migration and cell invasion. OPN mediates cell migration by acting as a soluble chemokine in the ECM and as a ligand for various surface receptors involved in process of migration. Several members of the integrin receptor family mediate OPN-induced migration; however, integrin alphav-beta3 is the most commonly associated with OPN-induced migration of metastatic breast cancer cells (Liaw et al. 1995, Seftor et al. 1999, Rathinam and Alahari 2010).

OPN-integrin alphav-beta3 induced cell migration has been implicated in number of signaling pathways; however, the hepatocyte growth factor (HGF) and its receptor c-Met signaling pathway is the best characterized for breast cancer cell migration (Tuck et al. 2000). Interestingly, OPN and integrin alphav-beta3 are often found overexpressed in metastatic breast tumors and tumor-isolated cells compared to non-metastatic tumors/cells. For example, invasive breast cancer cell line MDA-MB-435 expresses high levels of OPN and also migrate towards soluble OPN via integrin alphav-beta3. Alternatively, non-metastatic breast cancer cell lines such as 21PT and 21NT, which lack integrin alphav-beta3 expression, use integrin alphav-beta1 and alphav-beta5 to migrate towards soluble OPN. Interestingly, All three breast cancer cell lines showed a significantly increased migration in response to rOPN treatment. Initially, OPN-induced migration was accompanied by an increase in c-Met kinase activity, then an increase in c-Met mRNA and protein expression was seen. (Tuck et al. 2000). Furthermore, OPN-integrin alphav-beta3 induced cell migration was also seen in several other cell types including, chondrosarcoma, gastric cancer cells and mesenchymal stem cells via a number of pathways, namely FAK, ERK1/2 and NF-kB (Fong et al. 2009, Liu et al.2014 , Zou et al. 2013).

Moreover, OPN also binds splice variants (CD44v3-v6) of the proteoglycan CD44 surface receptor. CD44 is heavily implicated in mediating cell migration. For example, OPN binding to CD44 binding mediates fibroblast cell migration and ablation of the OPN gene in OPN knockout models impairs CD44-dependent fibroblast migration (Zohar et al. 2000). Additionally, Gunthert and colleagues have demonstrated that overexpression of CD44v6 is sufficient to induce full metastatic behavior in pancreatic tumor cells that are normally non-metastatic (Gunthert, et al. 1991). OPN ligation to CD44 receptor has also been shown to mediate tumor cells migration by activating c-Met kinase and has been shown to induce hepatocellular cancer metastasis in vivo (Yoo et al. 2011).

In contrast to the process of migration, cellular invasion requires cells to degrade extracellular matrix and invade the surrounding tissue. OPN mediates cellular invasion and matrix degradation by upregulating two key protease families: the urokinase plasminogen activator (uPA) and matrix metallproteinases (MMPs). This was demonstrated in the late 1990s; Tuck et al (1999) were the first to report increased uPA expression and breast cancer cell invasion in response to recombinant OPN treatment. Thereafter, studies revealed that OPN mediates the secretion of uPA, MMP-2 and MMP-9 via integrin alphav-beta3-induced activation of PI3K/Akt and MAPK1/2 signaling pathways in breast cancer cells. More specifically, OPN-integrin alphav-beta3 activates PI3K/Akt signaling which then promotes IKK kinase activation. Once activated, IKK binds and degrades inhibitors of NF-kB, which then promotes NF-kB-induced expression of uPA and MMP-2 (Das et al. 2005). Accordingly, silencing OPN in hepatocellular carcinoma and gastric cancer cells significantly reduced OPN-NF-kB-mediated MMP-9, MMP-2 and uPA expression and impaired invasion in vitro and metastasis in vivo (Chen et al. 2011, Liu et al. 2014).

Osteopontin Role in Tumor Metastasis

Metastasis is the process of tumor cell dissemination to distant sites in the body. Formation of metastases requires tumor cells to alter their adhesion properties, invade the circulation system, extravagate into tissues, and proliferate at a secondary site (Woodhouse et al. 1997). OPN's role in mediating metastasis has been attributed to its ability to alter cell-adhesion properties and to promote cell migration, invasion and anchorage-independent survival (Tuck et al. 2007). OPN has also been shown to induce a metastatic phenotype in otherwise benign tumor cells (Oates et al. 1996). Moreover, subsequent studies have demonstrated OPN's critical role in mediating tumor metastasis. For example, transfecting mammary tumor cells with OPN resulted in increased tumor cell anchorage-independent survival and increased RGD-dependent adhesion in vitro. In vivo, those cells showed increased lymphovascular invasion, increased lymph node metastasis and lung micro-metastasis in comparison to control transfected cells (Allan et al. 2006). In contrast, knockdown of endogenous OPN reduces tumor cell invasiveness, migration and anchorage-independent growth in vitro and suppresses tumor formation and metastasis in vivo (Adwan et al. 2004, Shevde et al. 2006).

Osteopontin Role in Tumor Microenvironment

OPN is expressed by a variety of cell types including tumor cells, stromal cells and immune-system cells present in the tumor environment. Stromal cells in the tumor microenvironment play a key role in homing tumor cells and influencing tumor behavior via

signaling proteins and cytokines (Allinen et al. 2004). In particular, tumor-associated fibroblasts (TAF) are known to promote tumor growth, angiogenesis and metastasis. Recently it was demonstrated that tumor-derived OPN facilitates breast tumor metastasis by promoting the transformation of mesenchymal stromal cells (MSC) into TAF (Mi et al. 2011). Tumor-derived OPN binds to the integrin surface receptors of MSC and trans-activates the c-Jun pathway, which induces the expression of metastasis-associated cytokine known as CCL5. Additionally, OPN-induced expression of CCL5 was strongly associated with increased tumor metastasis to the lungs. MSC cells extracted from metastatic sites showed an OPN-dependent expression of CCL5 and TAF cell markers including α-smooth muscle actin, tenascin-c, CXCL12 (or stromal cell-derived factor 1), fibroblast-specific protein-1, MMP-2 and MMP-9 (Mi et al. 2011). On the contrary, stromal-derived OPN is implicated in promoting cellular neoplastic transformation and the expansion of initiated tumor cells in early lesions. For example, Luo et al have demonstrated that stromal- derived OPN promotes pre-neoplastic keratinocytes proliferation and survival through the CD44 receptor and MAPK pathway (Luo et al. 2011). Subsequent studies revealed that TAFs are the main source of stromal-derived OPN and that OPN expression is a determining factor to their role in promoting tumor progression (Liu et al. 2012). This suggests that different cell types contribute to the presence of OPN in a tumor environment. The increased OPN expression by different cells in the tumor environment may further enhance OPN effect on tumor progression and metastasis.

In a tumor microenvironment, OPN's primary immune target is macrophages. OPN interaction with macrophages promotes a number of processes including cytokine expression, angiogenesis, and cell migration (Jain et al. 2008, Kale et al. 2013). OPN is

known to bind integrin alpha9-beta1 present on macrophages and activate MAPK signaling pathway. Activated MAP kinase induces cyclooxygenase-2 (COX-2)-dependent prostaglandin E2 (PG2) expression. PG2 promotes both endothelial cells migration and tumor cell migration. In contrast, OPN has been shown to induce the activation of tumor associated T-cells and natural killers (NK) cells. Having said that, OPN is also able to inhibit T–cells and NK cells-induced apoptosis (Tuck et al. 2007).

RATIONALE

In the past three decades and a half since it was first identified, OPN has gained considerable interest as a potential molecular target for breast cancer therapy. This is in part attributed to the accumulated supporting evidence implicating OPN tumor progression of several cancer types including breast cancer. High levels of OPN expression in primary tumor tissues and plasma of breast cancer patients is associated with increased tumor burden, increased metastasis and poor prognosis (Chakraborty et al. 2006; Tuck et al., 2000, Weber et al., 2010). Corresponding experimental studies have demonstrated that knockdown of OPN significantly decreases mammary tumor cell proliferation, survival and migration in vitro and tumor growth and metastasis in vivo (Dai et al. 2010, Shevde et al. 2006). Claudin-low subtype of breast cancer was the lastly identified molecular subtype of triple negative cancers. It has a prevalence of 7-14% and is associated with poor prognosis. Our laboratory assessed OPN's expression in a murine claudin-low mammary model and has determined that murine mammary tumors express significantly higher levels of OPN, 15-fold higher, than normal mammary tissues (Campbell et al. 2011). This differential expression of OPN furthered our interest in exploring OPN's implication in claudin-low tumor progression. Thus, the purpose of this study is to characterize the role of OPN in claudin-low mammary tumor progression.

Based on the literature and previous findings in our lab, the following was hypothesized:

High expression levels of OPN are associated with increased proliferation, migration and survival of the murine mammary claudin-low cell line, RJ348.

To test this hypothesis three objectives were identified:

Objective 1: Downregulate OPN expressions in RJ348 cells and examine cellular proliferation, survival and migration in vitro

Objective 2: Investigate potential molecular pathways involved in OPN signaling in RJ348 cells including its main receptor integrin Alphav and other downstream effectors

Objective 3: Establish stable OPN knockdown in RJ348 cells and examine its long-term effect on cellular migration and invasion in vitro.

Model System

A murine claudin-low mammary tumor cell line isolated in our lab named RJ348 is utilized in this project. RJ348 mammary tumor cells were isolated from a recurrent tumor derived from a MTB-IGFIR transgenic mouse. Briefly, MTB-IGFIR transgenic mice overexpress the full-length human type I insulin-like growth factor receptor (IGF-IR) cDNA specifically in the mammary epithelia in a doxycycline-dependent manner. Overexpression of the IGF-IR resulted in palpable mammary tumor formation by 10 weeks of age. The majority of established tumors regressed in response to IGF-IR downregulation; however, approximately 21% of the mammary tumors recurred in the absence of IGF-IR overexpression (Jones et al. 2007). DNA microarray analysis of the recurrent mammary tumors revealed a 15-fold increase in OPN gene expression compared to normal mammary tissue. Thus, the focus of this study is to characterize the role of OPN in a mammary tumor cell line (RJ348) established from a recurrent mammary tumor.

MATERIALS AND METHODS

Cell Culture

The RJ348 murine mammary tumour cells were grown in Dulbecco's modified eagle medium (DMEM) (GIBCO, Burlington, ON) containing the following supplements: 10% tetracycline-free fetal bovine serum (FBS) (Clontech, Mountain View, CA), 1mM sodium pyruvate (GIBCO, Burlington, ON), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (GIBCO, Burlington, ON) 4 mM glutamine (GIBCO, Burlington, CA), 2 mM hydrocortisone, 5 μg/ml estrogen, 5 μg/ml prolactin, 10 μg/ml EGF, 10 μg/ml insulin, and 1% antibiotic-antimycotic (GIBCO, Burlington, ON). For experiments utilizing recombinant OPN(R&D systems, Burlington, ON) concentration of 5μg/ml was utilized to treat RJ348 cells. Cells were maintained at 37°C and 5% carbon dioxide.

Western Blot Analysis

RJ348 cells were lysed and homogenized in lysis buffer (Appendix II). Homogenous cells' lysates were centrifuged at 14,000-xg for 20 minutes at 4°C, and proteins samples were then reclaimed. Protein concentration was quantified using a Bradford assay (Bio-Rad, Hercules, CA, USA) (Bradford, 1976). Protein lysates (40 -60 μg of protein) were reduced in reducing buffer (Appendix II) and separated using 10% sodium dodecyl sulfate (SDS)- polyacrylamide gels. Separation was achieved using an Xcell II min cell system (Invitrogen, Burlington, ON, Canada) by applying 127V for ~ 2.5 hours to the system. Proteins were then transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) by applying 27V for

approximately 2.5 hours using a wet transfer method. The membranes were then blocked in 5% skim milk in Tris-buffered saline containing 0.01% Tween 20 (TBST) at 4°C overnight. The blots were then incubated in a 1:500 dilution of primary antibody for OPN, integrin alphav and integrin beta-3 and a 1:1000 dilution of primary antibody for b-actin, ERK1/2, pERK1/2, Akt, and pAkt in 5% milk in TBST at 4°C overnight. Primary antibodies utilized include anti-OPN (AKm2A1), Anti-integrin beta-3 (C-20) from Santa Cruz Technologies (Santa Cruz, MA, USA), anti-integrin alphav (ab76609) from Abcam Inc (Cambridge, MA), anti-phospho-Akt (Ser473), p44/42 MAPK (Erk1/2) and anti-β-actin all were obtained from Cell Signalling Technology (Beverly, MA, USA). Blots were then washed three times in TBST for a period of ~10 minutes and incubated in a 1:2000 dilution of the appropriate secondary antibody in 5% milk in TBST – anti-mouse for OPN (Cell Signalling Technology, Beverly, MA, USA), anti-goats for integrin beta-3 (Santa Cruz Technologies, Santa Cruz, MA, USA) and anti-rabbit for the rest of the antibodies (Cell Signalling Technology, Beverly, MA, USA). The blots were then washed three times for 10 minutes in TBST. Western Lightning Chemiluminescence substrate (Perkin Elmer, Wellesley, MA, USA) and Clarity Western ECL Substrate (BIO-RAD, Mississauga, ON) were applied to the membrane for a period of 1 minute. Protein detection was imaged on a FluorChem 9900 gel documentation imaging system (Alpha Innotech, San Leandro, CA, USA) and ChemiDoc™ XRS+ System (BIO-RAD, Mississauga, ON).

Transient Knockdown (RNAi)

RJ348 cells were transfected with one of three stealth RNAi sequences directed against OPN or Integrin alphav and a guanine-cytosine (GC) control sequence for a negative controls . All sequences were obtained from Invitrogen (Burlington, ON, Canada) and were used at a final concentration of 100 nM (Burlington, ON, Canada). Cells were transfected using Lipofectamine 2000 transfection reagent (5ul) and Opti-MEM media (500ul) (Invitrogen, Burlington, ON, Canada). Approximately 5 to 6 hours post transfection media was replaced with RJ348 media and cells were incubated at 37°C with 5% carbon dioxide. Cell lysates were collected 24, 48 and 72 hours post-transfection.

RNA Extraction

RJ348 cells were grown in 60 mm culture plates, transfected with targeted-siRNA or GC control (as previously described) and subsequently total RNA was isolated from cells following the *mir*Vana miRNA Isolation Kit instructions (Ambion, Austin, TX, USA). Briefly, cells were lysed using lysis buffer solution and stabilized with a homogenate additive. Post homogenization, acid-phenol, chloroform, was added to the lysate suspension, and samples were then centrifuged at 10,000xg for 5 minutes at room temperature. Thereafter, the aqueous phase was recovered and 100% ethanol was added to the recovered samples. The lysate-ethanol mixture was then passed through a glass-fibre filter, and subsequently washed with two different low-ionic strength working solutions mixed with 100% ethanol. Finally, 100µl of RNase free water heated to 95°C was added to filters and then spun at 10,000 x g for 30 seconds to recover the RNA. The concentration of RNA

(ng/µl) for each sample was measured using the NanoDrop (Thermo Scientific, Wilmington, DE, USA)

Reverse Transcription

Post quantification, equal concentration of RNA samples were added to nuclease-free microcentrifuge tubes with the following components: 1 µl Oligo(dT) 12-18 Primer, 1 µl dNTP mix, and up to 12 µl of nuclease-free water. The mixture was transferred to a PTC- 100 Peltier Thermal Cycler (Bio-Rad, Mississauga, ON), heated to 65°C for 5 minutes and then a master mixture solution was added to each tube, master mixture is composed of the following: 4 µl 5X First-Strand Buffer, 2 µl 0.1 M DTT, and 1 µl RNasin. The mixtures were then incubated at 42°C for 2 min, followed by the addition of 1 µl of SuperScript II Reverse Transcriptase to each tube. The samples were left to incubate at 42°C for 50 minutes, followed by 70°C for 15 minutes to inactivate the reaction. Final samples are then either utilized for RT-PCR or stored in −20

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was used to measure OPN and integrin alphav expression in RJ348 cells treated with OPN and alphav-targeting RNAi. qRT-PCR was carried out on CFX 96-touch RT-PCR detection system (Bio Rad, Mississauga, ON), using a Platinum SYBR Green. The appropriate concentrations of cDNA produced in the reverse transcription step above were amplified in a 20ul reaction volume with ul of cDNA and 19ul of primer-specific prepared master mixture solution containing 7ul of RNAase free water; 1ul of BSA, 1ul of the appropriate primer and 10ul of Plantinum SYBR Green. Reaction mixtures were then amplified with the following qPCR parameters: Presoaking at 95° C

for 10 minutes followed by 42 amplification cycles of denaturation at 95° C for 10 s, annealing at 60° C for 10 s and elongation at 72 ° C for 10 s. Lastly, a melting curve was generated by taking fluorescent measurements every 0.2° C for 15 s from 55° C until 95° C to ensure a single PCR product. Efficiency of each primer set was determined using 10-fold serial dilutions of a representative cDNA sample and expression of each gene was then normalized to HPRT mRNA levels with efficiency correction using CFX manger software (Bio Rad, Mississauga, ON).

Scratch Wound Migration Assay

RJ348 cells where seeded in 6-well cell culture dishes and grown to ~50-60% confluency. Cells were then transfected with targeted-RNAi, or a GC control sequence as previously described. Using a pipette tip, a scratch wound was created down the centre of each plate 48 hours following the transfection. Pictures were taken using an inverted light microscope (Olympus IX71inverted microscope) equipped with Q imaging micropublisher 3.3 RTV imaging system every 12 hours, until the scratch wound was no longer visible. Image J software was used to quantify the decrease in wound/gap area (area from one edge of the wound to the other) and Excel software was used to quantify the % of wound closure using the following formula: % of wound closure= [(area at=0hrs –area at=Δhrs)/ area at=0h]×100%).

Trans-well Invasion Assay

Trans-well Falcon inserts with 8.0um pore size coated with matrigel were purchased from BD Bioscience (BD Bioscience, Mississauga, ON, Canada). Approximately 3000 cells were suspended in 200ul of serum and growth factor/hormone-free RJ348 media in the top insert. The bottom well under the insert was filled with 300ul of RJ348 media containing serum, growth factors and hormones. Cells were cultured at 37°C and 5% carbon dioxide for 48 hours. Media was then aspirated from the lower chamber. The bottom of the insert was fixed with 5% glutaraldehyde in 1xPBS, for 10 minutes, washed with water and stained with 0.5 % toluidine blue staining solution for 10-20 minutes at room temperature. The inner surface of the upper chamber was then wiped clean and cells that had migrated to the bottom of the insert were visualized using a motorized inverted system microscope (Olympus IX81, IX2 series) equipped with image pro plus imaging system.

