ONCOSTATIN M CONTRIBUTES TO BREAST TUMOR METASTASIS

by

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DEDICATION

To my wife who has offered me so much support and convinced me to not give up numerous times during graduate school. This is also dedicated to my two young sons who keep me focused on the goals at hand and cheer me up whenever I am down.

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ABSTRACT

The process of breast tumor metastasis has been examined to a great extent in recent years, but connecting specific protein-protein interactions to respective metastatic events remains challenging. The primary cause of death in patients with breast cancer is not from the primary tumor itself but rather from tumor metastases, which cause over 90% of all mortalities. In order for tumor cells to metastasize, they must undergo a phenotypic change known as the epithelial-mesenchymal transition (EMT). An EMT allows for the intravasation of tumor cells into nearby blood vessels or lymphatic channels. Once the primary tumor cells are within the network of vessels, they undergo a homing process to specific organs within the body. For breast cancer, secondary metastatic locations include the liver, brain, lung, and bone. It is known that signaling by proteins called cytokines influences breast tumor cells to adopt a more metastatic and aggressive phenotype. Cytokine signaling often changes protein functioning in the surrounding tumor environment and can lead to increased vasculature, which provides nutrients to a growing tumor, or phenotypic changes to individual cells on the invasive edge of a proliferating tumor. Transmembrane proteins, such as cluster of differentiation 44 (CD44), contribute to cell migration, cell detachment, and cellular docking during metastasis. The upregulation of CD44 expression by cytokine signaling is observed in less aggressive tumor cells, while more aggressive tumor cells constitutively express high basal levels of CD44. Both EMT and CD44 induced by cytokines are involved in metastasis during specifically timed events in the metastatic cascade.

TABLE OF CONTENTS

DEDICATION iv

ACKNOWLEDGEMENTS v

ABSTRACT vii

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF ABBREVIATIONS xvi

CHAPTER ONE: INTRODUCTION 1

Breast Cancer 1

Breast Cancer Subtypes 1

Metastatic Breast Cancer 2

Oncostatin M (OSM) 3

Cluster of Differentiation 44 (Cd44) 5

Cancer Stem Cells (CSCs) and Breast Cancer Stem Cells (BCSCs) 7

Circulating Tumor Cells (CTCs) 8

In Vivo Models of Human Breast Cancer 8

Patient survival 9

Summary 9

CHAPTER TWO: OSM CONTRIBUTES TO BREAST CANCER METASTATIC POTENTIAL THROUGH CD44-INDUCED CELL MIGRATION AND DETACHMENT 14

Abstract 15

Introduction 16

Materials and Methods 19

Cell Culture and Cytokine Stimulation 19

Immunofluorescence Staining and Microscopy 19

qRT-PCR 20

Western Blot Analysis 20

Flow Cytometry 21

shRNA and Chemical Inhibitors 21

Detachment Assay 22

Scratch Assay 22

Results 23

OSM Induces Both CD44 Expression and Cleavage 24

OSM-Induced EMT is Independent of CD44 26

OSM-Induced Tumor Cell Detachment is Dependent on CD44 27

OSM-Induced Tumor Cell Migration is Dependent on CD44 28

Discussion 28

Conclusion 30

CHAPTER THREE: CD44 IS IMPORTANT FOR TUMOR CELL EXTRAVASATION DURING THE METASTATIC CASCADE 42

Abstract 43

Introduction 44

Materials and Methods 45

Cell Culture 45

BioFlux System....46

Cd44 Knockdown Mouse Model of Breast Cancer....46

Bioluminescence *in Vivo* Imaging of Live Animals....47

Flow Cytometry....47

Cell Sorting....47

Estrogen Pellet Implantation in Mice Receiving Mcf-7 Er+ Cells....48

Bcsc Mouse Model of Breast Cancer....48

Oncomine Data....48

Results....49

Cd44 Is Necessary for Tumor Cell Docking fo Endothelial Cells....49

Decreased Cd44 Expression Leads to an Increase in Breast Circulating Tumor Cells (Ctcs)....50

A Low Number of $Cd44^{+/}Cd^{24-/Low}$ Breast Cancer Cells (BCSCs) Initiate Tumor Formation....51

Cd44 Is Crucial For the Survival Of Human Patients Expressing Low Osmr....52

Discussion....53

Conclusion....55

REFERENCES....63

APPENDIX A....79

LIST OF TABLES

Table 1: $CD44^{+}/CD24^{-/low}$ MCF-7 luc BCSCs are capable of tumor initiation *in vivo*. Cells were collected after cell sorting was performed. Following cell sorting, $CD44^{+}/CD24^{-/low}$ cells were compared to MCF-7 luc parental cells. 61

Table 2 Primers 62

LIST OF FIGURES

Figure 1.1: Breast tumor cells metastasize through a series of events. Angiogenesis occurs, which allows for intravasation of invasive and migratory tumor cells into the vasculature. Cells in circulation eventually create a secondary tumor at a distal location. (Ma and Weinberg, 2008) (66) 10

Figure 1.2: CD44 is composed of exons capable of alternative splicing. Rearrangement of CD44 exons creates CD44v isoforms that pose larger molecular weights compared to the standard CD44 (CD44s). (Sen and Yip, 2009) (67)..... 11

Figure 1.3: CD44 is alternatively spliced while retaining CD44s exons. CD44v6 and CD44v8-10 are commonly present on breast tumor cells. (Xu H. et. al, 2015) (45)..... 12

Figure 1.4. CD44 is post-translationally cleaved by MMPs. CD44 is cleaved by MMPs directly adjacent to the cell membrane below the variable region of exons. The cleavage relieves the extracellular domain (ECD) of being bound to the cellular membrane. As a second event γ-secretase cleavage occurs within the membrane producing an intracellular domain (ICD). (Adapted from Louderbough and Schroeder, 2011) (68) 13

Figure 2.1. OSM induces EMT and a BCSC phenotype in human breast cancer cells. A. MCF-7 luc human breast cancer cells were treated with OSM (25 ng/mL) for 72 hours. OSM induces an increased mesenchymal phenotype and a slight decrease and redistribution in E-cadherin expression, as measured by immunofluorescence using antibodies for E-cadherin (green) and actin (red) (DAPI = blue; 63x magnification). B. MCF-7 cells were treated with OSM (25 ng/mL) for 1 hour, and qPCR was performed with primers for the EMT transcription factors Slug (n = 3, +/- SEM) and Snail (n = 3, +/- SEM) (Slug *p<.05, paired t test). C. MCF-7 cells were treated with OSM (25 ng/mL) for 24, 48, and 72 hours, and cell lysates were assessed by western blot analysis for E-cadherin and α-catenin. D and E. ER+ MCF-7 and T47D cells were treated with OSM (25 ng/mL) for 72 hours and flow cytometry was performed for $CD44^{+}/CD24^{-/low}$. While OSM induces a small non-significant increase the $CD44^{+}/CD24^{-/low}$ BCSC population in MCF-7 cells, in T47D cells, OSM induces the BCSC population 3-fold (+/- SEM for both) (**p<0.005, paired t-test). 32

Figure 2.2 OSM induces CD44s (70 – 85 kDa), CD44 isoforms (above 85 kDa), and cleaved products (below 35 kDa) in ER+ cells but not ER- cells. Cells were treated with (25 ng/mL) OSM for 24, 48, and 72 hours, and whole cell lysates were collected for immunoblotting using an anti-CD44 antibody. A. MCF-7 (ER+). B. T47D (ER+). C. MDA-MB-231 (ER-)... 34

Figure 2.3. Reduced CD44 expression does not abrogate OSM-induced EMT in ER+ cells. A. Both MCF-7 luc-shCD44 and MDA-MB-231 luc-shCD44 cells show knockdown of CD44s protein expression compared to non-targeting control (shNTC) cell lines by western blot analysis. B. Quantification of CD44 knockdown (n = 3, +/- SEM, paired t test $p < 0.005$). C. MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for one hour, and qPCR analysis for Slug and Snail was performed. Knockdown of CD44 expression did not decrease OSM-induced Slug (C), but Snail decreased in shNTC compared to shCD44 cells (D) (n=3, +/- SEM, One Way Anova $p < 0.005$). E. MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for 72 hours, and EMT and E-cadherin was observed via immunofluorescence (20x magnification scale bar in = 30 nm). F. MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for 72 hours to observe EMT morphology by phase contrast images (20x magnification scale bar = 200 μm)..... 35

Figure 2.4. Reduced CD44 expression decreases OSM-induced tumor cell detachment in ER+ cells. A. ER+ MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) over a 5-day period, and detached cells were counted on days 1, 3, and 5. Data is represented as fold-change (*$p<0.05$, **$p<0.005$, ***$p<0.001$, one-way ANOVA) B. ER- MDA-MB-231 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) over a 5-day period, and detached cells were counted on days 1, 3, and 5. data is represented as fold change (***$p<0.001$, one-way ANOVA) (n=3, +/- SEM). 36

Figure 2.5. Reduced CD44 expression decreases OSM-induced tumor cell migration in ER- cells to a greater extent than in ER+ cells. A. ER+ MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for 3 days, and wound healing was analyzed using ImageJ software. Data was interpreted as fold change (*$p<0.05$, one-way ANOVA) (n=3, +/- SEM). B. ER- MDA-MB-231 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for 3 days, and wound healing was analyzed using Image J software. Data was interpreted as fold change (***$p<0.001$, one-way ANOVA) (n=3, +/- SEM). 37

Figure 2.6. Model for OSM-induced migration and detachment. OSM signaling induces an EMT and upregulates CD44 expression and cleavage in ER+ breast tumor cells. Once bound to its receptor, OSM induces cells to undergo EMT and subsequent detachment and migration. OSM signaling

also increases expression of the transmembrane protein CD44 and its cleavage, possibly via MMPs. OSM-induced CD44 is involved in migration and detachment as well as other downstream events during the metastatic cascade. 38

Supplemental Figure 2.1. ..... OSM induces an EMT in MCF-7 cells. MCF-7 breast tumor cells were treated with OSM (25 ng/mL) for 72 hours. OSM induces an increased mesenchymal phenotype, a redistribution in E-cadherin expression (green, upper panels, scale bar = 20 nm), and a slight decrease in □-catenin expression (green, lower panels scale bar = 20 nm), as measured by immunofluorescence (DAPI = blue). 39

Supplemental Figure 2.2. ...OSM has no effect on different CD44 variant mRNA levels in breast cancer cells. A. MCF-7 cells were treated with OSM (25 ng/mL) for 0.5, 1, 4, and 8 hours followed by RNA collection and qPCR analysis for CD44s (n = 3, +/- SEM). B. MCF-7 cells were treated with OSM (25 ng/mL) for 8 hours followed by RNA collection and qPCR analysis for different CD44 splice variants. (n = 3, +/- SEM). 40

Supplemental Figure 2.3. OSM-induced CD44 cleavage is reduced by a general MMP inhibitor in ER+ breast tumor cells. A. T47D cells were treated with 10 nM of GM6001 (global MMP inhibitor) for 48 hours prior to the addition of OSM (25 ng/mL) for an additional 48 hours. Whole cell lysates were collected and CD44 western blot analysis was performed to observe cleaved products. OSM induces CD44 in T47D cells after 48 hours; inhibition of MMP activity reduces OSM-induced CD44 cleavage. B. Quantitation of the 15 kDa – 35 kDa bands by ImageJ demonstrate that GM6001 significantly reduces OSM-induced CD44 cleaved products (n = 3, +/- SEM) (*$p<0.05$, unpaired t-test). 41

Figure 3.1: CD44 increases tumor cell docking in MDA-MB-231 luc cells in an *in vitro* model. A. MDA-MB-231 luc-shNTC and shCD44 cells were stained with Calcein AM for 15 minutes in a cell culture flask in the incubator. Cells were removed from the flask via trypsin and placed in a conical on ice. 250,000 cells suspended in 0.5 mL media was slowly added to the BioFlux apparatus where a single layer of HMEC endothelial was present. Using 1 dyne of shear stress, cells were pumped through the hollow tube containing endothelial cells. Flow rate was paused followed by a wash cycle to remove any undocked cells and fluorescent cell counts were performed. B. MDA-MB-231 luc-shNTC and shCD44 docked cells counted and statistically analyzed. (n=5, +/- SEM) ($p<0.005$) $p = 0.0026$ 56

Figure 3.2: Orthotopic injection of breast cancer cells with decreased CD44 expression leads to increased CTC numbers and decreased WBC maturation. A. Nude mice were injected with either MDA-MB-231 luc-

shCD44 or shNTC control cells, and tumor development was observed over a 12-week period. shCD44 and shNTC groups were split into two groups, and half received rhOSM (1 μg) peri-tumorally 3x/week. B. Following the termination of the experiment, blood was drawn for each animal for CTC analysis using qPCR for Alu amplification. C. White blood cell counts for each murine group at the termination of the experiment. A significant difference of p = <0.001 when comparing shNTC –rhOSM to shCD44 –rhOSM. (n=3, +/- SEM). 57

Figure 3.3: BCSCs promote tumor initiation at low numbers. A. BCSCs and parental MCF-7 luc cells were collected and orthotopically injected into mammary fat pads of nude mice to observe tumor initiation. MCF-7 luc cells were treated with OSM (25 ng/mL) for 72 hours followed by performing flow cytometry analysis B. OSM increased BCSC phenotype, which was subsequently used for the second animal experiment and data is shown in Table 1. (n = 3, +/- SEM)..... 58

Figure 3.4: Patients who express low levels of OSMR have a better survival rate if CD44 levels are high. An Oncomine™ microarray data set from invasive ductal carcinoma patients (n = 172) was sorted to analyze only those who had low levels of OSMR (n = 86). After isolating OSMR low data, it was then broken into categories of CD44 low and CD44 high. Statistical analysis between the two groups was performed by using a log rank test, p = 0.0046. 59

Figure 3.5: OSM and CD44 promote metastasis in different ways. OSM binds to its receptor, signaling downstream events such as EMT, migration, and detachment. OSM also activates the cleavage of CD44 through MMPs leading to increased migration and detachment (see Chapter 2). CD44 also leads to breast tumor cell docking and extravasation. 60

LIST OF ABBREVIATIONS

BCSC	Breast Cancer Stem Cell
BLI	Bioluminescence
CSC	Cancer stem cell
CD44	Cluster of Differentiation 44
CTC	Circulating Tumor Cell
DCIS	Ductal Carcinoma In Situ
DFS	Disease Free Survival
ECD	Extracellular domain
ECM	Extracellular matrix
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor-alpha
ERK	Extracellular Regulated Kinase
Estrogen/E2	17-Beta-Estradiol
HA	Hyaluronic acid
Her2	Human Epidermal Growth Factor Receptor-2
ICD	Intracellular domain
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
IL-6	Interleukin-6
IL6R	Interleukin-6 Receptor

JAK	Janus Kinase
LIF	Leukemia Inhibitory Factor
LIFRβ	Leukemia Inhibitory Factor Receptor
MAPK	Mitogen Activated Protein Kinase
MET	Mesenchymal-epithelial transition
MFI	Mean Fluorescence Intensity
MMP	Matrix metalloproteinases
NF-κB	Nuclear Factor Kappa-B
OSM	Oncostatin M
OSMRβ	Oncostatin M Receptor Beta
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol-3-Kinase
PR	Progesterone Receptor
Q-PCR	Quantitative Polymerase Chain Reaction
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
SD	Standard Deviation
SEM	Standard Error of the Mean
shCD44	Short hairpin RNA against CD44
shNTC	Short hairpin RNA non- targeting control
shRNA	Short hairpin RNA
STAT3	Signal Transducer and Activator of Transcription
TAM	Tumor-Associated Macrophage
TGFβ	Transforming Growth Factor-Beta

TME	Tumor microenvironment
TNBC	Triple Negative Breast Cancer
TNFα	Tumor Necrosis Factor

CHAPTER ONE: INTRODUCTION

Breast Cancer

According to the American Cancer Society, roughly 252,710 new cases of invasive breast cancer will be diagnosed in women in the United States in 2017 (1). The American Cancer Society projects mortality from the disease will reach roughly 40,610 women during this year alone (1), and the risk of a woman developing breast cancer at some point in her life is currently 1 in 8 according to breastcancer.org. The epithelial cells lining lactiferous ducts are the most common location for the formation of breast cancer and lead to invasive ductal carcinoma (IDC). IDC occurs in ~80% of breast cancer cases (2), as compared to invasive lobular carcinoma that makes up roughly 10% of breast cancer cases and originates from glandular tissue (3). Inflammatory breast cancer and a few other rare types make up the remaining 10%. IDC develops through a series of steps. Normal breast epithelium progresses to atypical breast hyperplasia that changes to ductal carcinoma *in situ*, which may be followed by malignant IDC. In some cases , metastatic disease will develop (4).