Immunofluorescence

RJ348 Cells were plated onto sterile coverslips and grown to approximately 60-80% confluency. RNAi was performed and after 24 hours cells were washed with PBS and fixed for 1 hour at room temperature in 10% buffered formalin. Coverslips with fixed cells were then washed with PBS and permeabilized with 0.1% Triton X in PBS for 10 minutes at room temperature (this step was not performed for Annexin V antibody staining). Fixed cells were then washed once again with PBS, blocked in 5% BSA for 10 minutes, and then incubated overnight at 4°C with the primary antibody. Antibodies included a 1:200 dilution of Ki67 (Abcam, Cambridge, MA, USA) and anti-caspase3 cleaved form (Millipore, Etobicoke, ON, Canada). A 1:100 dilution of rabbit

secondary antibody conjugated to Alexa-fluor 488 (green) (Invitrogen, Burlington, ON, Canada) for both Ki67 and cleaved caspase-3 was applied for 1 hour at room temperature. Cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, Oakville, ON). Prolong Gold (Invitrogen, Burlington, ON, Canada) was used to suppress photobleaching and preserve the fluorescent signal. Images were captured with an Olympus BX-61 fluorescent microscope equipped with MetaMorph imaging software system. Positively stained cells were then counted manually.

Stable Transfections

Lenti-vector plasmids containing siRNA targeting the OPN gene were purchased from the Applied Biological Materials Inc (Applied Biological Materials Inc, BC, Canada). All four plasmids contain a CMV promoter, a GFP tag and a puromycin selection marker. Lenti-vector plasmid containing scrambled siRNA strand was used as a negative control. Cells were transfected with either OPN targeting plasmids or negative control plasmids at a final concentration of 10ug DNA per 10cm culture dish. Cells were transfected using Lipofectamine 2000 (30ul) and opti-MEM media (3ml) (Invitrogen, Burlington, ON, Canada). Six hours post transfection; media was replaced with RJ348 media and cells were incubated at 37°C and 5% carbon dioxide. Puromycin kill curve was generated for RJ348 cells which demonstrated an optimal selection concentration of 7ug/ml of puromycin. Therefore, transfected cells were selected using 7ug/ml of media of puromycin.

Puromycin resistant cells were then selected. Western blot and RT-PCR analysis were used to determine the degree of OPN knockdown.

Statistics

The student's t test was used to compare two means and an analysis of variance (One Way ANOVA) followed by a Tukey's HSD test was used to compare multiple means. Differences were considered to be significant at $p < 0.05$.

RESULTS

RJ348 Cells Express OPN

OPN protein levels were initially examined in RJ348 cells. Western blot analysis detected two protein isoforms in RJ348 cells' lysate weighting ~58kDa and ~62kDa relative to a protein molecular weight standard (Figure 1).

OPN Expression Downregulated by RNA Interference (Transient Knockdown)

RNAi reduced OPN expression in RJ348 cells. Previous experiments in our lab showed three different OPN-targeting RNAi oligonucleotide sequences namely, RNAi 1, RNAi 2 and RNAi 3 capable of reducing OPN protein levels. RNAi 1, however, induced the greatest knockdown and therefore was used in this study. Changes in OPN protein levels following treatment with OPN RNAi 1 are shown in figure 2A. Densitometric analysis revealed a visible knockdown of OPN in OPN RNAi treated cells in comparison to cells treated with guanine-cytosine (GC) control sequence. Knockdown efficiency was approximately 74%, 24 hours post transfection; 89%, 48 hours post transfection; and 88%, 72 hours post transfection (Figure 2B). Furthermore, RT-PCR analysis showed a significant knockdown of OPN mRNA levels, 48 hours post transfection in RJ348 cells treated with OPN RNAi compared to GC control treated cells (Figure 2C). Based on these results all subsequent experiments were carried out at 48 hours post transfection, unless otherwise indicated.

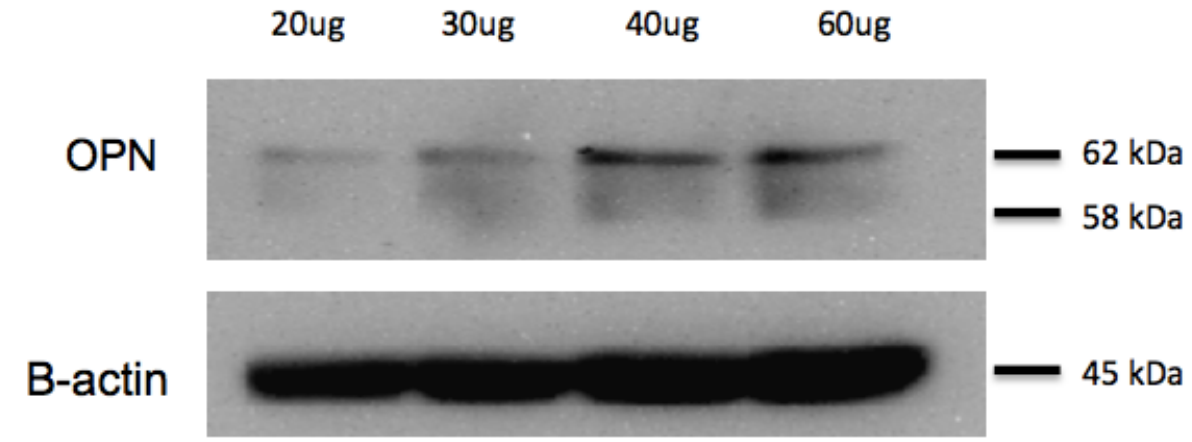

Figure 1. Western blot analysis of OPN protein levels expressed by RJ348 cells. Protein concentrations loaded were 20ug, 30ug, 40ug and 60ug per lane.

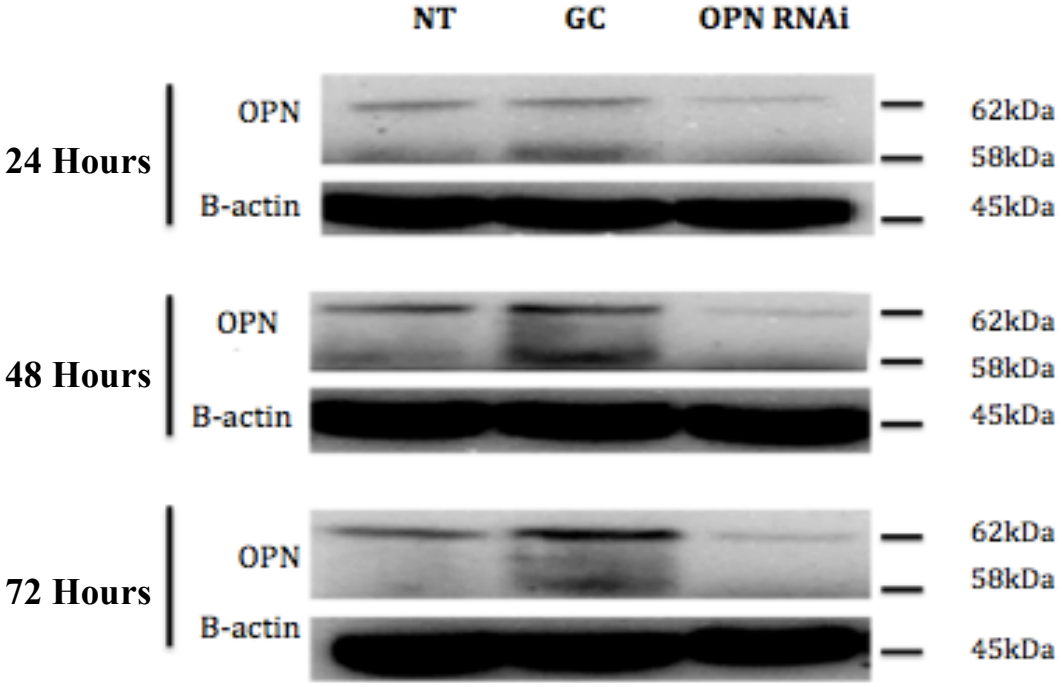

Figure 2A. Western blot analysis of OPN protein expression in OPN RNAi treated and GC control treated RJ348 cells at 24 hours, 48 hours and 72 hours post transfection. OPN protein levels were visibly lower in RJ348 cells treated with OPN RNAi compared to GC control treated cells at 24, 48 and 72 hours post transfection.

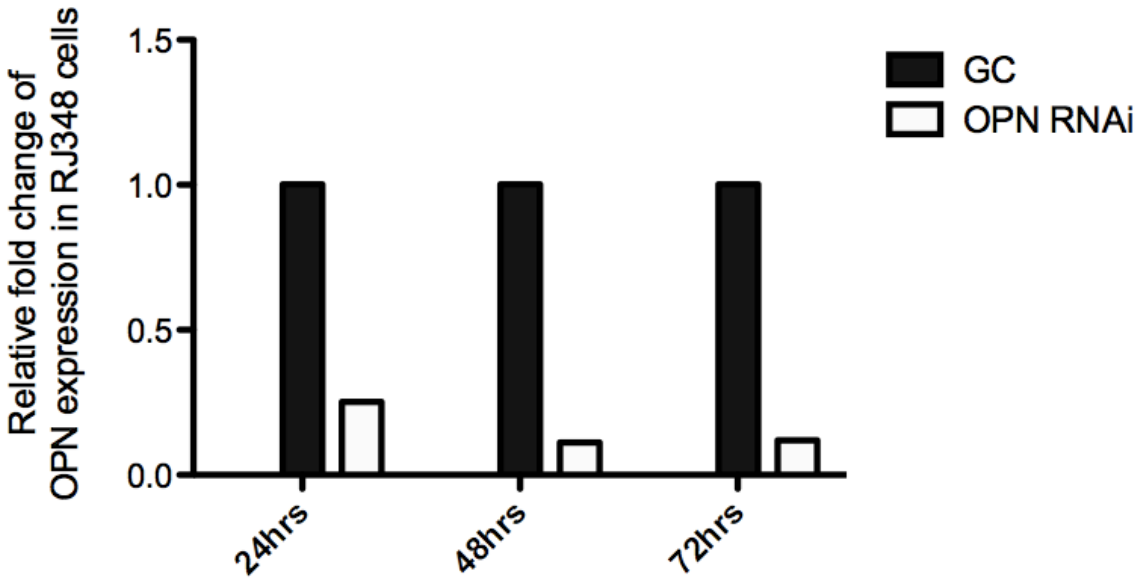

Figure 2B. Densitometric analysis of OPN protein levels in OPN RNAi treated RJ348 cells. Results revealed a significant knockdown of OPN expression with an efficiency of ~ 74%, 24 hours post transfection, 89%, 48 hours post transfection and 88%, 72 hours post transfection in RJ348 cells treated with OPN RNAi compared to GC control treated cells. Bar graphs represent mean of two independent trials.

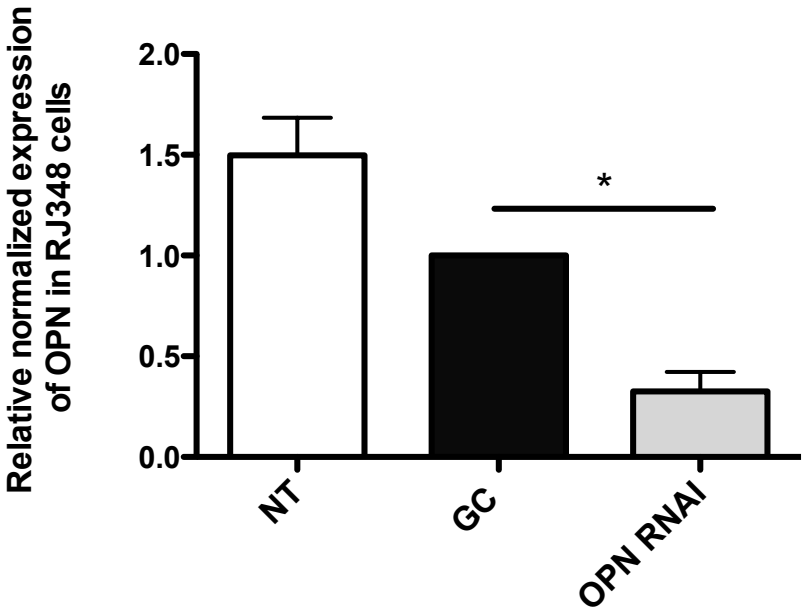

Figure 2C. RT-PCR analysis of OPN mRNA levels in OPN RNAi treated RJ348 cells at 48 hours post transfection. Cells treated with OPN RNAi showed a significant decrease in OPN mRNA level compared to GC control treated RJ348 cells. Levels of OPN mRNA were normalized to the housekeeping gene *hprt1*. Data was analyzed using One Way ANOVA Test ($P < 0.0014$) followed by Tukey's HSD test. Bar graphs represent mean ± SEM of three independent trials. Asterisk (*) represents statistical significance. Abbreviation: NT= no treatment, GC= guanine cytosine control.

Transient knockdown of OPN Impaired RJ348 cell Migration and Invasion

To evaluate the effect of OPN knockdown on RJ348 cell migration, scratch wound assays ware performed. Post-scratch, wound closure was captured by microscopic imaging every 12 hours until the wound was completely closed in the GC control cells. Results revealed significantly decreased migration of RJ348 cells treated with OPN RNAi compared to GC control treated cells, at 24 and 48 hours post-scratch. The greatest effect of OPN knockdown on cell migration (~55% decrease) was seen at 48 hours post-scratch (Figure 3).

OPN knockdown RJ348 cells were then treated with recombinant OPN to examine exogenous OPN's ability to rescue the effect of OPN knockdown on cell migration. OPN knockdown RJ348 cells were treated with recombinant OPN at a concentration of 5ug/ml at time of the scratch (T=0). Results demonstrated a visible increase in cell migration in OPN knockdown RJ348 cells treated with recombinant OPN compared to cells treated with OPN RNAi only (Figure 4).

To further examine the effect of OPN knockdown on cell invasion, a trans-well assay was utilized. Briefly, trans-well assay examines the ability of cells to invade through matrigel-coated chambers containing microporous filter that acts as a basement membrane analog. RJ348 cells suspended in serum-free media are placed in the top chambers, and serum-containing media is placed in the bottom chambers, that act as a chemoattractant and recruits invasive cells to migrate through the basement membrane analog. Quantification of the number of cells that invaded through the matrigel in OPN RNAi treated cells compared to GC control treated cells demonstrated a significant decrease in RJ348 cell invasion in response to OPN knockdown (Figure 5).

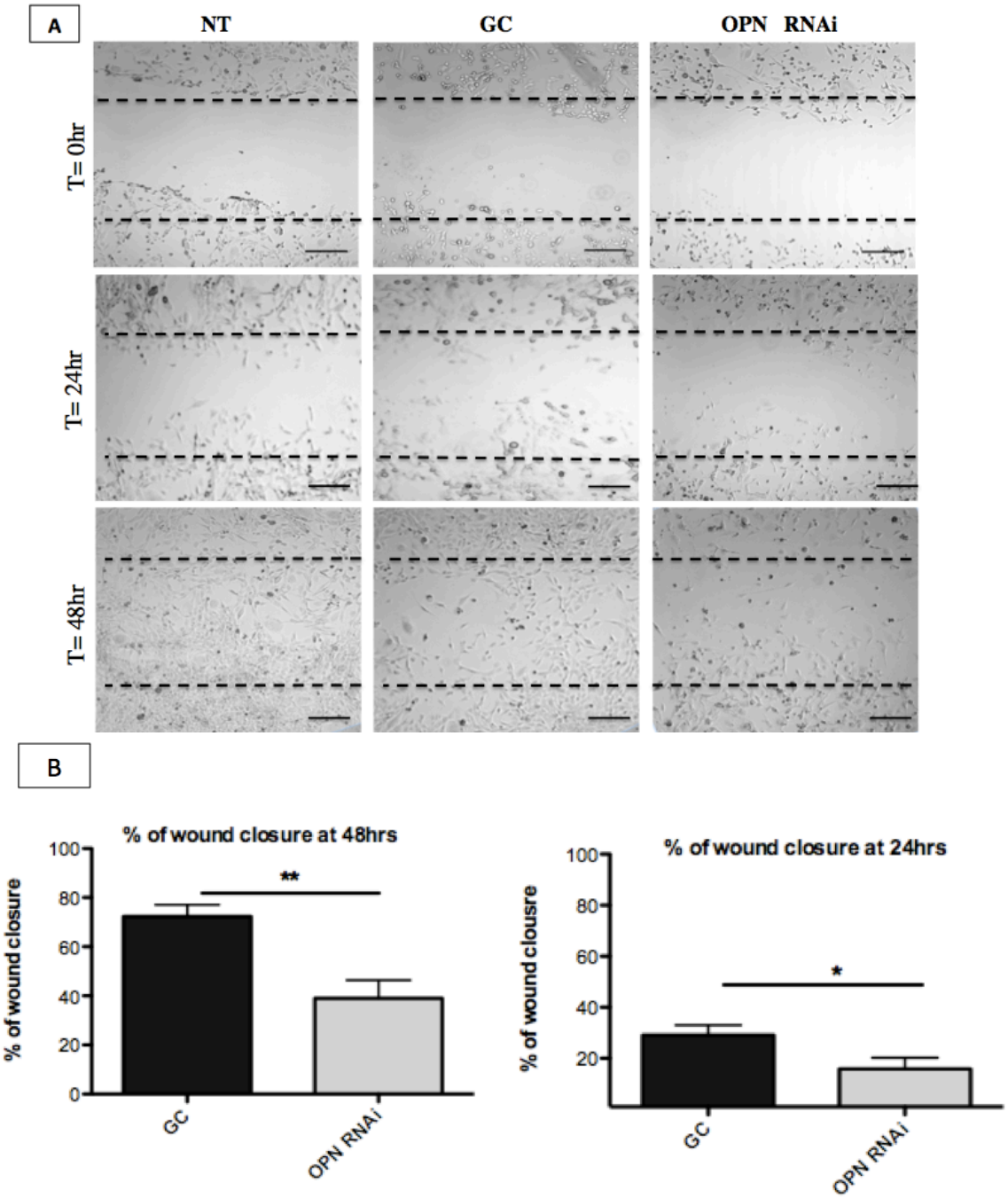

Figure 3. Scratch wound migration assay analysis of OPN knockdown RJ348 cells at the time of scratch T=0 (48 hours post transfection), 24 hours and 48 hours post scratch. **(A)** Visible decrease in RJ348 migration was observed at 24 and 48 hours post scratch in OPN RNAi treated cells in comparison to GC control treated and untreated cells. **(B)** Quantification of the percentage of wound closure post scratch revealed a significant decrease, ~54%, in wound closure at 24hr (p=0.043) and ~55% at 48hr (p=0.0096) in OPN RNAi treated RJ348 cells compared to GC control treated cell. Data was analyzed using unpaired T test. Bar graphs represent mean ± SEM of three independent trials. Asterisks (*) represent statistical significance. Scale bar=200um.

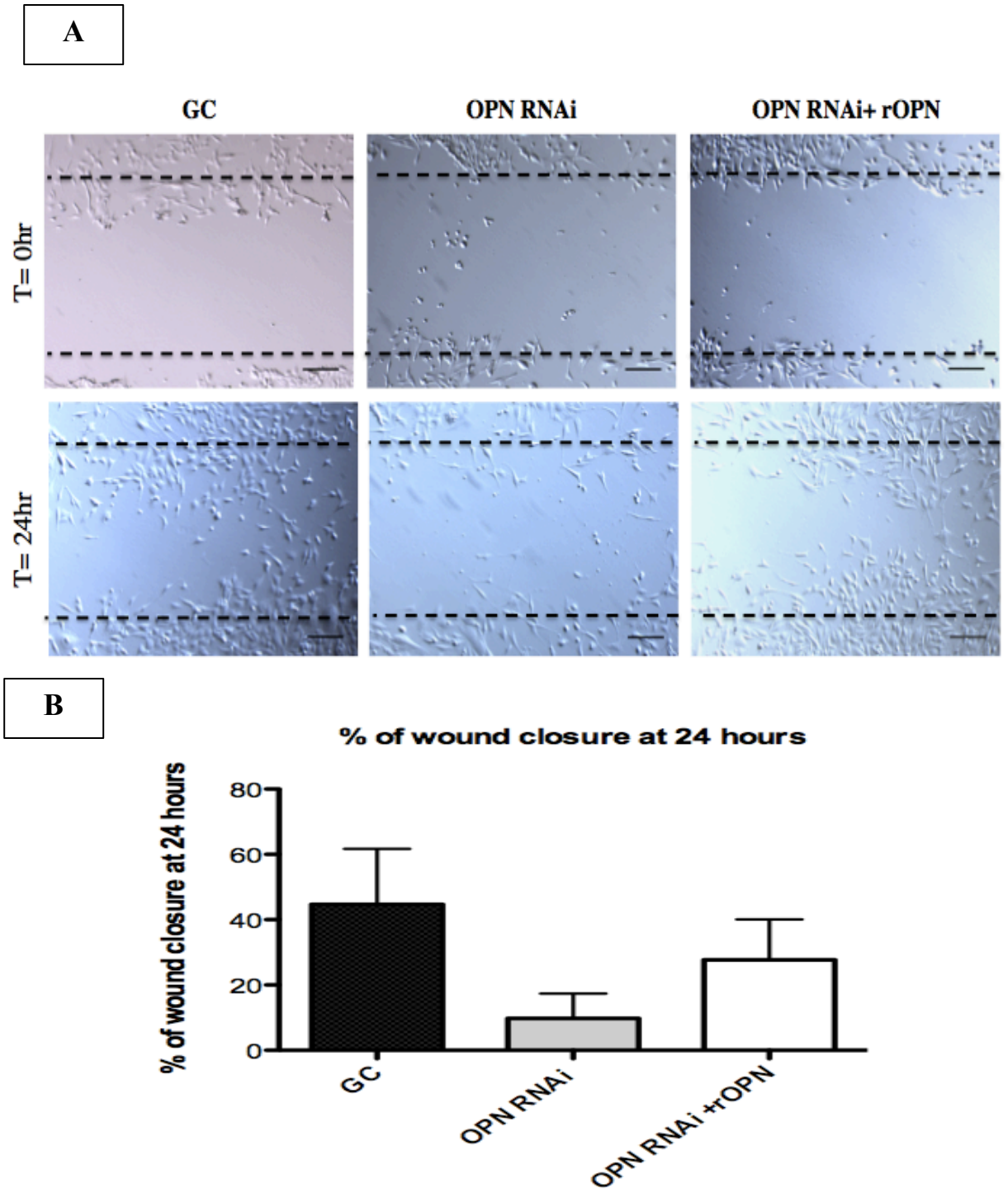

Figure 4. Wound healing migration assay analysis of OPN RNAi treated RJ348 cells receiving recombinant OPN at a concentration of 5ug/ml. **(A)** RJ348 cells treated with OPN RNAi and recombinant OPN (rOPN) show a visible increase in cell migration at 24hrs post scratch in comparison to RJ348 cells treated with OPN RNAi only. **(B)** Quantified percentage of wound closure was moderately higher in RJ348 cells treated with OPN RNAi+ rOPN compared to OPN RNAi treated cells. Data was analyzed using One Way ANOVA test ($p>0.05$), followed by tukey's HSD test. Bar graphs represent Mean ±SEM of three independent trials. Scale bar=100um.

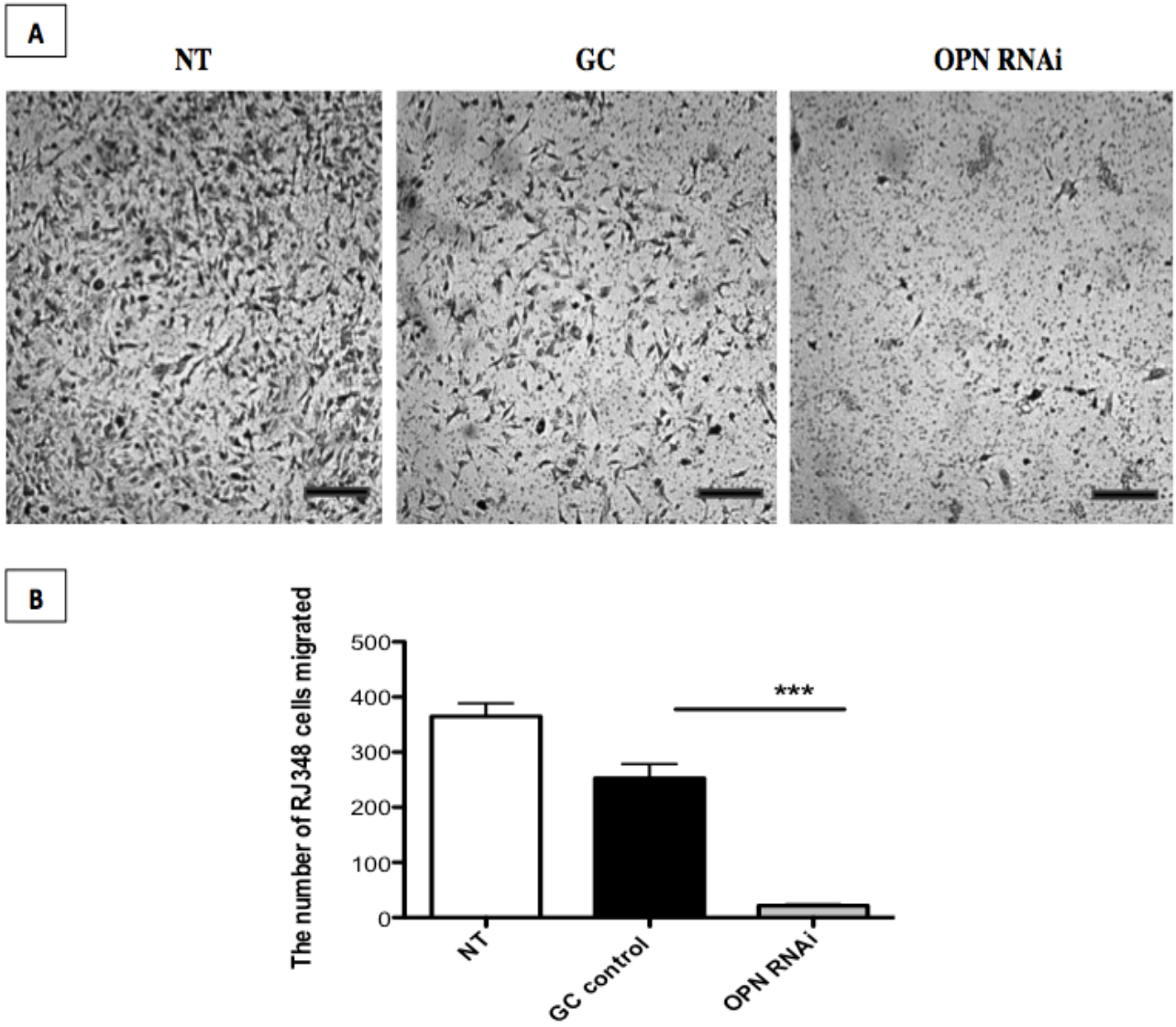

Figure 5. Trans-well invasion assay analysis on OPN RNAi treated RJ348 cells at 48 hours post seeding. **(A)** A visible decrease in RJ348 cell invasion was observed in OPN RNAi treated RJ348 cells in comparison to GC control treated and untreated cells. **(B)** Quantification analysis of OPN RNAi treated RJ348 cells compared to GC control treated RJ348 cells. OPN knockdown significantly decreased invasion of OPN RNAi treated RJ348 cells compared to GC control treated cells. Data was analyzed using One Way ANOVA test ($p=0.0001$), followed by Tukey's HSD test. Bar graphs represent mean ±SEM of three experimental trials. Asterisk (*) represents statistical significance. Scale bar= 300um.

Transient Knockdown of OPN Decreased RJ348 cell Proliferation

To evaluate the effect of OPN knockdown on cellular proliferation, immunofluorescence labeling of the proliferation marker Ki67 was carried out on RJ348 cells treated with OPN RNAi. Briefly, Ki67 is a nuclear antigen that is expressed in all phases of the cell cycle with the exception of the G0 resting phase. Cellular expression of Ki67 is an indicator of cell cycle progression and cellular proliferation (Urruticoechea et al. 2005). RJ348 cells treated with OPN RNAi showed a lower number of Ki67-positively staining cells (Figure 6 D-F) compared to GC control treated cells (Figure 6 A-C). Approximately 10% of OPN RNAi treated cells and 19% of GC control treated cells stained positive for antigen marker Ki67. Approximately 50% decrease in cellular proliferation was seen in response to OPN knockdown (Figure 6 G).

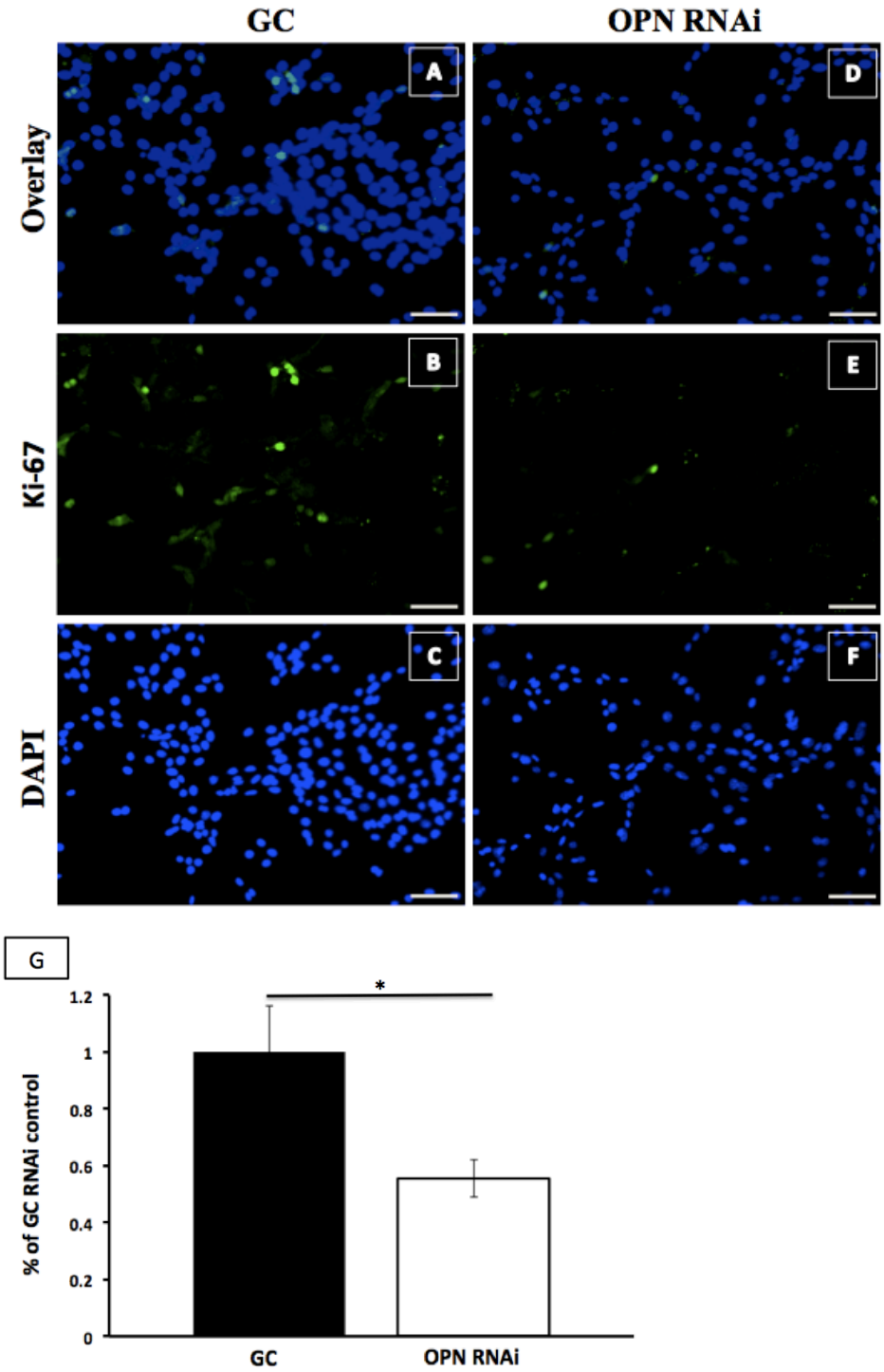

Figure 6. Immunofluorescence analysis of proliferation marker Ki67 in OPN knockdown RJ348 cells. Visibly lower number of Ki 67-positive RJ348 cells treated with OPN RNAi **(D-F)** compared to GC control treated cells **(A-C)**. **(G)** Quantitative analysis of Ki-67 positive staining in OPN RNAi treated cells compared to GC control treated cells. A decrease of ~50% was observed in OPN knockdown RJ348 in comparison to GC control cells. Data was analyzed using unpaired T Test ($p=0.034$) . Bar graphs represent mean ±SEM of three trials. Asterisk (*) indicates statistical significance. Scale bar= 100um.

Transient Knockdown of OPN Increased RJ348 Cell Apoptosis

One of the key events in the process of apoptosis is the cleavage of caspase-3, which has been shown to be a reliable indicator of apoptosis. RJ348 cells were labeled with an immunofluorescent antibody against cleaved caspase-3 to evaluate the effect of OPN knockdown on cellular apoptosis. Results showed a significantly higher number of cleaved caspase-3-positive staining cells in OPN-RNAi (Figure 7 D-F) treated RJ348 cells in comparison to GC control treated cells (Figure 7 A-C). Approximately 7% of OPN-RNAi treated cells and 4% of GC RNAi treated RJ348 cells stained positive for cleaved caspase-3 (Figure 7 G). Knockdown of OPN resulted in ~60% increase of cellular apoptosis.

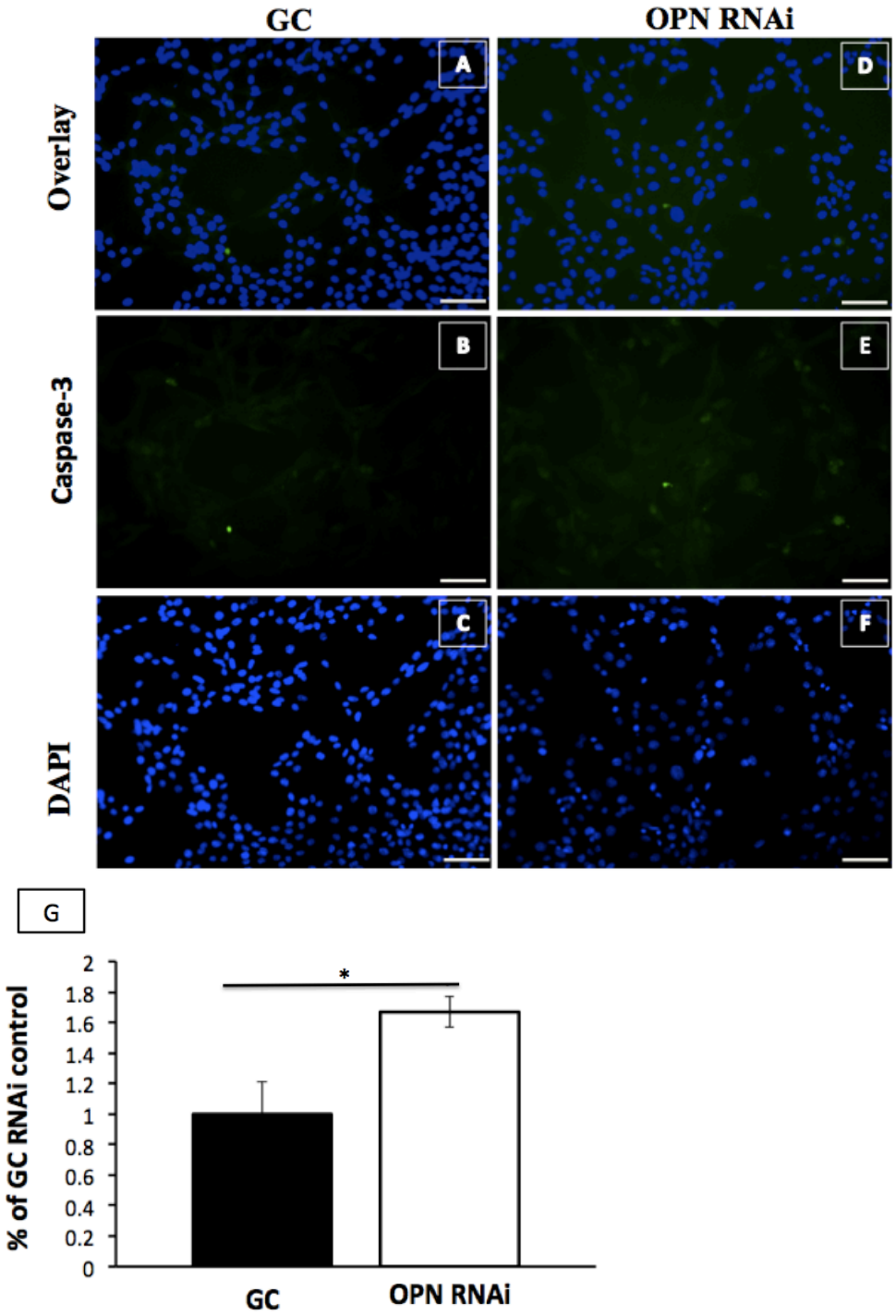

Figure 7. Immunofluorescence assay analysis of cleaved caspase-3 in OPN knockdown RJ348 cells. RJ348 cells treated with OPN RNAi had significantly higher caspase-3 positive cells **(D-F)** in comparison to GC control cells **(A-C)**. **(G)** Illustrates quantitative analysis of caspase-3 positive cells in OPN RNAi treated cells in comparison to GC control cells. An increase of ~60% in apoptosis was observed in OPN knockdown RJ348 in comparison to GC control cells. Data was analyzed using unpaired T test (p= 0.039). bar graphs represent mean ±SEM of three trials. Asterisk (*) indicates statistical significance. Scale bar= 100um.