Breast Cancer Subtypes

Breast cancer cannot be treated as a single, straightforward disease; instead each individual diagnosis is categorized into a subtype based on the origination of the tumor (ductal versus lobular) and the hormone and growth factor receptors present on the tumor cells. It is the specific subtype of breast cancer that dictates the use of certain therapeutic

interventions as well as the prognosis for the patient. Patients with luminal A, luminal B, human epidermal growth factor receptor 2 (HER2), and triple negative breast cancer (TNBC) are four of the most common subtypes of breast tumors (5). Luminal A breast cancer presents with low Ki-67 expression and is estrogen receptor-positive (ER+), progesterone receptor-positive (PR+) or negative (PR-), and HER2 negative. Luminal A is considered a highly treatable subtype (6). It also is the most common subtype, resulting in 150,000 cases each year (7). Luminal B expresses lower levels of hormone receptors and higher levels of proliferation markers compared to luminal A (8). The subtype HER2 is HER2+ and is found in roughly 20% of breast cancers (9). The final subtype is TNBC (ER- PR- HER2-), which does not possess any of the above mentioned receptors and is considered one of the most difficult subtypes to treat based on the limited amount of therapeutic intervention possible due to the absence of all three receptors (10).

Metastatic Breast Cancer

It is widely known that breast cancer is easily treatable if caught in early stages and if the tumor cells are ER+ (11). Most breast cancer is lethal as a result of local invasion and metastasis of tumor cells from the primary tumor to other tissues such as the lung, bone, brain and liver (12, 13). Surgery and other treatments can be used to remove or inhibit the primary breast tumor. But there is a risk of not eliminating or destroying every cancer cell within the tumor, which can lead to recurrence and metastasis (14–16). The stepwise events involved in metastasis are commonly referred to as the metastatic cascade (Fig. 1). These events happen in sequential order and begin as soon as the primary tumor cells undergo changes that allow for a more aggressive nature. Primary tumor cells lose cell-cell adhesion proteins allowing for individual cellular migration and

invasion towards the vasculature. This is followed by intravasation of the tumor cell into the fluid network of the bloodstream (17). Once in the bloodstream, tumor cells circulate, dock, and undergo extravasation. Finally, the cells colonize a secondary site, where they establish a metastatic tumor at a distal location.

A crucial step in the metastatic cascade is the epithelial-mesenchymal transition (EMT), a key event where a cell undergoes a phenotypic change from an epithelial to a more aggressive mesenchymal phenotype. This process is absolutely necessary during development, wound healing, and also tumor progression. Epithelial markers and mesenchymal markers fluctuate during the transition. Two important epithelial markers are the proteins E-cadherin and α-catenin, which participate in cell-cell adhesion (18, 19). Without EMT, cuboidal epithelial cells would not be able to become mobile, which is necessary for tumor cells to invade and metastasize. Cytokine signaling influences EMT through the activation of transcription factors that repress epithelial markers.

Oncostatin M (OSM)

Oncostatin M (OSM) is a pleiotropic, interleukin-6 (IL-6) family cytokine, which is important in inflammation and necessary for immune function. As an inflammatory cytokine, OSM can accumulate in the tumor microenvironment (TME) (20). Tumor cells, as well as monocytes, macrophages, and neutrophils, secrete OSM (21). OSM activates the JAK/STAT, MAPK, and PI3K/AKT pathways (22, 23) via binding its receptors, OSM receptor (OSMR) or leukemia inhibitory factor receptor (LIFR). OSMR and LIFR are heterodimers that each consist of a common gp130 subunit dimerized with either OSMRβ or LIFRβ (24). Our studies, as well as other published data, have shown that OSM inhibits proliferation of breast cancer cells while increasing metastatic capacity

Patient biopsy studies have demonstrated that increased OSMRβ expression on breast tumor cells is also associated with tumor progression (25), suggesting tumor cells have increased signaling from the OSM accumulated in the TME. As an inflammatory cytokine, OSM plays a role in other cancers besides breast cancer, such as prostate, cervical, and ovarian carcinoma, where OSM promotes tumor proliferation (26–29). OSM also plays a role in hepatic and endometrial carcinoma, as an inducer of a more aggressive metastatic phenotype while decreasing proliferation of tumor cells (30, 31).

Recent work has shown that OSM is directly capable of suppressing estrogen receptor (ER) expression, which decreases the chance that hormone-blocking therapeutics will be able to delay tumor progression (32). Cancer-associated adipose tissue is also capable of increasing breast cancer progression through OSM signaling and the JAK/STAT3 pathway (33). OSM is not just a molecule involved in cancer progression, as it can be found to have positive functions in addition to those associated with inflammation. For example, OSM is also involved in the central nervous system (CNS) acting as a neuroprotective cytokine during excitotoxic injury *in vitro* (34). Overall, OSM signaling events have varying outcomes during inflammation, as well as across different cancers.

After its initial discovery, OSM was thought to be a potential therapeutic for the treatment of breast cancer patients because of the inhibitory effects on proliferating breast tumor cells. As more studies were published, it was discovered that OSM was not only capable of inhibiting proliferation but was a promoter of metastasis as well (35). Now breast tumor therapeutics are being developed to target OSM, its receptor, and its downstream signaling.

OSM signaling upregulates proteins involved in metastasis. OSM has been shown to play a role in a number of events in the metastatic cascade such as EMT, cellular detachment, migration, invasion. OSM induces a metastatic phenotype by encouraging primary tumor cells to undergo EMT through STAT3 signaling (36). Cellular detachment is necessary for breast tumor cells to metastasize, and OSM has been shown to be a strong inducer of this process (37). Breast tumor cells freed from their primary tumor mass must adopt an invasive characteristic in order to become mobile and enter into the blood stream (38). Our work suggests that OSM increases the number of circulating tumor cells and metastasis to lung (manuscript in preparation), and our published work demonstrates that OSM is needed for osteolytic breast cancer metastasis to bone (35). In summary, OSM is an important inflammatory cytokine for promoting tumor metastasis.

Cluster of Differentiation 44 (Cd44)

Cluster of differentiation 44 (CD44) is a transmembrane glycoprotein involved in a variety of cellular processes such as survival and growth, migration and invasion, lymphocyte activation, and cell adhesion (39, 40). The canonical binding of CD44 to hyaluronic acid (HA) in the extracellular matrix (ECM) is responsible for CD44's downstream effects during tumor progression among many other cellular functions (41). HA exists in the ECM as a high molecular weight glycosaminoglycan (42). During tumor progression, high molecular weight HA is cleaved to low molecular weight hyaluronic acid by hyaluronidase and is capable of binding CD44 and inducing cleavage of the protein, which in turn promotes cellular migration and reduces the stationary nature of a cell (43).

Human CD44 protein contains 19 exons, 9 of which are found in the portion just outside the cell membrane (Fig. 2) (44). It is within the variable region where CD44 gets its complexity and specificity for certain types of cancer. The standard form of the protein (CD44s) contains exons 1-5 and 16-20 with the variable region completely absent. CD44s alone poses minimal threat in tumor progression relevance and is therefore not a highly reliable biomarker itself. Identification of which specific CD44 variants are present is a TME is one way to gain a better understanding of metastatic potential and general aggressiveness of a primary tumor.

When CD44 alternative splicing occurs, it produces a variety of CD44 variant proteins (CD44v) (Fig. 3) (45), and it is exons 6-15 that are capable of undergoing alternative splicing (46–48). Variant exons 6-15 are termed v1, v2, v3, etc. through v10 (49), and a key feature of the exon rearrangements is that there are extensive combinations. For example, CD44v6 contains only variant exon 6 plus the CD44 standard and is associated with highly aggressive tumors (50). CD44v3-10 contains the standard exons plus variant exons v3-v10 and is less common than CD44v6 (51). ER+ cell lines possess an epithelial phenotype and contain CD44v8-10, known as CD44E (CD44 epithelial) (52). Regardless of the combination of exons comprising CD44v, exons from the CD44s remain present despite alternative splicing.

Whether CD44s or a CD44v protein, the transmembrane CD44 is susceptible to cleavage by MMPs and γ-secretase at different locations near the cellular membrane (Fig. 4). During post-MMP cleavage, the extracellular domain (ECD) of CD44 is removed from the membrane leaving a bound remaining portion of CD44 (53). Subsequent cleavage by γ-secretase occurs to this membrane bound portion and releases the

intracellular domain (ICD), which can then translocate to the nucleus where it participates in transcriptional activation (54). The processing of CD44 can lead to changes in outcomes involved in cellular adhesion and migration and ultimately to tumor invasion and metastasis. (55, 56).

Cancer Stem Cells (CSCs) and Breast Cancer Stem Cells (BCSCs)

The stemness of a tumor cell allows for the loss of regulated cell division when necessary and the expression of self-renewal genes (57, 58). Cancer stem cells (CSCs), rapidly proliferating tumor cells, and a host of non-tumor cells make up the heterogeneous population of a primary tumor (59). Current therapies target rapidly dividing cells within and around a primary tumor. CSCs are therefore not targeted because of their non-rapidly proliferating characteristic while in a heterogeneous tumor population (60).

Cell surface markers used to specifically identify breast cancer stem cells (BCSCs) are the presence of CD44 (cluster of differentiation 44) and the lack of CD24 (cluster of differentiation 24). This population of BCSCs is known as $CD44^{+}/CD24^{-/low}$ (61). While little is known regarding BCSCs and metastasis, it is known that inflammatory cytokines increase the BCSC population from human breast cancer cells (62). The BCSC population is minor compared to the total tumor cell population, but BCSCs are speculated to be one of the reasons that recurrence occurs following treatment.

BCSCs are capable of intravasating into a lymphatic or blood vessel where it becomes possible for metastasis to take place (63). Following extravasation, it is speculated that BCSCs are capable of secondary tumor initiation by activating genes

responsible for proliferation (64). Along with BCSCs, primary tumor cells possess the ability to survive within the circulation where they are referred to as circulating tumor cells.

Circulating Tumor Cells (CTCs)

The bloodstream provides a method of transportation for metastatic cells during breast tumor metastasis. Circulating tumor cells (CTCs) in the blood stream are exposed to an environment different than that of the primary tumor location. Although the cells are neither proliferating nor highly active in cell-cell communication, they are able to find a very specific docking location where extravasation then occurs. A variety of detection methods for detecting CTCs are available, but therapeutic elimination of CTCs has remained elusive. However, CTCs can be studied by mimicking *in vivo* conditions of live cells in the bloodstream using a BioFlux machine (Fluxion Biosciences, San Francisco, CA). Flow rates can be controlled to replicate similar shear stress forces that CTCs would be exposed to in the bloodstream while in transit. Real time video may be recorded to provide observations of CTC behavior, such as docking and rolling in a fluidic environment provided insight into the process preceding extravasation.

In Vivo Models of Human Breast Cancer

Murine models are widely used in cancer research because they closely replicate human cancer characteristics. The athymic nude mouse is commonly used for tumor studies because it is unable to reject the implantation of human cancer cells (65). Orthotopic mouse models of human breast cancer involve the injection of tumor into the mammary fat pad to simulate human breast tumor growth in its natural environment and subsequent metastasis.

Patient survival

Although murine models are necessary for gaining insights that lead to novel therapeutics for the treatment of breast cancer, human data must also be considered. Cancer transcriptome profiles, available from Oncomine™, may be used to gather data from patients based on the mRNA expression levels of genes such as OSM, OSMRβ, and CD44 expression levels. Survival analyses may be performed to determine the best and worst potential outcomes based on whether a patient expresses high or low levels of each gene.

Summary

In summary, the metastatic cascade involves a number of ordered events leading to secondary tumor formation. OSM signaling contributes to the process by promoting an EMT in ER+ primary breast tumor cells, which turns a less aggressive cell into a more aggressive tumor cell. Following EMT, cells become migratory and detach from their original location in order to intravasate into the blood stream. CTCs and BCSCs travel to their secondary location where they dock and extravasate before establishing a metastatic tumor. CD44 contributes to the docking process as the CTCs and BCSCs home to their secondary location, such as bone, liver, lung and the brain.

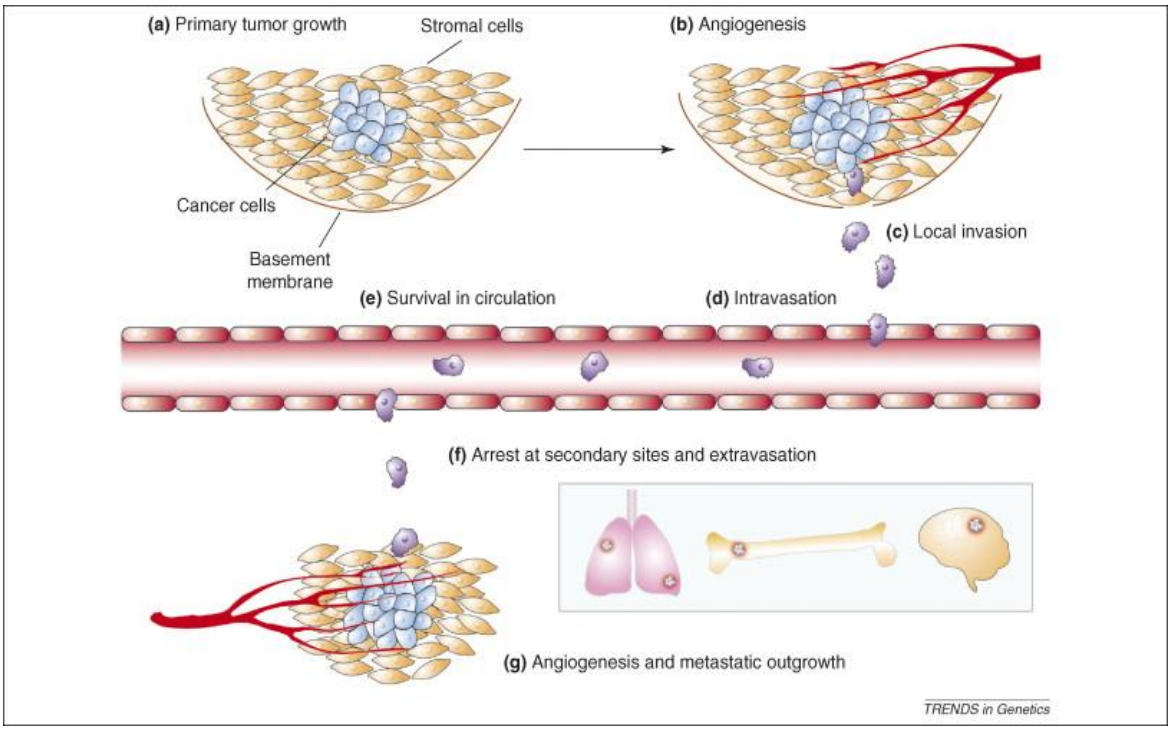

Figure 1.1: Breast tumor cells metastasize through a series of events. Angiogenesis occurs, which allows for intravasation of invasive and migratory tumor cells into the vasculature. Cells in circulation eventually create a secondary tumor at a distal location. (Ma and Weinberg, 2008) (66)