Alternation in Signaling Kinases Activity Associated with Decreased OPN Expression

To evaluate the effect of OPN knockdown on downstream effectors and examine pathways involved in OPN signaling, western blot analysis was carried out on RJ348 cell lysate post OPN knockdown. The change in protein levels of Akt, pAkt, ERK1/2, pERK1/2, MMP-2 and MMP-9 was evaluated in OPN RNAi treated cells in comparison to GC control RNAi treated RJ348 cells (Figure 8). Densitometric analyses revealed no difference in AKT or ERK1/2 expression levels between OPN RNAi treated and GC control RNAi treated RJ348 cells (Figure 8 A). A visible increase in pERK1 and pAkt levels was observed in OPN RNAi treated cells in comparison to GC control RNAi treated cells (Figure 8 B). Approximately 2-fold increase of pERK1 was observed in OPN RNAi treated cells in comparison to GC control RNAi treated RJ348 cells. Protein expression of pAkt was increased by 7-fold in OPN RNAi treated cells in comparison to GC control RNAi treated RJ348 cells.

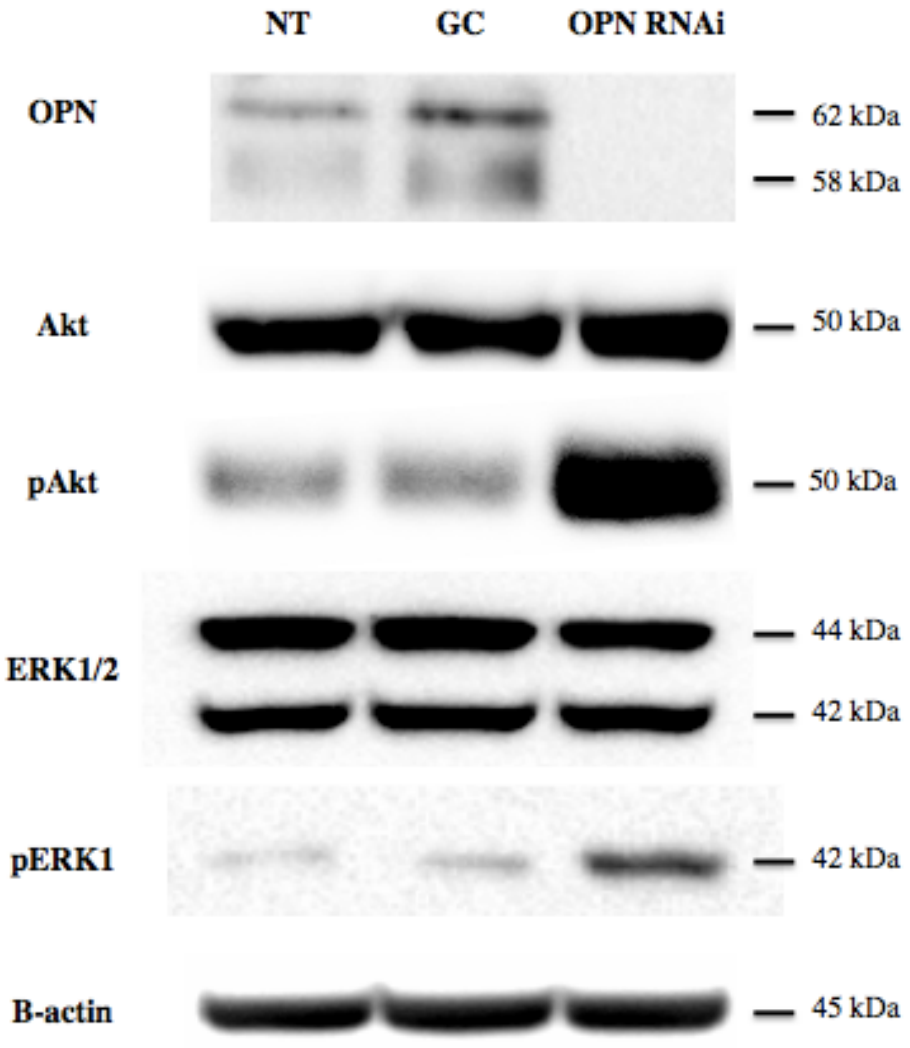

Figure 8. Western blot analysis of Akt, pAkt, ERK1/2 and pERK12 signaling proteins levels post OPN knockdown in RJ348 cells. No visible change in total Akt and ERK1/2 levels was observed in OPN RNAi treated cells compared to GC control treated and non-treated cells. pAkt and pERK1/2 protein levels were greater in OPN RNAi treated cells compared to GC control and no treatment cells. Data is based on two independent trials.

RJ348 Cells Express Integrin Alphav Cell Surface Receptor

To examine the presence of OPN's main cell surface receptor, integrin alphav-beta3, western blot analysis was carried out on RJ348 cell lysate. Integrin alphav subunit was detected with a molecular weight of ~123 kDa. On the other hand, integrin beta3 subunit could not be detected on the blots (Figure 9). To evaluate the effect of alphav knockdown on RJ348 cells migration and invasion, integrin alphav mRNA levels were downregulated by RNAi. Transient knockdown of integrin alphav subunit was achieved using an integrin alphav-targeted siRNA oligonucleotide sequence that was previously shown effective in our laboratory. Integrin alphav knockdown efficiency obtained was ~99% in integrin alphav RNAi treated cells compared to GC control treated cells (Figure 9).

Transient Knockdown of Alphav Decreased RJ348 Cell Adherence and Migration

To evaluate the effect of alphav knockdown on RJ348 cell migration, scratch wound assays were performed. Post-scratch wound closure was captured via microscopic imaging every 12 hours until the wound was completely closed in the control cells. Results revealed a visible decrease in the percentage of wound closure in alphav RNAi treated cells in comparison to GC treated and non-treated cells. Additionally, results also demonstrated a drastic loss of cell attachment and adherence to the plate in alphav RNAi treated cells compared to GC treated and non-treated cells (Figure 10).

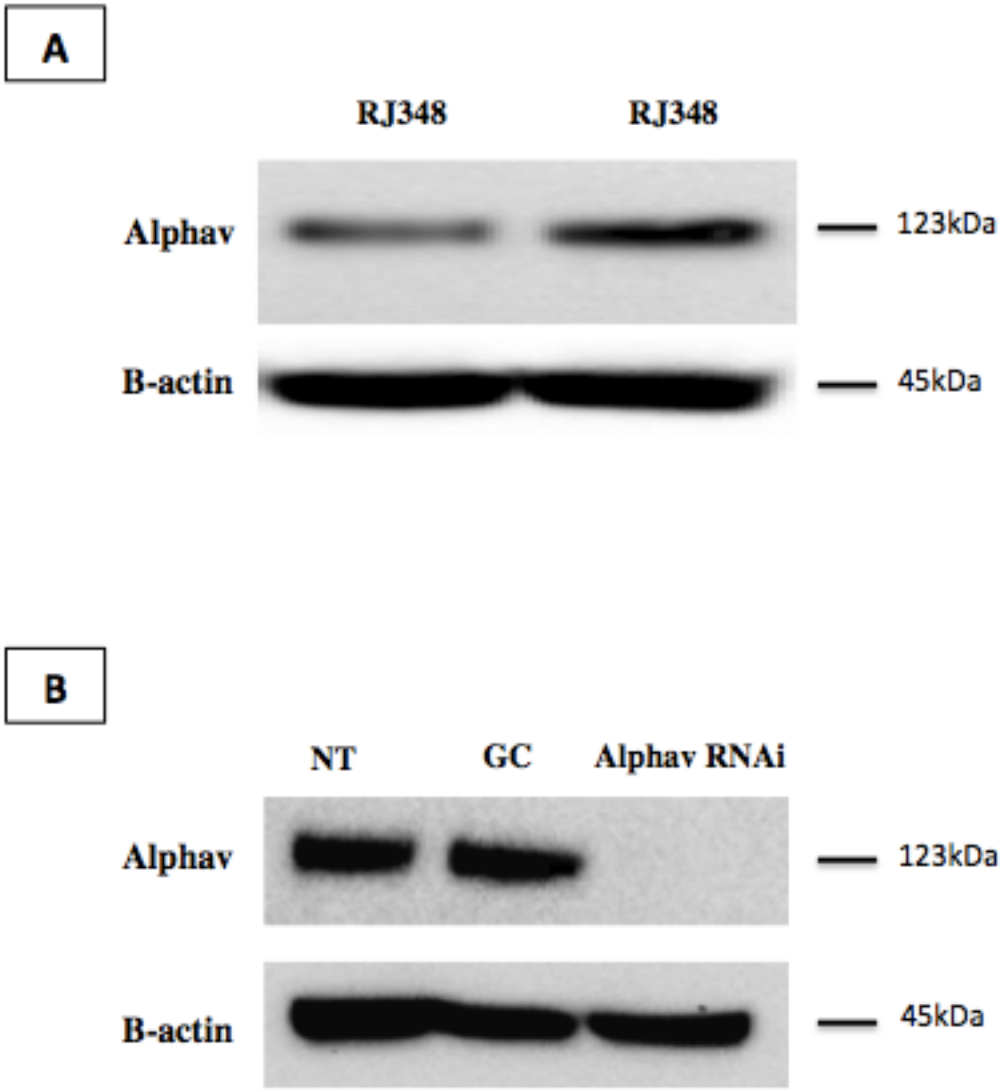

Figure 9. Western blot analysis of integrin alphav expression in RJ348 cells **(A)** Integrin alphav protein weighting ~123kDa was detected in RJ348 cell lysate, both lanes are non-treated RJ348 cell lysate **(B)** A visible knockdown of integrin alphav protein level in integrin alphav RNAi treated RJ348 cells at 48hr post transfection in comparison to GC RNAi control treated and non- treated RJ348 cells. Integrin alphav knockdown efficiency obtained was ~99% in alphav RNAi treated cells compared to GC control treated cells. Data is based on two independent trials.

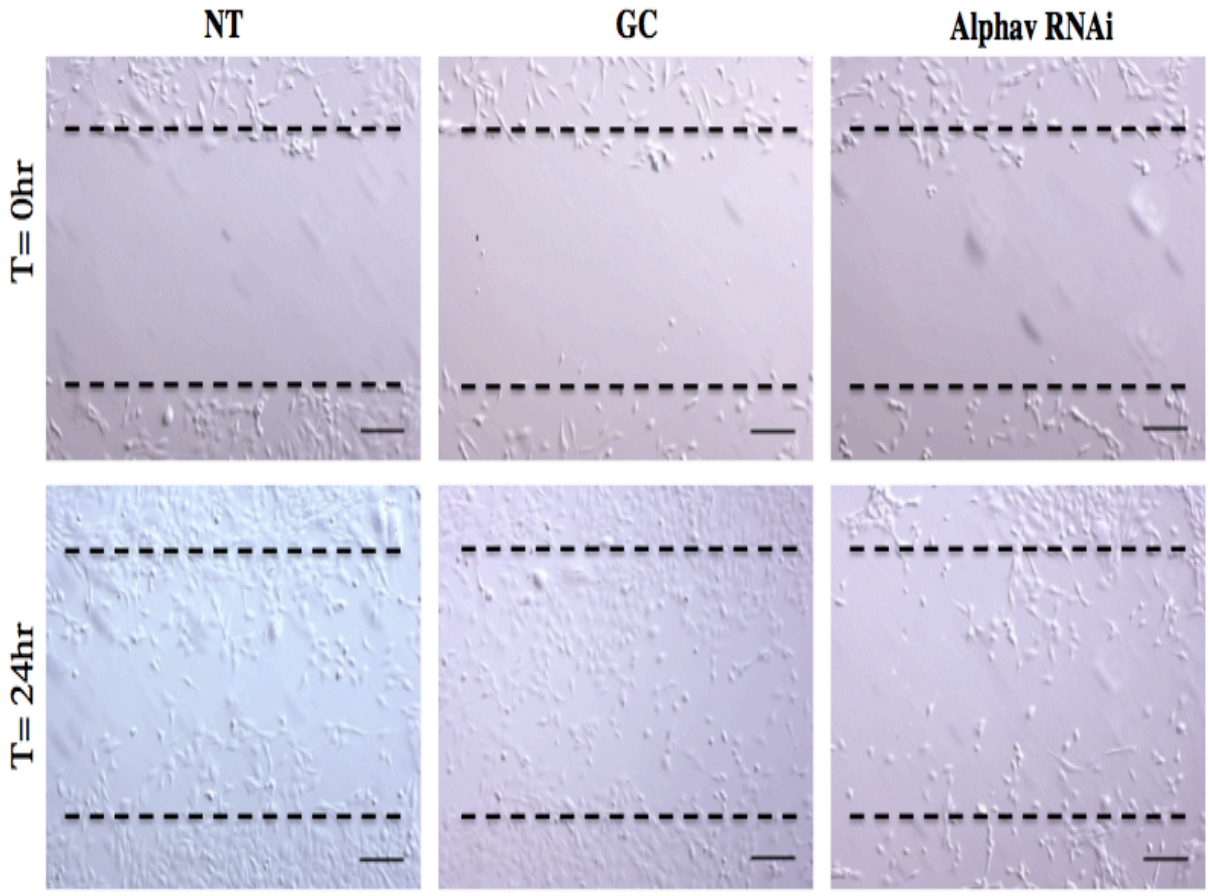

Figure 10. Scratch wound migration assay analysis on alphav RNAi treated RJ348 cells at the time of scratch T=0 (48 hours post transfection) and 24 hours post scratch. A Visible decrease in percentage of wound closure was observed at 24-post scratch in alphav RNAi treated cells compared to GC control treated and untreated cells. In alphav RNAi treated cells there is also a visible loss of cell attachment in comparison to GC treated and non-treated cells. Data is based on two independent trials. Scale bar= 100um.

Stable Transfections of RJ348 cells

To examine the long-term effect of OPN knockdown on tumor progression, RJ348 cells were transfected with DNA plasmids to obtain a stable OPN knockdown cell line. RJ348 cells were successfully transfected with cDNA plasmids containing siRNA oligonucleotide sequence targeting OPN mRNA. cDNA Plasmids also contained a puromycin resistant gene as a selection marker and a GFP tag to monitor cell selection. Post transfection cells where treated with 7ug/ml of puromycin to select for and sustain cells expressing plasmid of interest. Approximately 98% of selected RJ348 cells were GFP positive (Figure 11). However, OPN protein levels were not reduced in RJ348 cells expressing OPN –targeting plasmids (Figure 12). Real-time PCR analysis was also carried out on stable-RJ348 cells to evaluate the level of OPN knockdown. Results showed no reduction in OPN mRNA levels in any of the plasmid-expressing RJ348 colonies compared to NC plasmid-expressing RJ348 cells (Figure 12).

Migration Pattern of RJ348 Cells Stably Expressing OPN-Targeting plasmids

To validate the previously reported RT-PCR and western blot findings (Figure 12), scratch wound migration assays were carried out on RJ348 cells expressing Plasmid A (A-OPN), Plasmid B (B-OPN), Plasmid C (C-OPN), Plasmid D (D-OPN), NC plasmid and non-treated cells. Results showed no reduction in migration of RJ348 cells expressing plasmid A (A-OPN), Plasmid B (B-OPN), plasmid C (C-OPN) and Plasmid D (D-OPN) compared to NC plasmid treated cells. In fact, cells expressing plasmid C (C-OPN) and plasmid B (B-OPN) showed a moderately higher % of wound closure compared to the other treatment groups and NC plasmid treated cells. (Figure 13-1 and Figure 13-2).

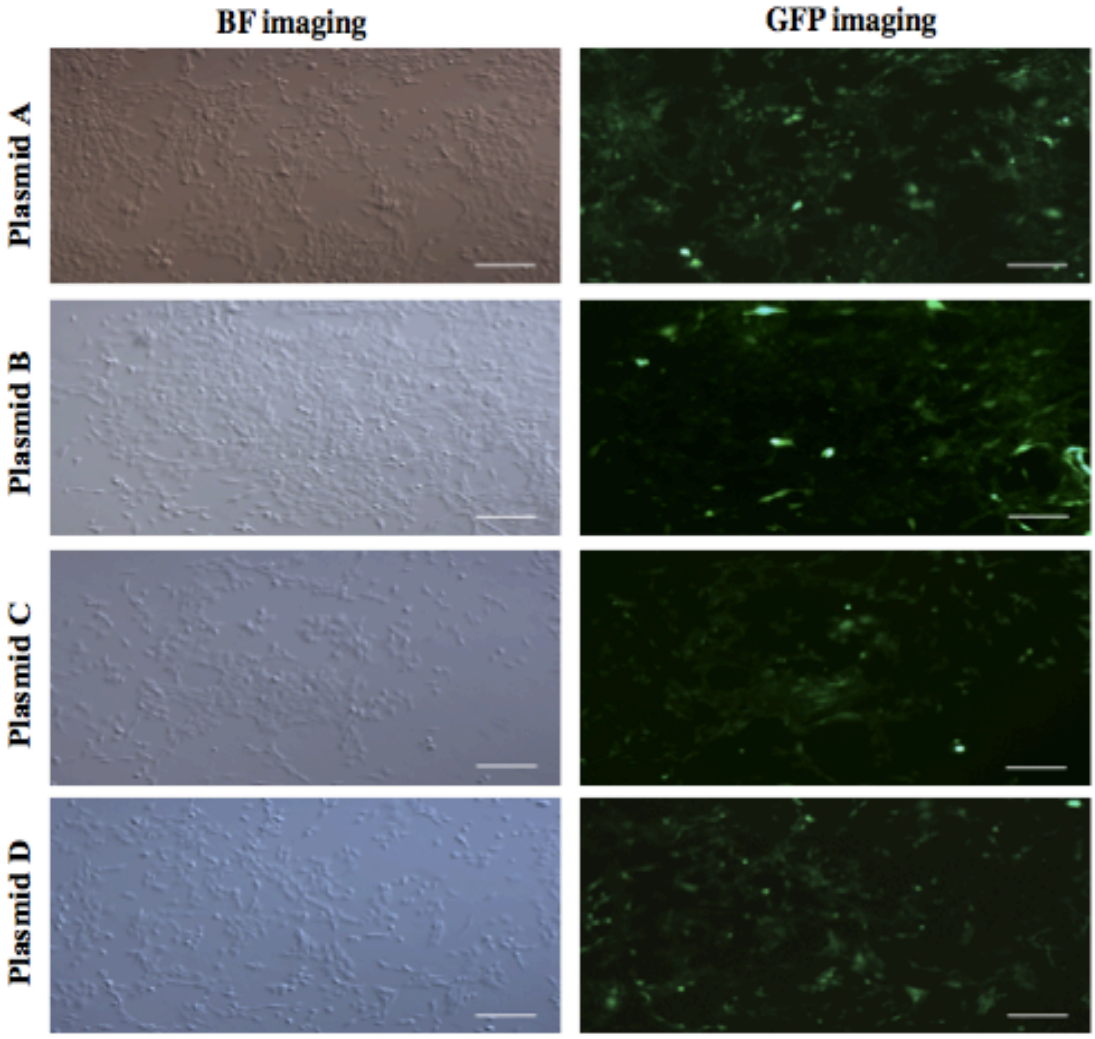

Figure 11. Fluorescence and brightfield imaging of RJ348 cells stably expressing the OPN-targeting plasmids, 6 weeks post selection. The left panel of the figure represents bright-field imaging of RJ348 cells expressing A-OPN, B-OPN, C-OPN and D-OPN knockdown plasmids. The right panel represents green fluorescence imaging corresponding to the bright-field images. Scale bar= 100um.

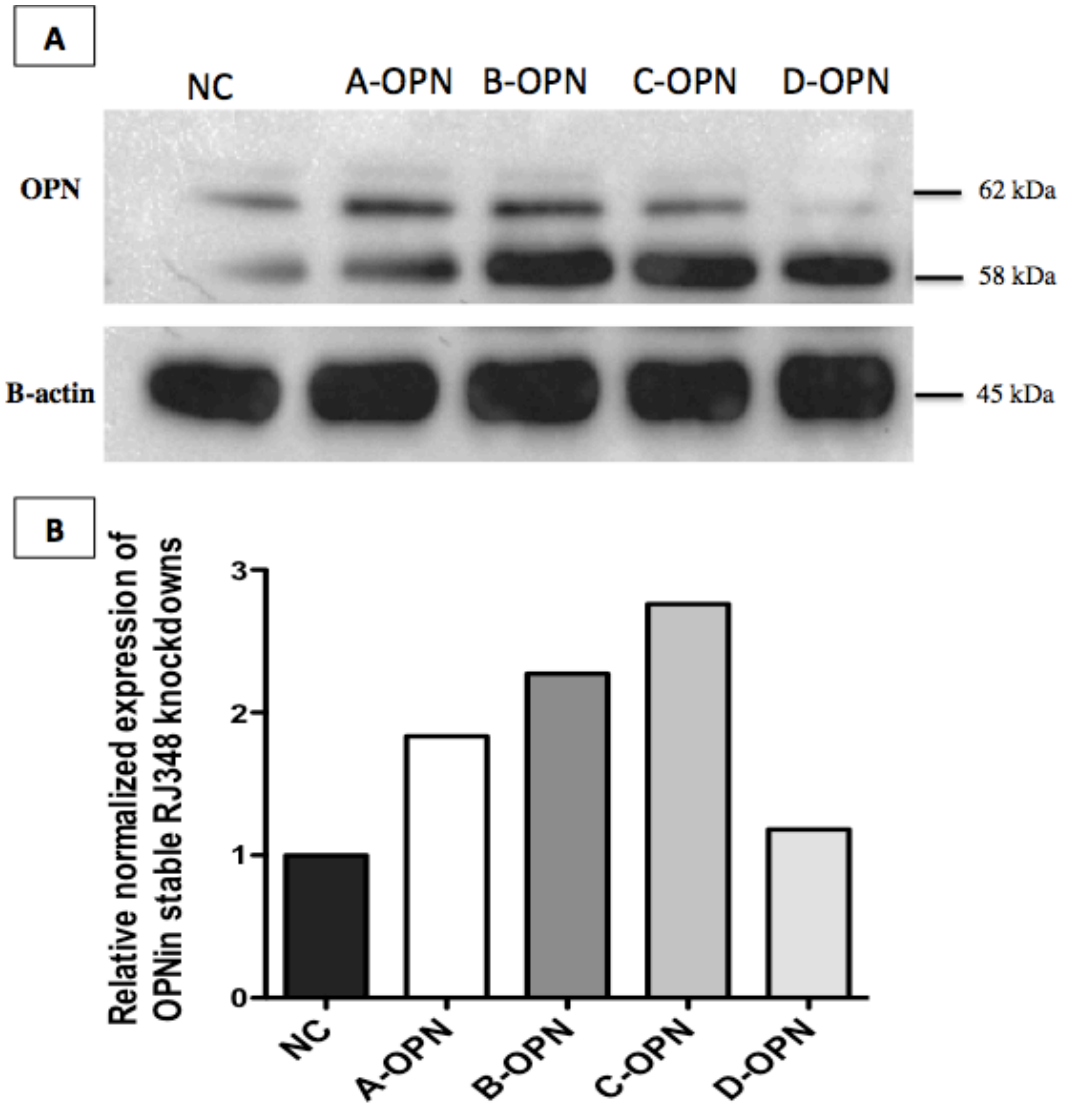

Figure 12. OPN expression levels in RJ348 cells stably expression plasmid A-OPN, B-OPN, C-OPN and D-OPN, 6 weeks post selection **(A)** Western blot analysis of OPN stable knockdown plasmid-expressing RJ348 cells compared to NC plasmid-expressing cells. OPN protein levels were the same all OPN targeting-plasmid treated groups compared to NC control plasmid-treated cells **(B)** OPN mRNA expression in RJ348 cells transfected with A-OPN, B-OPN, C-OPN and D-OPN plasmids 6-week post-colonies selection. RJ348 cells expressing A-OPN, B-OPN, C-OPN and D-OPN plasmids show a slight increase in OPN mRNA expression in comparison to RJ348 cells expressing NC control plasmid. Bar graphs represent mean of three independent trials.

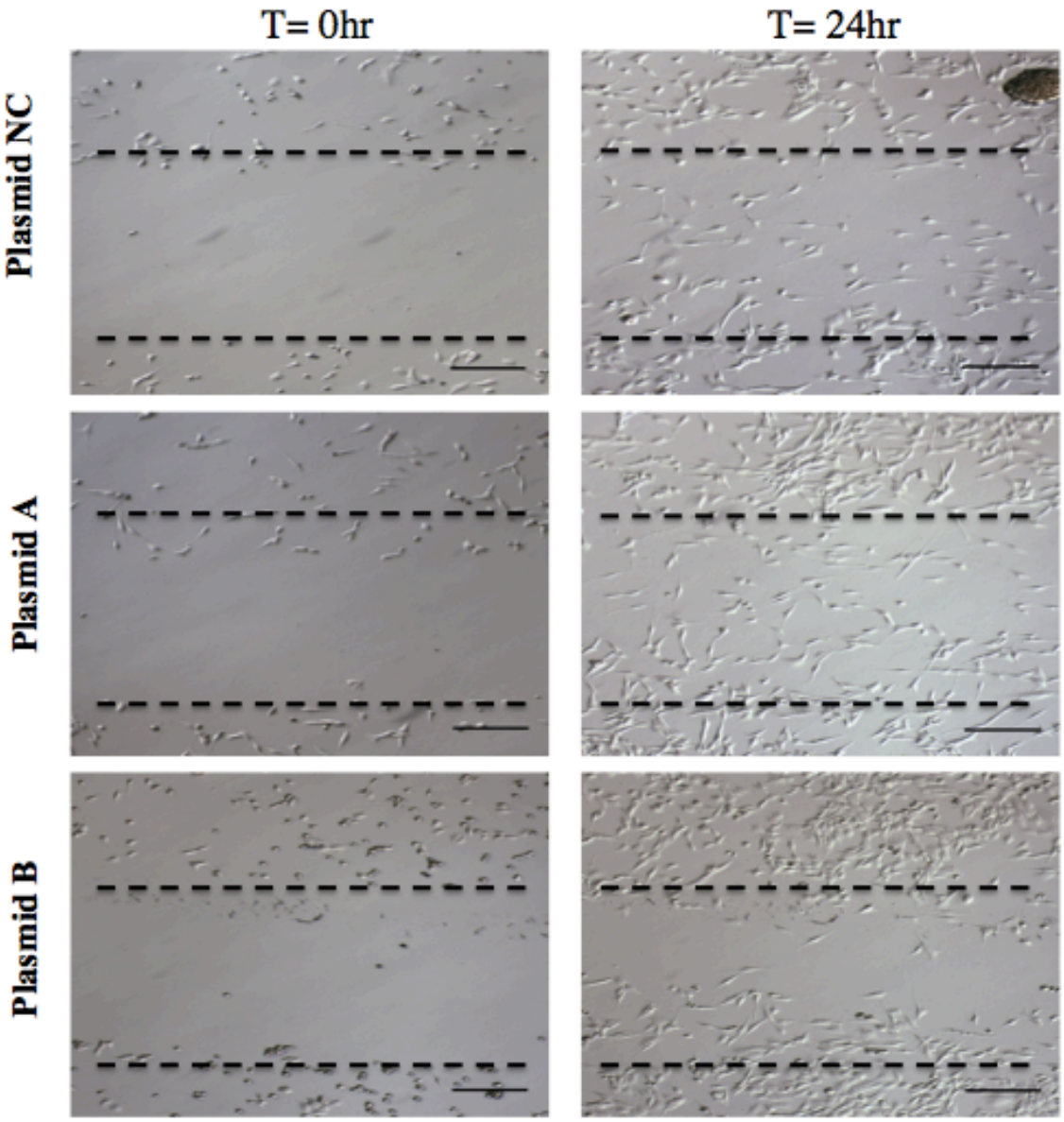

Figure13-1. Scratch wound migration analysis of RJ348 cells expressing plasmid A and plasmid B compared to NC plasmid RJ348 cells. There is no visible decrease in cell migration in RJ348 cells expressing plasmid A (A-OPN) or Plasmid B (B-OPN)) in comparison to NC plasmid treated RJ348 cells. Scale bar= 100um.

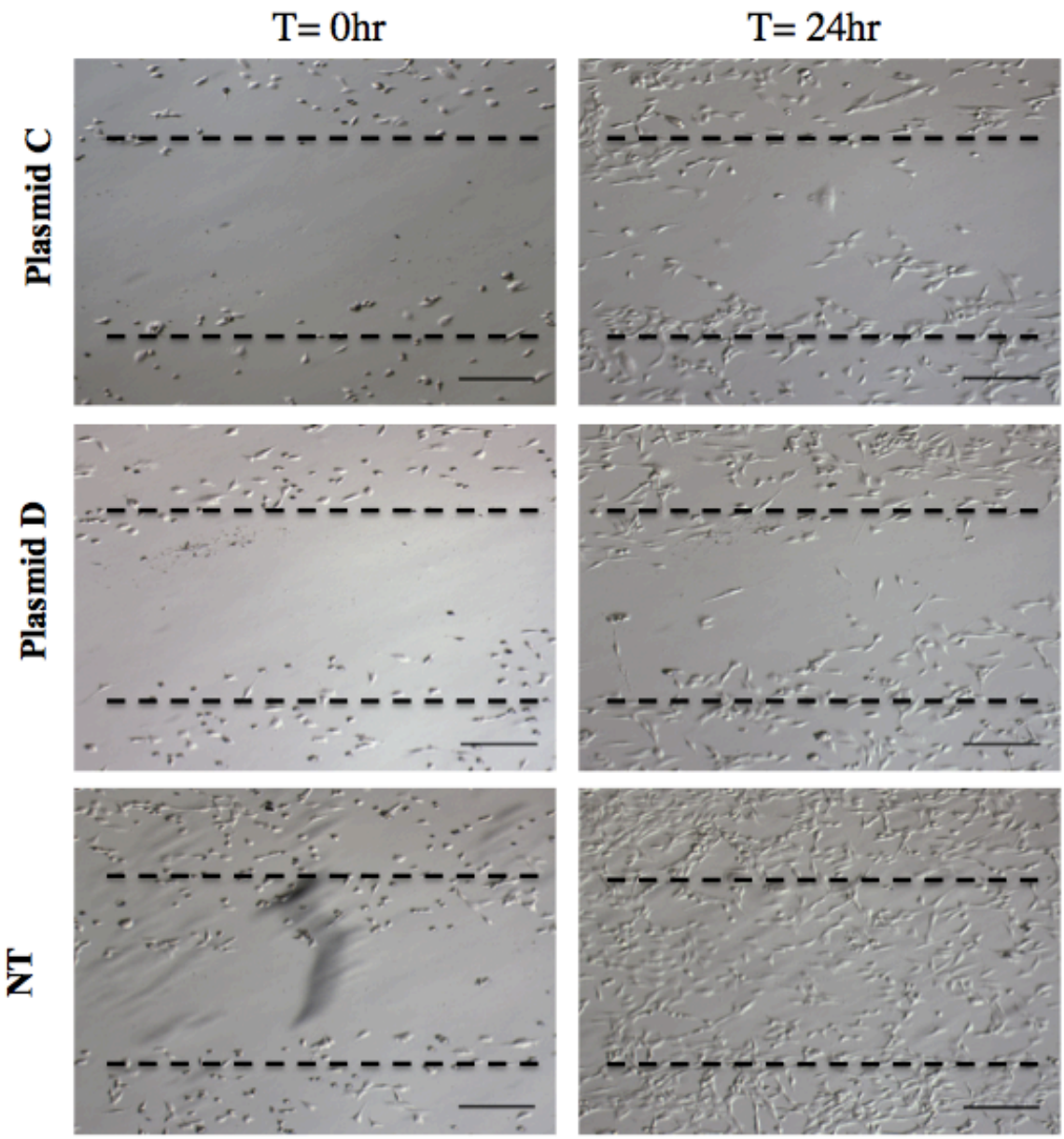

Figure 13-2. Scratch wound migration analysis of RJ348 cells expressing plasmid C (C-OPN), plasmid-D (D-OPN) and non-treated cells. D-OPN cells and C-OPN plasmid treated RJ348 cells showed a slightly higher percentage of wound closure than NC plasmid treated cells. All four OPN-targeted plasmids and NC treated plasmid show less migration rate in comparison to non-treated cells. Scale bar= 100um.

DISCUSSION

Given its elevated expression in various types of cancer, OPN appears to play a key role in the process of tumorigenesis. In breast cancer, OPN expression levels are significantly higher in malignant tumors compared to benign tumors and normal breast tissues (Rudland et al. 2002, Wang et al. 2003). OPN's increased expression is strongly associated with tumor invasiveness and metastasis (Chakraborty et al. 2006; Tuck et al., 2000, Weber et al., 2010). Furthermore, studies found significantly higher levels of OPN in patients with metastatic breast cancers and strongly associated higher OPN levels with poor survival (Bramwell et al. 2006). Based on the accumulated evidence supporting OPN's implication in breast cancer, our laboratory screened for OPN expression in a murine claudin-low mammary tumor model. DNA microarray analysis determined increased OPN expression by approximately 15-fold in claudin-low mammary tumor tissues compared to normal mammary tissue. Thereafter, the role of OPN in claudin-low mammary tumorigenesis was examined more closely via claudin-low tumor derived-cells, namely RJ348 cells.

To examine the role of OPN in claudin-low mammary tumorigenesis, we first assessed the effects of OPN transient knockdown on cellular migration, invasion, proliferation and apoptosis in vitro. Scratch wound assay results demonstrated that transient knockdown of OPN significantly impaired RJ348 cell migration compared to GC control treated cells (Figure 4). The greatest effect of OPN knockdown on RJ348 migration was at 48 hours post scratch, showing approximately 50% reduction in RJ348 migration compared to control cells. Our findings complement other in vitro studies examining the role of OPN in cell migration. For example, when studying the role of OPN in human glioblastoma tumorigenesis, Lamour et al assessed the effects of OPN transient knockdown on U87-MG

and U373-MG cell migration in vitro. Results demonstrated a significant impairment in glioma cell migration in response to OPN knockdown (Lamour et al. 2010). Similarly, Likui et al demonstrated that knockdown of OPN in colorectal cancer cells (Lovo), resulted in a significant decrease in cell migration across a scratch wound area (Likui et al. 2011). In breast cancer cells, Shevede et al suggest that knockdown of OPN in the metastatic breast cancer cell line, MDA-MB-435, also impairs cell migration (Shevede et al. 2006). On the other hand, a number of recent studies suggest that MDA-MB-435 cells may be of a melanoma origin and therefore, are not a true breast cancer cell line. The controversy remains about the origin of MDA-MD435 cells with no definitive conclusion on the true origin of this cell line (Chambers, 2009).