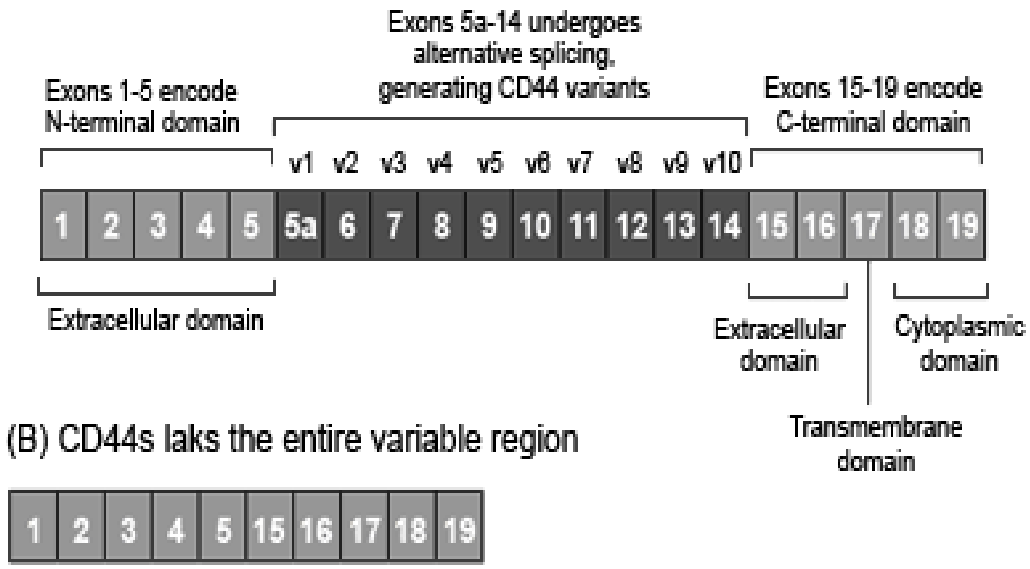

Figure 1.2: CD44 is composed of exons capable of alternative splicing. Rearrangement of CD44 exons creates CD44v isoforms that pose larger molecular weights compared to the standard CD44 (CD44s). (Sen and Yip, 2009) (67)

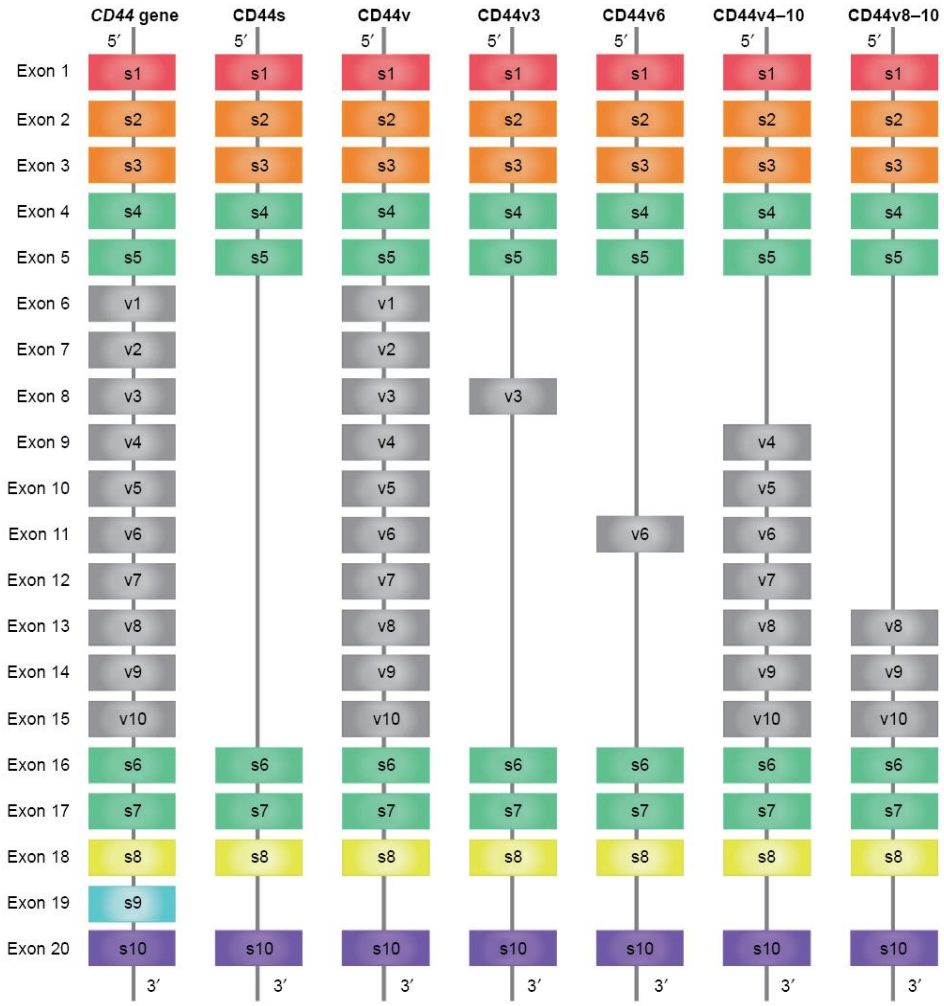

Figure 1.3: CD44 is alternatively spliced while retaining CD44s exons. CD44v6 and CD44v8-10 are commonly present on breast tumor cells. (Xu H. et. al, 2015) (45)

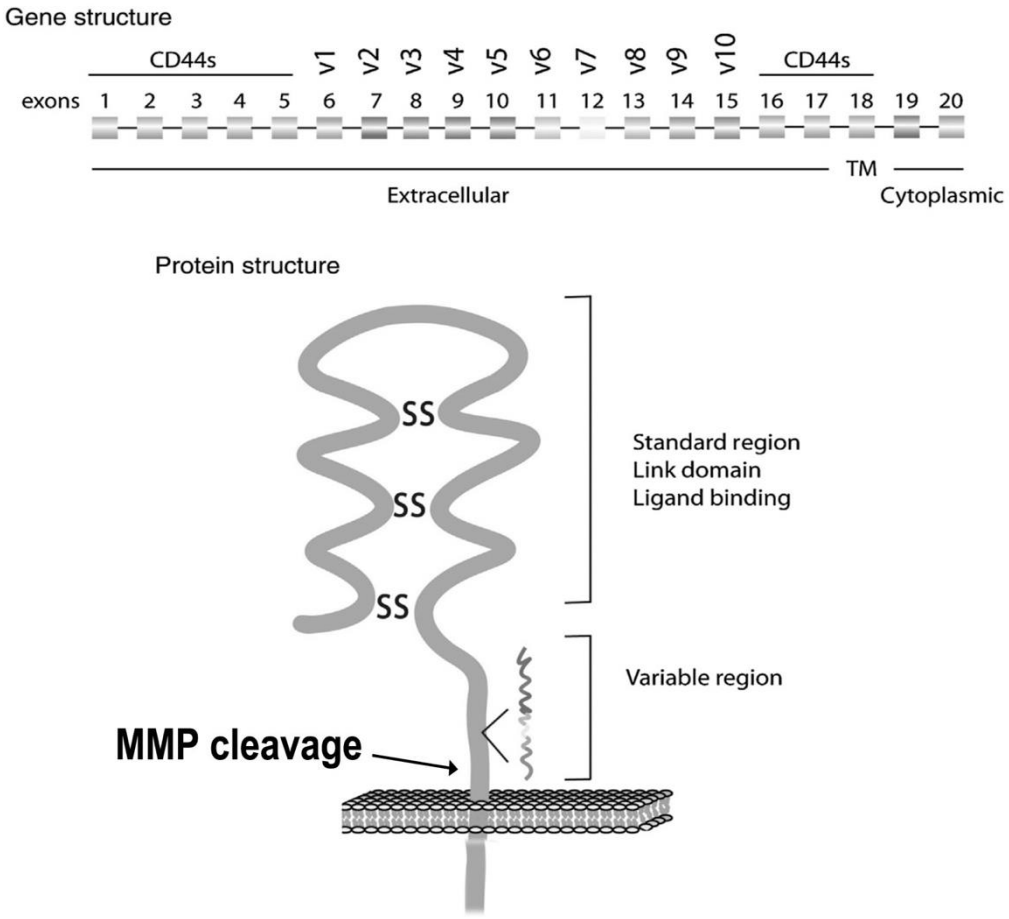

Figure 1.4. CD44 is post-translationally cleaved by MMPs. CD44 is cleaved by MMPs directly adjacent to the cell membrane below the variable region of exons. The cleavage relieves the extracellular domain (ECD) of being bound to the cellular membrane. As a second event γ-secretase cleavage occurs within the membrane producing an intracellular domain (ICD). (Adapted from Louderbough and Schroeder, 2011) (68)