Nevertheless, despite its importance in measuring cell migration across a wound area, scratch wound assay does not provide sufficient information on cells' capability of invasion. The process of cell invasion is a complex process that requires cells to secrete specific proteases and activate a number of pathways to mediate the degradation of the basement membrane and ECM. The apparatus used for scratch wound assay lacks the complexity of a true tumor-cell environment. Therefore, a more relevant assay with an apparatus that mimics the tumor cell environment is required to measure tumor cell invasion. For this purpose, we utilized a trans-well invasion assay to further assess the effect of OPN knockdown on RJ348 cell invasion. RNAi-mediated knockdown of OPN significantly impaired RJ348 invasion ability. The average number of RJ348 cells treated with OPN RNAi that successfully invaded through the matrigel was significantly lower, than GC control treated cells (Figure 6). The negative effect of OPN knockdown on cell invasion was also seen in other in vitro studies. A recent study conducted by Zhang et al demonstrated

that RNAi-mediated knockdown of OPN in breast cancer cells, MDA-MB-231, significantly decreased cell invasion (Zhang et al. 2014). OPN's implication in cell invasion has also been seen in other cancer types. For example, Lamour et al (2010) demonstrated that transient knockdown of OPN decreased glioma cell invasion by ~3-fold in compared to GC control cells. Similarly. Similarly, using lung cancer (A549) cells, Polat et al demonstrated that knockdown of OPN via RNA interference significantly impaired cell invasion (Polat et al. 2013).

While it is well accepted that OPN plays a significant role in cell migration and invasion, the effect of OPN on cancer cell growth and proliferation remains controversial. There are a considerable number of studies that suggest a role for OPN in cellular proliferation and other contradicting studies that suggest otherwise. Our study further implicates OPN in the process of proliferation. Proliferation assay results demonstrated that knockdown of OPN in RJ348 significantly decreased cellular proliferation. There was ~ 44% less Ki67 positive staining cells in OPN RNAi treated cells in comparison to GC control treated cells (Figure 6). Additionally, our results are in agreement with other studies that support OPN's implication in tumor cell growth. To list a few, Lamour et al (2010) showed that knockdown of OPN in glioma (U87-MG) cells resulted in a 6-fold decrease in cellular proliferation compared to GC control treated cells. Similarly, Likui et al (2011) demonstrated that knockdown of OPN in colon cancer (lovo) cells resulted in a significant decrease in cellular proliferation in comparison to control cells. Contrary to this, a number of studies have demonstrated that OPN expression has no effect on cellular proliferation and tumor growth. Zhang et al (2003) demonstrated that induced-overexpression of OPN in mammary tumor (MT2994) cells does not affect cellular growth rate in vitro (Zhang et al.

2003). Similarly, MCF-7 breast cancer cells stably overexpressing OPN showed no change in cellular growth rate compared to GC control cells (He et al. 2005). It is important to highlight that both of these studies are based on an induced OPN-overexpression model in cell lines that do not normally express high levels of OPN. Meanwhile, our findings, along with the corresponding studies, are based on invasive breast cancer cell lines that express high levels of OPN endogenously. One explanation to the observed contradicting effect of OPN knockdown on cellular proliferation may be attributed to the difference between de novo-gene upregulation and induced-gene overexpression modeling systems. For example, tumor-derived OPN expression is regulated by several TFs and signaling molecules, some of which intertwine to form a complex signaling system. Despite, the integration of plasmid-derived OPN into the cells' genome, the expression pattern and signaling complexity normally present in cells naturally overexpressing OPN may not be present nor perpetuated in cells with induced OPN overexpression. Additionally, tumor cells that overexpress OPN may have an increased activity of different pathways that are directly associated with cell growth and proliferation. Those pathways may not be activated in cells with induced-OPN overexpression. As a result, no significant change in tumor cell growth is observed in response to induced-OPN overexpression.

Transient knockdown of OPN in RJ348 cells significantly increased apoptosis. Using the death marker cleaved caspase-3, immunofluorescence assay revealed a 69% increase in apoptosis in RJ348 cells treated with OPN RNAi compared to GC control treated cells (Figure 7). These results are in agreement with other in vitro studies utilizing RNAi for OPN knockdown. For example, in a recent study, Zhang and associates demonstrated that

RNAi-mediated OPN knockdown in the metastatic breast cancer cell line, MDA-MB-231, resulted in a significant increase in cell apoptosis (2014). Similarly, Zhao et al demonstrated that OPN knockdown in hepatocellular carcinoma cells significantly increased mitochondrial-induced cellular apoptosis.

The presence and activation of integrin alphav-beta3 receptor is critical for OPN's signaling in mammary tumorigenesis (Tuck et al. 2000, Rangaswami et al. 2006, Sloan et al. 2006). Correspondingly, integrin alphav-beta3 expression was assessed in RJ348 cells. Western blot analysis demonstrated that RJ348 cells express integrin alphav protein (Figure 9).

Once expression was confirmed, the effect of integrin alphav knockdown on RJ348 cell migration was evaluated. Integrin alphav expression was downregulated with an efficiency of ~ 98% in RJ348 cells and scratch wound assays were carried out at 24hours and 48hours post transfection. Various attempts were made to optimize the scratch wound migration assay on integrin alphav knockdown RJ348 cells. Unfortunately, knockdown of integrin alphav had a drastic effect on cell adhesion properties and resulted in loss of cell-cell attachment and adherence to the plate. Therefore, there was a large variability in the percentage of wound closure in alphav RNAi treated cells in comparison to GC control. However, there was a visible decrease in cell migration in alphav knockdown cells in comparison to control (Figure 10). It is difficult to draw a conclusion based on these findings, as the decrease in percent of wound closure may be due to integrin alphav knockdown and/or a result of loss of adherence.

Despite several efforts made to map out OPN's signaling pathways in malignant tumors, there are numerous gaps in the literature on OPN's exact mechanisms and signaling. Our study explored a number of potential signaling pathways that are highly regulated by OPN receptors, namely integrin alphav and CD44 surface receptors. This was achieved by examining the effect of OPN knockdown in the RJ348 cells on the expression and activation of various signaling molecules involved in those signaling pathways, including Akt, pAkt, ERK1/2 and pERK1/2. Results demonstrated no significant changes in total Akt and ERK1/2 levels in OPN knockdown cells compared to control cells. However, a large increase in pERK1/2 and pAkt levels was observed in OPN knockdown RJ348 cells compared to GC control cells (Figure 8 A and B). The observed effect of OPN knockdown on total Akt and ERK1/2 levels has also been demonstrated by several other in vitro studies. Robertson et al showed that knockdown of OPN in PC3 prostate cancer cells does not alter total Akt and ERK1/2 levels. Similarly, previous findings in our lab have shown no significant change in total Akt and ERK1/2 levels in mammary tumor cells. In contrast to our current findings, these studies demonstrated a decreased pAkt and pERK1/2 levels in OPN knockdown prostate cancer and breast cancer cells in comparison to control cells. In fact, our study is the first to report increased activation of Akt (pAkt levels) and ERK1/2 (pERK1/2 levels) in response to OPN knockdown. The increase may be a result of cells compensating for OPN knockdown. For example, in the case of Akt kinase activity, studies have demonstrated that Akt can act as both a downstream effector of OPN signaling via integrin alphav activation and an upstream regulator of OPN gene expression (Dai et al. 2009). Activated Akt can induce the transactivation of OPN gene by binding to an Akt responsive element in the OPN gene promoter mapped to a region between base −600 and

base −777 (Zhang et al. 2003). Thereby, increased levels of pAkt may be attributed to cells efforts to rescue the drastic decrease in OPN levels. In contrast, the upregulation of pERK1/2 levels in OPN knockdown may be a result of increased activity of other ERK1/2-inducing signaling molecules. For example, activated p38 MAPK was shown to be upregulated in response to OPN knockdown in MDA-MB-231 invasive breast cancer cells (Pang et al. 2011). On the other hand, a different study has shown that activated p38 MAP kinase induces ERK1/2 activation in epithelial cells and that inhibiting any of those kinases induce a robust transactivation of the other (Sharma et al. 2003). Consistent with these findings, increased ERK1/2 activity in OPN knockdown RJ348 cells may be a result of increased p38 MAPK activation as previously reported by Sharma and associates.

The long-term effect of OPN knockdown on claudin-low tumor progression was also examined in RJ348 cells. Lenti-vector plasmids with OPN-targeting siRNA oligosequences were utilized to create stable OPN-knockdown RJ348 cell lines. Although, cells were successfully transfected with the plasmids and a number of RJ348 colonies were isolated, there was no evident knockdown of OPN in RJ348 colonies stably expressing the OPN-targeting plasmids (Figure 9 and 10). In fact, some of the selected colonies showed a slight increase in OPN expression compared to RJ348 cells expressing negative control plasmid. Although results were unexpected, they can be attributed to a number of experimental limitations. For example, the siRNA sequences utilized in the plasmids may not be efficient. This is not surprising as it was recently reported by Taxman et al that one major challenge remaining today in the use of RNA therapeutics is designing an effective siRNA sequence (Taxman et al. 2010). In addition, all individual siRNA sequences used in the plasmids are

targeting murine OPN splice variant 5 (NCBI Reference Sequence: NM_001204233.1). Meanwhile, there are 4 other splice variants of the transcribed murine OPN gene, which could be more prominent in RJ348 cells than splice variant 5. This may render the siRNA function obsolete, because they are very specific and even the slightest alternation in the target's sequence could impair their function. Alternatively, the lack of OPN knockdown may also be a result of selective silencing of the siRNA-linked promoter. This explanation is compelling because the OPN-siRNA sequence is linked to a separate promoter than the GFP sequence. This also suggests that GFP-positive RJ348 cells may not be expressing the siRNA sequence of interest, as they are linked to a different promoter.

Summary and conclusion

In this study we examined the role of OPN in claudin-low mammary tumorigenesis. Using RNAi, OPN mRNA and protein levels were successfully downregulated in RJ348 mammary tumor cells. The effect of OPN downregulation on RJ348 cell proliferation, survival, migration and invasion was then evaluated. Results demonstrated that transient knockdown of OPN expression in RJ348 claudin-low mammary tumor cells significantly decreased cell migration and invasion in vitro. Additionally, decreased migration induced by OPN knockdown was partially rescued by the addition of recombinant OPN. Furthermore, downregulating OPN expression levels via RNAi resulted in a significant decrease in cell proliferation and survival in vitro. We have also demonstrated an increase in pAkt and pERK1/2 kinase levels. Although, these results were

not anticipated, they provide an insight to a potential role of Akt and ERK1/2 signaling in inducing OPN expression. Furthermore, RJ348 cells were successfully transfected with OPN targeting lenti-siRNA vectors, and a number of colonies were selected. However, OPN expression levels were not knocked-down in the selected colonies. This in particular caused a major limitation to evaluating the long-term effect of OPN knockdown on claudin-low mammary tumorigenesis. On the other hand, the lack of OPN knockdown in selected colonies sheds a light on the differential expression of OPN splice variants in RJ348 murine mammary cells. Since the lenti-siRNA vectors used were specifically targeting OPN splice variant 5, this study suggests that OPN splice variant 5 may not be expressed abundantly in the RJ348 cell line, and that other splice variants, may be more prominent.

Study limitations

One of the prominent limitations to this study is the lack of an in vivo model. Examining the role of OPN in claudin-low breast cancer growth and metastasis in vivo was a major objective in this study. However, due to the lack of evident OPN knockdown in the established stable RJ348 cell lines, this part of the study was not feasible. Establishing stable OPN knockdown model is extremely important to assess the long-term effects of OPN knockdown on claudin-low tumor progression. Another limitation is the lack of proper MMP-2 and MMP-9 antibodies. Several efforts were made to evaluate MMP-2 and MMP-9 protein expression in RJ348s; unfortunately, none of the antibodies used were effective to detect the corresponding bands on western blots. Additionally, Integrin beta3 subunit protein was not detected in western blot analysis. This may be due to the poor specificity of the

antibody used. Therefore, further optimization is required to detect the beta3 subunit in RJ348 cells.

Future Directions

As previously mentioned, establishing a stable OPN knockdown RJ348 cell line is a valuable extension to the findings reported in this thesis and the current knowledge on OPN's implication in claudin-low breast cancer. One major limitation to modeling claudin-low breast cancer in vivo is the lack of a murine claudin-low mammary tumor model. Currently, RJ348 is the only characterized murine cell line as a human like claudin-low breast cancer. Having said that, a number of human claudin-low breast cancer cell lines were utilized to explore claudin-low tumor progression in vivo; however, limited clinical relevance remains a major draw back of these models. This is mainly due to the absence of functional immune system in the designated mouse models, which is known to be heavily involved in claudin-low breast cancer progression.

Given that RJ348 is a murine cell line, it can be utilized in immunocompetent mice, thereby providing a more clinically relevant model to human claudin-low breast cancer. The use of RJ348 cells to examine the role of OPN in claudin-low tumors is in fact an ideal system considering that OPN is also involved in the immune system. Moreover, the increased expression of OPN in our mouse model and tumor-isolated cells makes it a valuable system to assess anti-OPN therapeutic drugs, such as andrographolide and other inhibitors.

Another valuable extension to this study is identifying the exact OPN isoforms detected on western blots. This may help identifying posttranslational modifications that OPN is exposed to. This is specifically important because OPN's activity is heavily dependent on posttranslational modifications. Thus, identifying those modifications may provide further insight on alternative strategies to attenuate OPN's function in tumor progression.

Lastly, examining other receptors mediating OPN's effects on RJ348 cells would provide further insight to OPN's signaling system. In this study, the effect of downregulating integrin alphav on RJ348 tumorigenic capacity was evaluated to assess its relevance to OPN knockdown. Several efforts were also exerted to examine co-localization of OPN and integrin alphav via immunofluorescence assay in RJ348 cells. Unfortunately integrin alphav antibody was not effective. An alternative method is immunoprecipitation, evaluating the presence of OPN- alphav dimers or other members of the integrin family would provide a more conclusive evidence on OPN signaling system.

REFERENCES

Ashkar, Samy, Georg F. Weber, Vassiliki Panoutsakopoulou, Marie E. Sanchirico, Marianne Jansson, Samer Zawaideh, Susan R. Rittling, David T. Denhardt, Melvin J. Glimcher, and Harvey Cantor (2000). "Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity." *Science*, *287*(5454), 860-864.

Atai, N. A., Bansal, M., Lo, C., Bosman, J., Tigchelaar, W., Bosch, K. J. Ard Jonker, Philip C. De Witt Hamer, Dirk Troost, Christopher A. McCulloch, VincentEverts, Cornelis J. F. Van Noorden[1] andJaro Sodek (2011). Osteopontin is up-regulated and associated with neutrophil and macrophage infiltration in glioblastoma. *Immunology*, *132*(1), 39-48.

Bai, W. L., R. J. Yang, R. H. Yin, W. Q. Jiang, G. B. Luo, R. L. Yin, S. J. Zhao, C. Li, and Z. H. Zhao.. (2012). "Molecular characterization and expression analysis of osteopontin cDNA from lactating mammary gland in yak (Bos grunniens)." *Molecular biology reports*, *39*(4), 3627-3635.

Behera, R., V. Kumar, K. Lohite, S. Karnik and G. C. Kundu (2010). "Activation of JAK2/STAT3 signaling by osteopontin promotes tumor growth in human breast cancer cells." *Carcinogenesis* 31(2): 192-200.

Bellahcene, A. and V. Castronovo (1995). "Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer." *Am J Pathol* **146**(1): 95-100.

Harry D. Bear, M.D., Ph.D., Gong Tang, Ph.D., Priya Rastogi, M.D., Charles E. Geyer, Jr., M.D., André Robidoux, M.D., James N. Atkins, M.D., Luis Baez-Diaz, M.D., Adam M. Brufsky, M.D., Ph.D., Rita S. Mehta, M.D., Louis Fehrenbacher, M.D., James A. Young, M.D., Francis M. Senecal, M.D., Rakesh Gaur, M.D., M.P.H., Richard G. Margolese, M.D., C.M., Paul T. Adams, M.D., Howard M. Gross, M.D., Joseph P. Costantino, Dr.P.H., Sandra M. Swain, M.D., Eleftherios P. Mamounas, M.D., and Norman Wolmark, M.D.. (2012). Bevacizumab added to neoadjuvant chemotherapy for breast cancer. *New England Journal of Medicine*, *366*(4), 310-320.

Berg, W. A., Gutierrez, L., NessAiver, M. S., Carter, W. B., Bhargavan, M., Lewis, R. S., & Ioffe, O. B. (2004). "Diagnostic accuracy of mammography, clinical examination, US, and MR imaging in preoperative assessment of breast cancer 1." *Radiology*, *233*(3), 830-849.

Bray, F., P. McCarron and D. M. Parkin (2004). "The changing global patterns of female breast cancer incidence and mortality." *Childhood* **4**(5).

Britt, K. (2012). Menarche, menopause, and breast cancer risk. *The lancet oncology*, *13*(11), 1071-1072.

Brooks, P., R. Clark and D. Cheresh (1994). "Requirement of vascular integrin alpha v beta 3 for angiogenesis." *Science* **264**(5158): 569-571.

Brooks, P. C., A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Klier and D. A. Cheresh (1994). "Integrin αvβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels." *Cell* **79**(7): 1157-1164.

Brooks, P. J., & Zakhari, S. (2013). Moderate alcohol consumption and breast cancer in women: from epidemiology to mechanisms and interventions. *Alcoholism: Clinical and Experimental Research*, *37*(1), 23-30.

Burdall, S. E., Hanby, A. M., Lansdown, M. R., & Speirs, V. (2003). "Breast cancer cell lines: friend or foe?." *Breast Cancer Research*, 5(2), 89-89.

Campbell, C. I., Thompson, D. E., Siwicky, M. D., & Moorehead, R. A. (2011). Murine mammary tumor cells with a claudin-low genotype. *Cancer Cell Int*, *11*(1), 28.

Carmeliet, P. and R. K. Jain (2011). "Molecular mechanisms and clinical applications of angiogenesis." *Nature* **473**(7347): 298-307.

Chae, Sujin, Hyoung-Oh Jun, EUNGYO LEE, SUKJIN YANG, Dong Chul Lee, Joon Ki Jung, Kyung Chan Park, Young Il Yeom, and KYUWON KIM. (2009). Osteopontin splice variants differentially modulate the migratory activity of hepatocellular carcinoma cell lines. *Angiogenesis*, *2*, 3.

Chambers, A. F. (2009). MDA-MB-435 and M14 cell lines: identical but not M14 melanoma?. *Cancer research*, *69*(13), 5292-5293.