CHAPTER TWO: OSM CONTRIBUTES TO BREAST CANCER METASTATIC POTENTIAL THROUGH CD44-INDUCED CELL MIGRATION AND DETACHMENT

(OSM-induced CD44 in metastatic potential)

Authors

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Abstract

Metastasis is the primary cause of mortality in breast cancer patients. Triple negative breast cancers (TNBC; estrogen receptor-negative, progesterone receptor-negative, and HER2-negative) are more aggressive and contribute to higher incidences of metastasis by possessing characteristics such as increased tumor cell migration and a high level of the transmembrane protein, cluster of differentiation 44 (CD44). Estrogen receptor-positive (ER+) breast cancer cells are less aggressive and do not migrate until undergoing an epithelial-mesenchymal transition (EMT) induced by growth factors or cytokines. These studies examine the effect of the interleukin-6 (IL-6) cytokine family member oncostatin M (OSM) on CD44 expression and activity in the metastatic potential of TNBC and ER+ breast cancer. ER+ T47D and MCF-7 human breast cancer cells treated with OSM demonstrate increased CD44 expression as well as CD44 cleavage. Conversely, ER- MDA-MB-231 human breast cancer cells do not show a change in CD44 expression in the presence of OSM. Knockdown expression of CD44 by shRNA did not prevent EMT in ER+ cells, but did prevent OSM-promoted metastatic processes such as cellular detachment and migration. In TNBC cells, CD44 knockdown expression only reduced tumor cell migration promoted by OSM. In summary, CD44 contributes to OSM-induced migration and detachment independently of OSM-induced EMT. These results suggest that regulation of CD44 by OSM contributes to the breast cancer metastatic cascade.

Introduction

Metastasis is the primary cause of mortality in cancer patients, leading to over 90% of cancer-related deaths (69). In women with metastatic breast cancer, survival is poor with a 5-year survival rate of only 26% (1). Continued studies addressing breast cancer metastasis are necessary if research is to contribute to improved patient outcome.

In order for primary tumor cells to metastasize they must undergo a series of stepwise events commonly referred to as the metastatic cascade. Tumor cells undergo a phenotypic change known as an epithelial-mesenchymal transition (EMT) (70–72) and lose cell-cell adhesion allowing for individual cellular migration and invasion towards the vasculature. This is followed by intravasation of the tumor cells into the fluid network of the bloodstream (17). Once the primary breast tumor cells are within the network of vessels they undergo a homing process to specific organs within the body (73, 74), such as the bone, lung, liver, and brain before extravasating and colonizing a secondary site (75–83). The tumor microenvironment (TME) plays a major role in the metastatic cascade (84), and signaling from cytokines present in the TME lead to tumor cell phenotypic changes necessary for metastasis to occur (85). Inflammatory cytokines of the interleukin-6 (IL-6) family such as oncostatin M (OSM) have been shown to be important in driving tumor invasion and metastasis (37).

OSM is a pleiotropic IL-6-family cytokine, which is important in inflammation exhibited during breast tumor development (20, 86, 87). Tumor cells including breast cancer cells, as well as monocytes, macrophages, and neutrophils, secrete OSM (88). OSM activates the JAK/STAT, MAPK, and PI3K/AKT pathways via binding its receptors, OSM receptor (OSMR) and leukemia inhibitory factor receptor (LIFR). OSMR

and LIFR are heterodimers consisting of either OSMRβ or LIFRβ dimerized with a common gp130 subunit (37, 89, 90). Our previous work and data from the literature have shown that OSM inhibits proliferation of breast cancer cells, while increasing metastatic potential (91, 92). OSM has been shown to cause EMT, cell detachment, migration, and an overall increase in tumor cell aggressiveness. Furthermore, our lab has demonstrated a direct for OSM in breast cancer metastasis to bone (93).Further investigations are needed to fully understand the mechanisms by which OSM induces metastatic potential.

Cluster of differentiation 44 (CD44) is a transmembrane glycoprotein involved in a variety of metastatic events including migration, cell adhesion, and invasion (39, 40, 94). CD44 contains a carboxy-terminal cytoplasmic tail and an amino-terminal region, which is outside the cell membrane and is folded together by post-translational glycosylation (95, 96).CD44 is a major receptor for binding hyaluronic acid (HA) in the extracellular matrix (ECM) (97), and it binds HA by its stem region that is directly above the surface of the membrane. CD44 can exist as multiple isoforms, which result from alternative splicing of variant exons (98).

The human CD44 gene contains 19 exons (44), and the standard form of the protein (CD44s) consists of exons 1-5 and 16-20 with variable region exons (exons 7-15) completely absent (46–48). CD44s alone poses minimal threat to tumor progression and is, therefore, not a reliable biomarker. When CD44 alternative splicing occurs, it produces a variety of CD44 variant proteins (45). Human variant exons 7-15 are termed v2, v3, v4 through v10 (49), and many CD44 variant (CD44v) isoforms exist (49). It is within the variable region where CD44 gets it complexity and specificity for certain types of cancer. For example, CD44v6 contains only variant exon 6 plus the CD44 standard

and is associated with highly aggressive tumors including breast (50). CD44v3-10 contains the standard exons plus variant exons v3-v10 and is less common than CD44v6 in breast cancer (51). Estrogen receptor-positive (ER+) breast cancer cell lines, which possess an epithelial phenotype, display CD44v8-10 known as CD44E (52). Regardless of the combination of exons comprising CD44v, exons from the CD44s remain present despite alternative splicing.

Whether CD44s or a CD44v protein, the transmembrane CD44 is susceptible to sequential cleavage by matrix metalloproteinases (MMPs) and γ-secretase at different locations near the cellular membrane (53, 99). First, MMPs such as MMP-2 and MMP–9 cleave CD44 just outside the membrane (100).During post-MMP cleavage, the extracellular domain (ECD) of CD44 is removed from the membrane leaving a bound remaining portion of CD44 (53). Subsequent cleavage by γ-secretase occurs to this membrane bound portion and releases the intracellular domain (ICD), which can then translocate to the nucleus where it participates in transcriptional activation (54). The processing of CD44 can lead to changes in outcomes involved in cellular adhesion and migration and ultimately to tumor invasion and metastasis. (55, 56).

Hyaluronic acid exists in the ECM as a relatively non-active high molecular weight glycosaminoglycan (42). During tumor progression, high molecular weight HA is cleaved to low molecular weight hyaluronic acid by hyaluronidase and is capable of binding CD44 and inducing cleavage of the protein, which in turn promotes cellular migration and reduces the stationary nature of a cell (43). CD44 has been shown to interact with ligands other than HA, such as collagens and osteopontin (OPN), and

depending on which protein CD44 interacts with contributes to differential regulation of cellular growth and migration (101).

In this study, we demonstrated a connection between OSM-induced breast cancer metastatic potential and CD44-induced cell migration and detachment. Here, we provided data showing that OSM both induced the expression of CD44s and CD44 variant proteins as well as promoted CD44 cleavage. Furthermore, our studies suggested that CD44 may be necessary for OSM-induced breast tumor cell detachment and migration, both important events in the metastatic cascade. These findings delineate an important mechanism by which OSM induces breast cancer metastatic potential.

Materials and Methods

Cell Culture and Cytokine Stimulation

MCF-7 and T47D ER+ luminal A human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD), maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and 100 U/mL each of penicillin and streptomycin. Cells were maintained at 37°C, 5% carbon dioxide, and 95% humidity. All media and supplements were obtained from Hyclone (Logan, UT). Human oncostatin M (OSM) (Peprotech, Rocky Hill, NJ), was added at 25 ng/mL.