Chambers, A. F., Behrend, E. I., Wilson, S. M., & Denhardt, D. T. (1991). Induction of expression of osteopontin (OPN; secreted phosphoprotein) in metastatic, ras-transformed NIH 3T3 cells. *Anticancer research*, *12*(1), 43-47.

Chellaiah, M. A., & Hruska, K. A. (2003). "he Integrin {alpha} v {beta} 3 and CD44 Regulate the Actions of Osteopontin on Osteoclast Motility." *Calcified tissue international*, *72*(3), 197-205.

Chen WY, Rosner B, Hankinson SE, Colditz GA, Willett WC (2011). "Moderate alcohol consumption during adult life, drinking patterns, and breast cancer risk" *JAMA* **306**:1884-1890

Chen, R. X., Xia, Y. H., Xue, T. C., Zhang, H., & Ye, S. L. (2011). Down-regulation of osteopontin inhibits metastasis of hepatocellular carcinoma cells via a mechanism involving MMP-2 and uPA. *Oncology reports*, *25*(3), 803-808.

Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans E, Godwin J, Gray R, Hicks C, James S(2005). "Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials." *Lancet*. **366**:2087-2106

Clarke, R. (1996). "Human breast cancer cell line xenografts as models of breast cancer—the immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines" *Breast cancer research and treatment*, *39*(1), 69-86.

Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans E, Godwin J, Gray R, Hicks C, James S(2005). "Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials." *Lancet*. **366**:2087-2106

Cuzick, J., Sestak, I., Baum, M., Buzdar, A., Howell, A., Dowsett, M., & Forbes, J. F. (2010). "Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial." *The lancet oncology*, *11*(12), 1135-1141.

Dai, J., Li, B., Shi, J., Peng, L., Zhang, D., Qian, W., ... & Guo, Y. (2010). A humanized anti-osteopontin antibody inhibits breast cancer growth and metastasis in vivo. *Cancer immunology, immunotherapy*, *59*(3), 355-366.

Dai, J., L. Peng, K. Fan, H. Wang, R. Wei, G. Ji, J. Cai, B. Lu, B. Li, D. Zhang, Y. Kang, M. Tan, W. Qian and Y. Guo (2009). "Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells." *Oncogene* **28**(38): 3412-3422.

Darby S, McGale P, Correa C, Taylor C, Arriagada R, Clarke M, Cutter D, Davies C, Ewertz M, Godwin J(2011). "Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials." *Lancet* **378**:1707-1716

Das, R., S. Philip, G. H. Mahabeleshwar, A. Bulbule and G. C. Kundu (2005). "Osteopontin: it's role in regulation of cell motility and nuclear factor kappa B-mediated urokinase type plasminogen activator expression." *IUBMB Life* **57**(6): 441-447.

Denhardt, D. T., Mistretta, D., Chambers, A. F., Krishna, S., Porter, J. F., Raghuram, S., & Rittling, S. R. (2003). Transcriptional regulation of osteopontin and the metastatic phenotype: evidence for a Ras-activated enhancer in the human OPN promoter. *Clinical & experimental metastasis*, *20*(1), 77-84.

Dsilva, S., Hiroaki, K., & Rothstein, T. (2012). AP-1 and NF-{kappa} B regulate osteopontin gene expression in anti-CD40 treated B cells. *The Journal of Immunology*, *188*, 161-16.

Early Breast Cancer Trialists' Collaborative Group (EBCTCG)(2005). "Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials." *Lancet* **365**:1687-1717.

Edlich RF, Winters KL, Lin KY (2005). "Breast cancer and ovarian cancer genetics" *J Long Term Eff Med Implants*. 15(5):533-45.

Fantozzi, A., & Christofori, G. (2006). "Mouse models of breast cancer metastasis." *Breast Cancer Res*, *8*(4), 212.

Fisher B, Anderson S, Bryant J, Margolese RG, Deutsch M, Fisher ER, Jeong JH, Wolmark N (2002). "Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer." *N Engl J Med* **347**:1233-1241

Fisher B, Jeong JH, Anderson S, Bryant J, Fisher ER, Wolmark N(2002). "Twenty-five-year follow-up of a randomized trial comparing radical mastectomy, total mastectomy, and total mastectomy followed by irradiation," *N Engl J* **347**:567-575

Fong, Y. C., S. C. Liu, C. Y. Huang, T. M. Li, S. F. Hsu, S. T. Kao, F. J. Tsai, W. C. Chen, C. Y. Chen and C. H. Tang (2009). "Osteopontin increases lung cancer cells migration via activation of the alphavbeta3 integrin/FAK/Akt and NF-kappaB-dependent pathway." *Lung Cancer* **64**(3): 263-270.

Gao, C., Guo, H., Mi, Z., Grusby, M. J., & Kuo, P. C. (2007). "Osteopontin induces ubiquitin-dependent degradation of STAT1 in RAW264. 7 murine macrophages." *The Journal of Immunology*, *178*(3), 1870-1881

Gianni L, Dafni U, Gelber RD, Azambuja E, Muehlbauer S, Goldhirsch A, Untch M, Smith I, Baselga J, Jackisch C (2011). "Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial." *Lancet Oncol*. **12**:236-244

Gotzsche, P. C., & Jorgensen, K. J. (2013). Screening for breast cancer with mammography. *Cochrane Database Syst Rev*, *6*(6).

Graessmann, M., Berg, B., Fuchs, B., Klein, A., & Graessmann, A. (2006). Chemotherapy resistance of mouse WAP-SVT/t breast cancer cells is mediated by osteopontin, inhibiting apoptosis downstream of caspase-3. *Oncogene,* *26*(20), 2840-2850.

Green, J. E., Shibata, M. A., Yoshidome, K., Liu, M. L., Jorcyk, C., Anver, M. R., ... &

Couldrey, C. (2000). “The C3 (1)/SV40 T-antigen transgenic mouse model of mammary cancer: ductal epithelial cell targeting with multistage progression to carcinoma.” *Oncogene*, *19*(8), 1020-1027.

Gunthert, U., M. Hofmann, W. Rudy, S. Reber, M. Zoller, I. Haussmann, S. Matzku, A. Wenzel, H. Ponta and P. Herrlich (1991). "A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells." *Cell* **65**(1): 13-24.

Guo, X., Zhang, Y. P., Mitchell, D. A., Denhardt, D. T., & Chambers, A. F. (1995). Identification of a ras-activated enhancer in the mouse osteopontin promoter and its interaction with a putative ETS-related transcription factor whose activity correlates with the metastatic potential of the cell. *Molecular and cellular biology*, *15*(1), 476-487.

He, B., Mirza, M., & Weber, G. F. (2005). An osteopontin splice variant induces anchorage independence in human breast cancer cells. *Oncogene*, *25*(15), 2192-2202.

Hennighausen, L., & Robinson, G. W. (2001). Signaling pathways in mammary gland development. *Developmental cell*, *1*(4), 467-475.

Hijiya, N., Setoguchi, M., Matsuura, K., Higuchi, Y., Akizuki, S., & Yamamoto, S. (1994). Cloning and characterization of the human osteopontin gene and its promoter. *Biochem. J*, *303*, 255-262.

Holliday, D. L., & Speirs, V. (2011). “Choosing the right cell line for breast cancer research.” *Breast Cancer Res*, 13(4), 215.

Hsieh, T. J., R. Chen, S. L. Zhang, F. Liu, M. L. Brezniceanu, C. I. Whiteside, I. G. Fantus, J. R. Ingelfinger, P. Hamet, and J. S. D. Chan, J. S. D. (2006). Upregulation of osteopontin gene expression in diabetic rat proximal tubular cells revealed by microarray profiling. *Kidney international*, *69*(6), 1005-1015.

Hutchinson, J. N., & Muller, W. J. (2000). “Transgenic mouse models of human breast cancer”. *Oncogene, 19*(53), 6130-6137

Hideyo Ihara, David T. Denhardt, Koichi Furuya, Teruhito Yamashita, Yukari Muguruma, Kunikazu Tsuji, Keith A. Hruska, Kanji Higashio, Shoji Enomoto, Akira Nifuji, Susan R. Rittling and Masaki Noda(2001). “Parathyroid hormone-induced bone resorption does not occur in the absence of osteopontin.” *Journal of Biological Chemistry*, *276*(16), 13065-13071.

Ishijima, Muneaki, Kunikazu Tsuji, Susan R. Rittling, Teruhito Yamashita, Hisashi Kurosawa, David T. Denhardt, Akira Nifuji, and Masaki Noda (2002). “Resistance to Unloading-Induced Three-Dimensional Bone Loss in Osteopontin-Deficient Mice.” *Journal*

of Bone and Mineral Research, *17*(4), 661-667.

Ishii Taeko, Shiro Ohshima, Tetsushi Ishida, Toru Mima, Youichiro Tabunoki, Hideyuki Kobayashi, Masahiro Maeda, Toshimitsu Uede, Lucy Liaw, Naokazu Kinoshita, Ichiro Kawase, Yukihiko Saeki (2004). "Osteopontin as a positive regulator in the osteoclastogenesis of arthritis." *Biochemical and biophysical research communications*, *316*(3), 809-815.

Jain, S., Chakraborty, G., Raja, R., Kale, S., & Kundu, G. C. (2008). Prostaglandin E2 regulates tumor angiogenesis in prostate cancer. *Cancer research*, *68*(19), 7750-7759.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: a cancer journal for clinicians*, *61*(2), 69-90.

Kawamura, K., Iyonaga, K., Ichiyasu, H., Nagano, J., Suga, M., & Sasaki, Y. (2005). "Differentiation, maturation, and survival of dendritic cells by osteopontin regulation." *Clinical and diagnostic laboratory immunology*, *12*(1), 206-212.

Keshtgar, M., T. Davidson, K. Pigott, M. Falzon, and A. Jones. "Current status and advances in management of early breast cancer." *International Journal of Surgery* 8, no. 3 (2010): 199-202.

Koh, Adeline, Andre Paes Batista Da Silva, Anil K. Bansal, Manju Bansal, Chunxiang Sun, Hyejin Lee, Michael Glogauer, Jaro Sodek, and Ron Zohar. (2007). "Role of osteopontin in neutrophil function. *Immunology*, *122*(4)," 466-475.

Lahmann PH, Hoffmann K, Allen N, van Gils CH, Khaw KT, Tehard B, Berrino F, Tjønneland A, Bigaard J, Olsen A (2004). "Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer And Nutrition (EPIC)". *Int J Cancer*. **111**:762-771

Li, X. D., Chen, J., Ruan, C. C., Zhu, D. L., & Gao, P. J. (2012). Vascular Endothelial Growth Factor–Induced Osteopontin Expression Mediates Vascular Inflammation and Neointima Formation via Flt-1 in Adventitial Fibroblasts. *Arteriosclerosis, thrombosis, and vascular biology*, *32*(9), 2250-2258.

Li, C. I., J. R. Daling, K. E. Malone, L. Bernstein, P. A. Marchbanks, J. M. Liff, B. L. Strom, M. S. Simon, M. F. Press, J. A. McDonald, G. Ursin, R. T. Burkman, D. Deapen and R. Spirtas (2006). "Relationship between established breast cancer risk factors and risk of seven different histologic types of invasive breast cancer." *Cancer Epidemiol Biomarkers Prev* **15**(5): 946-954.

Liaw, L., V. Lindner, S. M. Schwartz, A. F. Chambers and C. M. Giachelli (1995). "Osteopontin and β3 Integrin Are Coordinately Expressed in Regenerating Endothelium In Vivo and Stimulate Arg-Gly-Asp–Dependent Endothelial Migration In Vitro." *Circulation Research* **77**(4): 665-672.

Lipnik, Karoline, Helga Petznek, Ingrid Renner-Müller, Monika Egerbacher, Angelika Url, Brian Salmons, Walter H. Günzburg, and Christine Hohenadl. (2005). "A 470 bp WAP-promoter fragment confers lactation independent, progesterone regulated mammary-specific gene expression in transgenic mice." *Transgenic research*, *14*(2), 145-158.

Liu, J., Liu, Q., Wan, Y., Zhao, Z., Yu, H., Luo, H., & Tang, Z. (2014). Osteopontin promotes the progression of gastric cancer through the NF-κB pathway regulated by the MAPK and PI3K. *International journal of oncology*, *45*(1), 282-290.

Lund, S. A., Wilson, C. L., Raines, E. W., Tang, J., Giachelli, C. M., & Scatena, M. (2013)." Osteopontin mediates macrophage chemotaxis via α4 and α9 integrins and survival via the α4 integrin." *Journal of cellular biochemistry*, *114*(5), 1194-1202.

Malhotra, G. K., Zhao, X., Band, H., & Band, V. (2010). Histological, molecular and functional subtypes of breast cancers. *Cancer Biol Ther*, *10*(10), 955-960.

Maningat, P. D., Sen, P., Rijnkels, M., Sunehag, A. L., Hadsell, D. L., Bray, M., & Haymond, M. W. (2009). "Gene expression in the human mammary epithelium during lactation: the milk fat globule transcriptome." *Physiological genomics*, *37*(1), 12-22.

Mi, S.D. Bhattacharya, V.M. Kim, H. Guo, L.J. Talbot, P.C. Kuo (2011). "Osteopontin promotes CCL5-mesenchymal stromal cell-mediated breast cancer metastasis" *Carcinogenesis*(4): 477–487.

Minna Allinen, Rameen Beroukhim, Li Cai, Cameron Brennan, Jaana Lahti-Domenici, Haiyan Huang, Dale Porter, Min Hu, Lynda Chin, Andrea Richardson, Stuart Schnitt, William R Sellers, Kornelia Polyak (2004). Molecular characterization of the tumor microenvironment in breast cancer. *Cancer cell*, *6*(1), 17-32.

Moller, T., Olsson, H., Ranstam, J., & Collaborative Group on Hormonal Factors in Breast Cancer. (2002). Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50 302 women with breast cancer and 96 973 women without the disease. *Lancet, 360*(9328), 187-195.

Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., & Leder, P. (1988). "Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-< i> neu</i> oncogene." *Cell*, *54*(1), 105-115.

Nagatomo, T., S. Ohga, H. Takada, A. Nomura, S. Hikino, M. Imura, K. Ohshima, and T. Hara (2004). "Microarray analysis of human milk cells: persistent high expression of osteopontin during the lactation period." *Clinical & Experimental Immunology*, *138*(1), 47-53.

Narod SA, Tung N, Lubinski J, Huzarski T, Robson M, Lynch HT, Neuhausen SL, Ghadirian P, Kim-Sing C, Sun P, Foulkes WD (2014). "A prior diagnosis of breast cancer is a risk factor for breast cancer in BRCA1 and BRCA2 carriers" *Curr Oncol*. 21(2):64-8

Nemir, M., Bhattacharyya, D., Li, X., Singh, K., Mukherjee, A. B., & Mukherjee, B. B. (2000). "Targeted inhibition of osteopontin expression in the mammary gland causes abnormal morphogenesis and lactation deficiency." *Journal of Biological Chemistry*, *275*(2), 969-976.

Newcomb, P. A., A. Trentham-Dietz, J. M. Hampton, K. M. Egan, L. Titus-Ernstoff, S. Warren Andersen, E. R. Greenberg and W. C. Willett (2011). "Late age at first full term birth is strongly associated with lobular breast cancer." *Cancer* **117**(9): 1946-1956.

Oates, A. J., Barraclough, R., & Rudland, P. S. (1996). The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumour model. *Oncogene*, *13*(1), 97-104.

Osborne CK, Hobbs K, Trent JM (1987). "Biological differences among MCF-7 human breast cancer cell lines from different laborato- ries." *Breast Cancer Res Treat* 9(2):111-121.

Palmiter, R. D., Sandgren, E. P., Koeller, D. M., & Brinster, R. L. (1993). "Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice." *Molecular and cellular biology*, *13*(9), 5266-5275.

Pampena, D., Robertson, K., Litvinova, O., Lajoie, G., Goldberg, H., & Hunter, G. (2004). "Inhibition of hydroxyapatite formation by osteopontin phosphopeptides." *Biochem. J*, *378*, 1083-1087.

Pattengale, P. K., T. A. Stewart, A. Leder, E. Sinn, W. Muller, I. Tepler, E. Schmidt, and P. Leder (1989). "Animal models of human disease. Pathology and molecular biology of spontaneous neoplasms occurring in transgenic mice carrying and expressing activated cellular oncogenes." *The American journal of pathology*, *135*(1), 39.

Peppercorn, J., Perou, C. M., & Carey, L. A. (2008). Molecular subtypes in breast cancer evaluation and management: divide and conquer. *Cancer investigation*, *26*(1), 1-10.

Prat, Aleix, Joel S. Parker, Olga Karginova, Cheng Fan, Chad Livasy, Jason I. Herschkowitz, Xiaping He, and Charles M. Perou. M. (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res*,

12(5), R68

Prince, C. W., Oosawa, T., Butler, W. T., Tomana, M., Bhown, A. S., Bhown, M., & Schrohenloher, R. E. (1987). Isolation, characterization, and biosynthesis of a phosphorylated glycoprotein from rat bone. *Journal of Biological Chemistry*, *262*(6), 2900-2907.

Rangaswami, H., Bulbule, A., & Kundu, G. C. (2006). Osteopontin: role in cell signaling and cancer progression. *Trends in cell biology*, *16*(2), 79-87.

Rathinam, R. and S. Alahari (2010). "Important role of integrins in the cancer biology." *Cancer and Metastasis Reviews* **29**(1): 223-237.

Reeves, G. K., K. Pirie, J. Green, D. Bull and V. Beral (2009). "Reproductive factors and specific histological types of breast cancer: prospective study and meta-analysis." *Br J Cancer* **100**(3): 538-544.

Rhodes, D. J., Hruska, C. B., Phillips, S. W., Whaley, D. H., & O'connor, M. K. (2011). Dedicated Dual-Head Gamma Imaging for Breast Cancer Screening in Women with Mammographically Dense Breasts 1. *Radiology*, *258*(1), 106-118.

Rodrigues, L. R., Teixeira, J. A., Schmitt, F. L., Paulsson, M., & Lindmark-Mänsson, H. (2007). The role of osteopontin in tumor progression and metastasis in breast cancer. *Cancer Epidemiology Biomarkers & Prevention*, *16*(6), 1087-1097.

Ritte, R., A. Lukanova, A. Tjonneland, A. Olsen, K. Overvad, S. Mesrine, G. Fagherazzi, L. Dossus, B. Teucher, K. Steindorf, H. Boeing, K. Aleksandrova, A. Trichopoulou, P. Lagiou, D. Trichopoulos, D. Palli, S. Grioni, A. Mattiello, R. Tumino, C. Sacerdote, J. R. Quiros, G. Buckland, E. Molina-Montes, M. D. Chirlaque, E. Ardanaz, P. Amiano, B. Bueno-de-Mesquita, F. van Duijnhoven, C. H. van Gils, P. H. Peeters, N. Wareham, K. T. Khaw, T. J. Key, R. C. Travis, S. Krum-Hansen, I. T. Gram, E. Lund, M. Sund, A. Andersson, I. Romieu, S. Rinaldi, V. McCormack, E. Riboli and R. Kaaks (2013). "Height, age at menarche and risk of hormone receptor-positive and -negative breast cancer: a cohort study." *Int J Cancer* **132**(11): 2619-2629.

Robertson, B. W., Bonsal, L., & Chellaiah, M. A. (2010). Regulation of Erk1/2 activation by osteopontin in PC3 human prostate cancer cells. *Molecular cancer*, *9*(1), 260.

Rollo, Ellen E., Scott J. Hempson, Ajay Bansal, Ernest Tsao, Iman Habib, Susan R. Rittling, David T. Denhardt, Erich R. Mackow, and Robert D. Shaw (2005). "The cytokine

osteopontin modulates the severity of rotavirus diarrhea." *Journal of virology*, *79*(6), 3509-3516.

Sabatier, Renaud, Pascal Finetti, Arnaud Guille, José Adelaide, Max Chaffanet, Patrice Viens, Daniel Birnbaum, and François Bertucci (2014). Claudin-low breast cancers: clinical, pathological, molecular and prognostic characterization. *Molecular cancer*, *13*(1), 228.

Seftor, R. E., E. A. Seftor and M. J. Hendrix (1999). "Molecular role(s) for integrins in human melanoma invasion." *Cancer Metastasis Rev* **18**(3): 359-375.
Senger, D. R., Wirth, D. F., & Hynes, R. O. (1979). "Transformed mammalian cells secrete specific proteins and phosphoproteins." *Cell*, *16*(4), 885-893.

Shah, R., Rosso, K., & Nathanson, S. D. (2014). "Pathogenesis, prevention, diagnosis and treatment of breast cancer." *World journal of clinical oncology*, *5*(3), 283.

Sharma, G. D., He, J., & Bazan, H. E. (2003). p38 and ERK1/2 Coordinate Cellular Migration and Proliferation in Epithelial Wound Healing EVIDENCE OF CROSS-TALK ACTIVATION BETWEEN MAP KINASE CASCADES. *Journal of Biological Chemistry*, *278*(24), 21989-21997.

Shevde, Lalita A., Rajeev S. Samant, Jason C. Paik, Brandon J. Metge, Ann F. Chambers, Graham Casey, Andra R. Frost, and Danny R. Welch (2006). Osteopontin knockdown suppresses tumorigenicity of human metastatic breast carcinoma, MDA-MB-435. *Clinical & experimental metastasis*, *23*(2), 123-133.

Singletary, S. E., Allred, C., Ashley, P., Bassett, L. W., Berry, D., Bland, K. I., & Greene, F. L. (2002). "Revision of the American Joint Committee on Cancer staging system for breast cancer." *Journal of clinical oncology*, *20*(17), 3628-3636.

Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Press M, Mackey J, Glaspy J, Chan A, Pawlicki M (2001). "Adjuvant trastuzumab in HER2-positive breast cancer." *N Engl J Med*. **365**:1273-1283.

Sloan, E. K., Pouliot, N., Stanley, K. L., Chia, J., Moseley, J. M., Hards, D. K., & Anderson, R. L. (2006). Tumor-specific expression of αvβ3 integrin promotes spontaneous metastasis of breast cancer to bone. *Breast Cancer Research*, *8*(2), R20

Smetherman, D. H. (2013). "Screening, imaging, and image-guided biopsy techniques for breast cancer". *Surgical Clinics of North America*, *93*(2), 309-327.

Taxman, D. J., Moore, C. B., Guthrie, E. H., & Huang, M. T. H. (2010). Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. In *RNA Therapeutics* (pp. 139-156). *Humana Press*.

Tuck, A. B., D. M. Arsenault, F. P. O'Malley, C. Hota, M. C. Ling, S. M. Wilson and A. F. Chambers (1999). "Osteopontin induces increased invasiveness and plasminogen activator expression of human mammary epithelial cells." *Oncogene* **18**(29): 4237.

Tuck, A. B., Chambers, A. F., & Allan, A. L. (2007). Osteopontin overexpression in breast cancer: knowledge gained and possible implications for clinical management. *Journal of cellular biochemistry*, *102*(4), 859-868.

Tuck, A. B., B. E. Elliott, C. Hota, E. Tremblay and A. F. Chambers (2000) . "Osteopontin-induced, integrin-dependent migration of human mammary epithelial cells involves activation of the hepatocyte growth factor receptor (Met)." *Journal of Cellular Biochemistry* **78**(3): 465-475.

Tuck, A. B., F. P. O'Malley, H. Singhal, J. F. Harris, K. S. Tonkin, N. Kerkvliet, Z. Saad, G. S. Doig and A. F. Chambers (1998). "Osteopontin expression in a group of lymph node negative breast cancer patients." *Int J Cancer* **79**(5): 502-508.

Tuck, A. B., O'Malley, F. P., Singhal, H., Tonkin, K. S., Harris, J. F., Bautista, D., & Chambers, A. F. (1997). Osteopontin and p53 expression are associated with tumor progression in a case of synchronous, bilateral, invasive mammary carcinomas. *Archives of pathology & laboratory medicine*, *121*(6), 578-584.

Urruticoechea, A., Smith, I. E., & Dowsett, M. (2005). Proliferation marker Ki-67 in early breast cancer. *Journal of clinical oncology*, *23*(28), 7212-7220**.**

Ursin, G., L. Bernstein, S. J. Lord, R. Karim, D. Deapen, M. F. Press, J. R. Daling, S. A. Norman, J. M. Liff, P. A. Marchbanks, S. G. Folger, M. S. Simon, B. L. Strom, R. T. Burkman, L. K. Weiss and R. Spirtas (2005). "Reproductive factors and subtypes of breast cancer defined by hormone receptor and histology." *Br J Cancer* **93**(3): 364-371.

Valachis A, Polyzos NP, Patsopoulos NA, Georgoulias V, Mavroudis D, Mauri D (2010). “Bevacizumab in metastatic breast cancer: A meta-analysis of randomized controlled trials.” *Breast Cancer Res Treat*. 122(1):1–7

Weigelt, B., H. M. Horlings, B. Kreike, M. M. Hayes, M. Hauptmann, L. F. A. Wessels, D. de Jong, M. J. Van de Vijver, L. J. Van't Veer, and J. L. Peterse. (2008). Refinement of breast cancer classification by molecular characterization of histological special types. *The Journal of pathology*, *216*(2), 141-150.

Wu Y, Zhang D, Kang S (2013)." Physical activity and risk of breast cancer: a meta-analysis of prospective studies" *Breast Cancer Res Treat* **137**:869-882

Shi X, S. Bai, L. Li, X. Cao (2001) ."Hoxa-9 represses TGF-beta induced osteopontin gene transcription." *J. Biol. Chem*. 276(1) 850-855.

Yoo, B. K., Gredler, R., Chen, D., Santhekadur, P. K., Fisher, P. B., & Sarkar, D. (2011). c-Met activation through a novel pathway involving osteopontin mediates oncogenesis by the transcription factor LSF. *Journal of hepatology*, *55*(6), 1317-1324.

Yoshitake, H., Rittling, S. R., Denhardt, D. T., & Noda, M. (1999). "Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption." *Proceedings of the National Academy of Sciences*, *96*(14), 8156-8160.

Zhang, G. X., Zhao, Z. Q., Wang, H. D., & Hao, B. (2004). Enhancement of osteopontin expression in HepG2 cells by epidermal growth factor via phosphatidylinositol 3-kinase signaling pathway. *World Journal of Gastroenterology*, *10*(2), 205-208
Zhang, Q., Domenicucci, C., Goldberg, H. A., Wrana, J. L., & Sodek, J. (1990). Characterization of fetal porcine bone sialoproteins, secreted phosphoprotein I (SPPI, osteopontin), bone sialoprotein, and a 23-kDa glycoprotein. Demonstration that the 23-kDa glycoprotein is derived from the carboxyl terminus of SPPI. *Journal of Biological Chemistry*, *265*(13), 7583-758.

Zhang, G., He, B., & Weber, G. F. (2003). Growth factor signaling induces metastasis genes in transformed cells: molecular connection between Akt kinase and osteopontin in breast cancer. *Molecular and cellular biology, 23*(18), 6507-6519.

Zhang, H., Guo, M., Chen, J. H., Wang, Z., Du, X. F., Liu, P. X., & Li, W. H. (2014). Osteopontin Knockdown Inhibits αv, β3 Integrin-Induced Cell Migration and Invasion and Promotes Apoptosis of Breast Cancer Cells by Inducing Autophagy and Inactivating the PI3K/Akt/mTOR Pathway. *Cellular Physiology and Biochemistry*, *33*(4), 991-1002.

Zhao J, Li Dong, Bin Lu, Guobin Wu, Dongmei Xu, Jingjing Chen, Kai Li, Xin Tong*, Jianxin Dai, Side Yao, Mengchao Wu, Yajun Guo(2008). Down-regulation of osteopontin suppresses growth and metastasis of hepatocellular carcinoma via induction of apoptosis. *Gastroenterology*, *135*(3), 956-968.

Zohar, R., Suzuki, N., Suzuki, K., Arora, P., Glogauer, M., McCulloch, C. A. G., & Sodek, J. (2000). Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. *Journal of cellular physiology*, *184*(1), 118-130.

Zou, C., Luo, Q., Qin, J., Shi, Y., Yang, L., Ju, B., & Song, G. (2013). Osteopontin

promotes mesenchymal stem cell migration and lessens cell stiffness via Integrin β1, FAK, and ERK pathways. *Cell biochemistry and biophysics*, *65*(3), 455-462

APPENDIX I – SOURCE OF PRODUCTS AND MATERIALS

Acrylamide	BioRad Laboratories, Mississauga, ON
Agrose	Invitrogen, Burlington, ON
Alexa Fluor 488 Anti-rabbit IgG	Invitrogen, Burlington, ON
Alexa Fluor 594 Anti-goat IgG	Invitrogen, Burlington, ON
Aluminum potassium sulfate	Fisher Scientific, Whitby, ON
Amcon Ultra-15 Centrifugal filters	Millipore, Etobicko, ON
Ammonium Persulfate	Fisher Scientific, Whitby, ON
Anti-av antibody	Santa Cruz Biotechnology Inc., MA, CA
Anti-AKT antibody	Cell Signaling Technology, Danvers, MA
Antibiotic-Antimycotic	GIBCO, Burlington, ON
Anti-β3 antibody	Santa Cruz Biotechnology Inc., MA, CA
Anti-βactin antibody	Cell Signaling Technology, Beverly, MA
Anti-caspase 3 (Active form)	Millipore, Etobicoke, ON
Anti-ERK antibody	Cell Signaling Technology, Beverly, MA
Anti-goat biotin conjugated antibody	Cell Signaling Technology, Beverly, MA
Anti-Ki67 antibody	Abcam, Cambridge, MA
Anti-mouse HPR conjugated antibody	Cell Signaling Technology, Beverly, MA
Anti-OPN antibody	Santa Cruz Biotechnology Inc., MA, CA
Anti-phosphp-AKT antibody	Cell Signaling Technology, Beverly, MA
Anti-phospho-ERK1/ERK2 antibody	Cell Signaling Technology, Beverly, MA

Anti-Rabbit biotin conjugated antibody	Sigma, Oakville, ON
Anti-Rabbit HRP conjugated antibody	Cell Signaling Technology, Beverly, MA
Anti-VEFG antibody	Santa Cruz Biotechnology Inc., MA, CA
Aprotinin	Sigma-Aldrich Canada Ltd., Oakville, ON
Blasticidin	Invitrogen, Burlington, On
Bovine serum Albumin	Invitrogen, Burlington, On
Bromophenol Blue	Sigma, Oakville, ON
Cell culture Dishes (100mm)	Fisher Scientific, Whitby, ON
Citric Acid	Fisher Scientific, Whitby, ON
Chemiluminescence substrate	Perkin Elmer, Wellesley, MA
Costar 6 well culture dishes	Corning Life Science, Lowell, MA
Coverlips	Fisher Scientific, Whitby, ON
Dc Protein Assay	BioRad Laboratories, Mississauga, ON
Diaminobenzidine tetrahydrochloride	Sigma, Oakville, ON
Dihdrochloride hydrate (DAPI)	Sigma, Oakville, ON
Dithiothreitol(DTT)	Invitrogen, Burlington, On
DMEM Media	GIBCO, Burlingtone, ON
dNTP	New England Biolabs, Picketing, ON
Doxycycline diet	BioServ, Frenchtown, NJ
(EDTA)	Fisher Scientific, Whitby, ON
EGF	Sigma, Oakville, ON
Ethanol	Greenfield, Brampton, ON
Ethidium Bromide	Invitrogen, Burlington, ON

Exogenous Recombinant Osteopontin	R&D Systems, Minneapolis,, MN, USA
Falcon Cell Inserts	BD Bioscience, Mississauga, ON
Fetal Bovine Serum	Sigma, Oakville, ON
Fisherbrand Glass Slides	Fisher Scientific, Whitby, ON
Glacial Acetic Acid	Fisher Scientific, Whitby, ON
Glycerol	Fisher Scientific, Whitby, ON
Glycine	Fisher Scientific, Whitby, ON
Hepes	Sigma, Oakville, ON
Hydrochloric Acid	Fisher Scientific, Whitby, ON
Hydrocortisone	Sigma, Oakville, ON
Hydrogen Peroxide	Sigma, Oakville, ON
Insulin	Sigma, Oakville, On
Leupeptin	Sigma, Oakville, ON
Lipofectamine 2000	Invitrogen, Burlington, ON
Methanol	Fisher Scientific, Whitby, ON
Matrigel	Invitrogen, Burlington, ON
Nitrocellulose Membranes	Amercham, Piscataway, NJ
OPN RNAi sequence	Invitrogen, Burlington, ON
Opti-MEM media	GIBCO, Burlington, ON
Pepstatin A	Sigma, Oakville, ON
Phenylmethylsulfonyl Flouride (PMSF)	Fisher Scientific, Whitby, ON
Pottasium Chloride	Fisher Scientific, Whitby, ON
Potassium Phosphate	Fisher Scientific, Whitby, ON

Prolactin	Sigma, Oakville, ON
Prolong Gold antifade reagent	Invitrogen, Burlington, ON
Sodium Acetate	Fisher Scientific, Whitby, ON
Sodium Citrate	Fisher Scientific, Whitby, ON
Sodium Chloride	Fisher Scientific, Whitby, ON
Sodium Dodecyl Sulfate	Fisher Scientific, Whitby, ON
Sodium Flouride	Fisher Scientific, Whitby, ON
Sodium Phosphate dibasic anhydrous	Fisher Scientific, Whitby, ON
Sodium Pyruvate	Sigma, Oakville, On
Stealth RNAi	Invitrogen, Burlington, ON
Sucrose	Sigma, Oakville, ON
TEMED	GIBCO, Burlington, ON
Tratracyclin-Free Fetal Bovine Serum	Clontech, Mountain View, CA
Tris Base	Fisher Scientific, Whitby, ON
Triton X-100	Sigma, Oakville, ON
Trypsin-EDTA	Sigma, Oakville, ON
Tween-20	Fisher Scientific, Whitby, ON
Typan Blue Stain	Invitrogen, Burlington, ON

APPENDIX II – RECIPES FOR SOLUTIONS

Blocking Solution (5% for Immunohistochemistry)

BSA	0.5 g
PBS	10 ml

Blocking Solution (5% for Western Blots)

Skim Milk Powder	5 g
1X TBST	100 ml

Phosphate Buffered Saline

Sodium Chloride	16,0g
Potassium Chloride	0.4g
Sodium Potassium Dibasic Anhydrous	2.3 g
Potassium Phosphate Monobasic	0.4 g
pH to 7.4 with HCL	

Protein Lysis Buffer (500ml)

10 mM Tris-HCL pH 7.6	0.788 g
5 mM EDTA	0.7306 g

50 mM NaCl	1.461 g
30 mM Tetrasodium pyrophosphate	3.988 g
1% Triton X-100	5 ml
H_2O	to 500 ml

Add the following protease inhibitors before each use per 1 ml of lysis buffer

2 mg/ml Leupeptin	1µl
1 mg/ml Pepstatin	1 µl
2.5 mg/ml Aprotinin	2 µl
50 mM PMSF	20 µl
0.1 mM Sodium Orthovanadate	1 µl
1 M Sodium Flouride	50 µl

Reducing Buffer (3X)

10 % SDS	2 ml
Glycerol	1 ml
1M Tris-HCL	0.5 ml
Bromophenol Blue	0.01 g
H_2O	9 ml

Before use add 1/8 volume of 1.0 M DTT

Running Buffer (5X)

Tris	15.1 g
Glycine	72.1 g
10 % SDS	10 ml
H_2O	to 1 L

Transfer Buffer

Tris	7 g
Glycine	3.02 g
Methanol	200 ml
H_2O	to 1 L

Tris (1.0M)

Tris Base	12.12 g
H_2O	to 100 ml

Adjust pH to 6.8 with HCL

Tris (1.5M)

Tris base	18.16 g
H_2O	to 100 ml

Adjust pH to 8.8 with HCL

50 x Tris Acetate Buffer

Tris Base	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA	100 ml
H_2O	to 1 L

10X Tris Buffered Saline-Tween 20

Tris	24.2 g
NaCl	80 g
H_2O	to 1 L

Adjust to 7.4 with HCl

Then add 10 ml of Tween-20

Tris-Glycine SDS-Polyacrlylamide Gels (10%) for 1 gel

Resolving Gel

H_2O	4 ml
30% Acrylamide	3.3 ml
1.5 M Tris (pH 8.8)	2.5 ml
10% SDS	100 µl
10% Ammonium Persulfate	100 µl
TEMED	4 µl

Stocking Gel

H_2O	2.1 µl

30% Acrylamide	500 µl
1.5 M Tris (pH 6.8)	380 µl
10% SDS	30 µl
10% Ammonium Persulfate	30 µl
TEMED	3 µl