Immunofluorescence Staining and Microscopy

2.5×10^5 cells were plated onto glass coverslips (Thermo Fisher Scientific). After 24 hours, cells were treated with or without OSM for 5 days. After experimental treatment, cells were rinsed once with PBS and then fixed in ice-cold methanol for 20 minutes. Cells were rinsed 3 times with PBS for 10 minutes. Cells were blocked in 1X PBS/1% BSA/0.5%Triton X-100 (PBSAT) for 30 minutes and then incubated in PBSAT

overnight containing the primary antibodies for human E-cadherin (1:200, Abcam, Cambridge, UK), Alpha catenin (1:200; Abcam, Cambridge, UK), or Phalloidin 568 nm for actin (1:200, Thermo Fisher Scientific, Waltham, MA). Cells were washed 3 times in PBS and incubated with PBSAT containing goat anti-mouse Alexa Flour 488 (1:1000) and donkey anti-rabbit Alexa Flour 546 (1:500; Molecular Probes, Invitrogen, Carlsbad, CA) secondary antibodies for 2 hours. Cells were washed 3 times in PBS and mounted using Prolong Gold DAPI (Molecular Probes). Stains were visualized and confocal microscopy images were collected (Biomolecular Research Center, Boise, ID) using a Zeiss LSM 510 Meta system combined with the Zeiss Axiovert Observer Z1 inverted microscope and ZEN 2009 imaging software (Carl Zeiss, Inc., Thornwood, NY).

qRT-PCR

Total RNA was collected using RNA STAT-60^{TM} and isolated per the manufacture's protocol (Tel-Test; Friendswood, TX). cDNA was synthesized according to the manufacture's protocol (High Capacity cDNA Reverse Transcription Kit, Applied Biostems, In/Life Technologies). A LightCycler 96 (Roche Diagnostic Corporation, Indianapolis, IN) with settings of preincubation 1 cycle, 2 step amplification 45 cycles, and high resolution melting 1 cycle; all steps were set at 95° C for 120 seconds.

Western Blot Analysis

For whole cell lysates, 5 x 10^4 cells were seeded into 24-well plates (Thermo Fischer Scientific, Rockford, IL), unless otherwise stated. At the end of the experiment, plates were rinsed with ice-cold PBS, then placed on ice with 125 μL of RIPA buffer (Thermo Fischer Scientific) supplemented with 1:100 dilution of Sigma Protease Inhibitor Cocktail and 1:1000 phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis,

MO) for 15 minutes, mixing every 5 minutes. Plates were scraped and the contents transferred to an Eppendorf tube. Tubes were then centrifuged at 14,000 rpm for 20 minutes.

Lysates were electrophorated on a 7.5-10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed using primary antibodies for human E-cadherin (1:2000), β-actin (1:5000), α-catenin (1:2500) (Cell Signaling Secondary, Danvers, MA. CD44 (1:5000) (kindly provided by Dr. Knudson). Antibodies were HRP-conjugated donkey anti-rabbit (1:5000).

Flow Cytometry

A FACSCaliburTM flow cytometer (BD Biosciences, San Jose, CA) was used to observe populations of BCSCs present within a heterogeneous population of tumor cells. Breast tumor cells were treated with OSM (25 ng/mL) for 72 hours before performing flow cytometry with antibodies (BD Biosciences, San Jose, CA) to CD44 (1:5 FACS buffer), CD24 (1:5 FACS buffer), FITC (1:50 FACS buffer), and PE (1:50 FACS buffer) after blocking cells for 10 minutes with 1% BSA. Antibodies bound to their appropriate proteins for 30 minutes on ice in the dark. Following incubation, cells were washed 3x with FACS buffer (10% FBS, 0.1% sodium azide in PBS). Software analysis was performed using BD FACSCalibur software (CellQuest Pro, version 6.0).

shRNA and Chemical Inhibitors

Gene knockdown of CD44 and a non-targeting control sequence were created using shERWOOD shRNA lentiviral constructs (Transomic Technologies, Huntsville, AL). Viral transduction of MDA-MB-231 luc and MCF-7 luc cells was performed according to manufacturer's protocol. Puromycin was used for initial selection followed

by fluorescence microscopy to isolate transduced cell population; CD44 expression was confirmed by western blot analysis.

A MMP inhibitor (GM6001; Santa Cruz Biotechnology), was added to the cell growth media (10 nM) 2 days before cytokine treatment in serum-free media; followed by the addition of complete media and OSM for 2 more days.

Detachment Assay

A detachment assay was used to compare relative tumor cell detachment in the presence or absence of recombinant OSM (25 ng/mL). Cells were plated at 10,000 cells per well in a 24-well plate and allowed to attach overnight. Once plated, cells were placed in 1% FBS RPMI media for 4 hours without treatment. After a 4-hour incubation, cells were then treated with or without OSM in 1% media for the allotted time (1, 3, 5, or 7 days). To assess detached cells, media was collected and centrifuged. The pellets were resuspended in equal parts of RPMI media and Trypan Blue. Detached cells were then counted. Cells stained with Trypan blue were excluded from the counts. Proliferation was also measured, by trypsinizing adherent cells, pelleting cells, resuspending pellets in equal parts of RPMI media and Trypan blue, and counting cells using a hemocytometer. Detachment was measured by comparing the number of proliferating cells and detached cells in the presence or absence of OSM, and fold detachment was calculated.

Scratch Assay

A wound healing assay was used to measure mobility of breast cancer cell with or without OSM treatment. MCF-7 luc and MDA-MB-231 luc-shCD44 and shNTC control cells were plated at 200,000 cells per well in a 6-well plate with 10% FBS RPMI media. Once cells were 80-85% confluent, a scratch was made with a sterile p1000 micropipette

tip. Wells were washed with PBS followed by the addition of 10% FBS RPMI media each well, with or without OSM (25 ng/mL). Photomicrographs were taken at 72 hours. The area of each scratch was measured using ImageJ and the fold-migration was assessed by comparing day zero scratch area to day three scratch area.

Results

OSM induces EMT and a $CD44^{+}/CD24^{-/low}$ breast cancer stem cell-like phenotype

Estrogen receptor-positive (ER+) breast cancer cells, which are phenotypically epithelial in nature, undergo an EMT in response to metastatic inducers such as TGFβ and OSM (102, 103). Here, our studies showed that ER+ MCF-7 and MCF-7 luc (luciferase expressing) human breast cancer cells treated with OSM (25 ng/mL) underwent an EMT over a 3-day period, as displayed by a change in morphology where individual cells broke away from the initial colony and subsequently took on a more spindle-like mesenchymal appearance (Fig. 1A and Suppl. Fig. 1). Furthermore, immunofluorescence microscopy of MCF-7 luc cells demonstrated an OSM-induced decrease and redistribution of E-cadherin expression (Fig. 1A).

Previous studies have shown that developmental transcription factors such as Slug and Snail are responsible for repressing the expression of proteins associated with an epithelial phenotype such as E-cadherin (104). ER+ MCF-7 breast cancer cells treated with OSM (25 ng/mL) for one hour demonstrated an upregulation of both Slug ($p < 0.05$) and Snail by qRT-PCR (Fig. 1B). OSM treatment (25 ng/mL) of MCF-7 breast cancer cells also resulted in decreased expression of the cell-cell adhesion proteins E-cadherin and α-catenin over time, as measured by western blot analysis (Fig. 1C). These results demonstrated that OSM was able to initiate an EMT in ER+ breast cancer cells by

upregulating transcription factors associated with EMT, down regulating the expression of cell-cell adhesion proteins, and promoting a change in cell morphology from an epithelial to a mesenchymal phenotype.

Another common feature associated with tumor progression and overall metastatic burden is an increase in $CD44^{+}/CD24^{low/-}$ breast cancer stem cells (BCSCs) within a heterogeneous tumor cell population (105). $CD44^{+}/CD24^{low/-}$ cells have been shown to possess features associated with developmental stem cells as well as controlled cell division and a presence at the leading edge of a tumor (106). Treatment with OSM (25 ng/mL) for 72 hours increased the $CD44^{+}/CD24^{low/-}$ BCSC population in ER+ MCF-7 cells from less than 1% to over 1.5% (Fig. 1D) and in T47D cells from 1% to over 4% (Fig.1E). In untreated ER-negative (ER-) MDA-MB-231 luc cells, the $CD44^{+}/CD24^{low/-}$ BCSC cell population was so high (almost 90%) that OSM was unable to induce a BCSC phenotype (data not shown). Taken together, OSM induced both an EMT and a $CD44^{+}/CD24^{low/-}$ population of BCSCs in ER+ breast cancer cells, confirming previous results that OSM drives breast cancer metastatic potential.

OSM Induces Both CD44 Expression and Cleavage

Next, we decided to investigate whether OSM was able to induce CD44s and variant CD44 protein expression (Fig. 2). Our studies showed that treatment of ER+ MCF-7 cells with OSM (25ng/mL) for 24, 48, and 72 hours resulted in an increase in the expression of CD44s as well as high molecular weight (MW) CD44 isoforms, as measured by western blot analysis of whole cell lysates using an antibody specific to the cytoplasmic fragment of CD44 (Fig. 2A). Additionally, the expression of OSM-induced CD44s and high MW isoforms were most prevalent at 48 and 72 hours in ER+ T47D

cells (Fig. 2B). CD44s expression in the more aggressive ER- MDA-MB-231 cells showed a high basal level, which did not change in the presence of OSM during the same time period (Fig. 2C). Furthermore, OSM treatment did not alter high MW protein expression in MDA-MB-231 cells. To support our finding that OSM induced CD44s and variant CD44 protein expression in ER+ breast cancer cells, we investigated OSM-induced CD44 mRNA levels by qRT-PCR. While a non-significant increase in OSM-induced CD44s expression was observed at 8 hours in MCF-7 cells (Suppl. Fig. 2A), OSM did not significantly induce the mRNA levels of CD44 variants (v2 through v10) (Suppl. Fig. 2B). Together, these studies suggest that while OSM does not induce CD44 mRNA expression, it does result in increased levels of CD44s and variant proteins.

Proteolytic cleavage of CD44 by MMPs right outside the membrane and by γ-secretase within the cell membrane produce low molecular CD44 fragments (107, 108). Our studies demonstrated that OSM treatment of ER+ MCF-7 and T47D cells resulted in an increase in CD44 cleavage products (~35 kDa and below) (Fig. 2A and B), not seen in ER- MDA-MB-231 cells (Fig. 2C). Our lab has previously shown that OSM induces MMP activity in breast tumor cells, such as MMP-9 and MMP-2 (37). Inhibiting MMP activity using a global MMP inhibitor (GM6001) for 48 hours in ER+ T47D cells decreased the amount of OSM-induced CD44 cleavage (Suppl. Fig. 3A and B). To summarize, in ER+ breast cancer cells OSM promoted an increase in CD44s as well as variant protein levels and induced proteolytic cleavage of CD44, presumably through OSM-induced MMP activity.

OSM-Induced EMT is Independent of CD44

To explore the relationship between OSM-driven EMT and OSM-upregulation of CD44 expression, we established stable breast cancer cell lines with CD44 knockdown expression using shCD44 lentiviral transduction (Fig. 3A and B). ER+ MCF-7 luc-shCD44 cells showed a 80% reduction in CD44s expression by western blot analysis compared to control shNTC (non-targeting control) cells (Fig. 3A and B), while MDA-MB-231 luc-shCD44 cells showed an 85% reduction in CD44s (Fig. 3A and B). MCF-7 luc-shCD44 and control shNTC cells were treated with OSM (25 ng/mL) for 1 hour and qRT-PCR was performed for evaluate the EMT-driving transcription factors Slug and Snail. As expected, OSM treatment increased both Slug and Snail over 1.5-fold compared to untreated MCF-7 luc-shNTC (control) and MCF-7 luc-shCD44 (knockdown) cells (Fig. 3C, 3D). However, OSM treatment of knockdown MCF-7 luc-shCD44 cells resulted in no significant change in Slug expression compared to control shNTC cells, suggesting that CD44 is not involved in the upregulation of Slug by OSM (Fig. 3C). Interestingly, CD44 knockdown resulted in a significant increase in OSM-induced Snail expression compared to control shNTC cells, suggesting that the absence of CD44 increased OSM-induced Snail (Fig. 3D).

In the presence of OSM (25 ng/mL) for 72 hours, shNTC and shCD44 cells both showed a reduction in the cell-cell adhesion protein, E-cadherin by immunofluorescence (Fig. 3E). Interestingly, we did observe that both untreated and OSM-treated MCF-7 luc-shCD44 cells appeared to form much tighter colonies than control MCF-7 luc-NTC counterpart cells (Fig. 3E) Photomicrographs of MCF-7 luc-shCD44 and shNTC cells treated with OSM for 72 hours were also taken to observe the phenotypic EMT process

(Fig. 3F). A similar finding was seen, as MCF-7 luc-shCD44 cells appeared to form much tighter colonies than control MCF-7 luc-NTC cells. Taken together, these results suggested that OSM-induced EMT took place independently of CD44 in ER+ MCF-7 luc cells.

OSM-Induced Tumor Cell Detachment is Dependent on CD44

Our lab has previously shown that OSM induces tumor cell detachment, particularly in ER+ breast cancer cells (37). MCF-7 luc-shCD44 and MDA-MB-231 luc-shCD44 cells were treated with OSM (25 ng/mL), and fold-detachment was measured over a 5-day period (Fig. 4). OSM induced a 1.8- to 3-fold increase in cell detachment in ER+ MCF-7 luc-shNTC cells compared to untreated control cells (Fig. 4A; left). When MCF-7 luc-shCD44 cells were treated with OSM, no significant increase in tumor cell detachment was measured at days 1, 3, and 5 (Fig. 4A; right). Furthermore, a significant decrease in cell detachment was seen between OSM-treated control and MCF-7 luc-shCD44 cells at days 1 and 5 (Fig. 4), suggesting that knockdown of CD44 eliminated or delayed the ability of OSM to induce ER+ MCF-7 breast tumor cell detachment. The more aggressive ER- MDA-MB-231 luc-shNTC control cells did not detach in the presence or absence of OSM (Fig. 4B; left). Interestingly, MDA-MB-231 luc-shCD44 cells treated with OSM for 5 days showed a 6-fold increase in tumor cell detachment compared to control cells, similar to MCF-7 luc-shNTC cells (Fig. 4B). Therefore, it appears that MDA-MB-231 luc-shCD44 cells reverted from aggressive and basically OSM-unresponsive cells to less aggressive cells that responded to OSM when CD44 expression was reduced. Together, these results suggested that OSM-induced ER+ MCF-7 tumor cell detachment is dependent on CD44 expression.

OSM-Induced Tumor Cell Migration is Dependent on CD44

Tumor cells must become motile to undergo invasion and metastasis. Therefore, wound healing assays were performed using ER+ MCF-7 luc-shCD44 and MDA-MB-231 luc-shCD44 cells to quantitate tumor cell migration. MCF-7 luc-shNTC cells treated with OSM (25 ng/mL) for 3 days demonstrated a slight 1.5-fold increase in migration compared to untreated cells (Fig. 5A), Importantly, MCF-7 luc-shCD44 cells migrated to a significantly less extent when compared to shNTC control cells in the presence of OSM (Fig. 5A). When treated with OSM for 3 days, the ER- MDA-MB-231 luc-shCD44 cells showed a nearly 2.5-fold reduction in the number of migrating cells compared to MDA-MB-231 luc-shNTC cells (Fig. 5B). This striking difference demonstrates an importance for CD44 in OSM-induced ER- tumor cell migration. In conclusion, our findings suggest that OSM-induced CD44 appears to be important in tumor cell detachment and migration, both necessary for breast cancer cells undergoing metastasis.

Discussion

In this study, we show that OSM induces a breast cancer cell EMT that is not dependent on the presence of CD44. Rather, CD44 is necessary for tumor cell detachment and migration regulated by OSM. These results are significant if we are to understand the overall process of how OSM contributes to breast cancer metastatic potential.

Our findings here support previously published data demonstrating that OSM induces an EMT in breast cancer cells (109) and increases a BCSC-like phenotype (110). In this study, we show for the first time that OSM induces ER+ breast cancer cell CD44 protein expression, including CD44s and variant proteins. Other cytokines/growth factors

such as TGF-β have also been shown to be strong inducers of CD44 expression (100, 111). While it was our expectation that OSM treatment would lead to an induction in the expression of cancer-related variants such as CD44v6 (112), this was not the case. Our western blot data demonstrated that the addition of OSM resulted in an increase in the expression of variant proteins already present before treatment. In TNBC cells, such as MDA-MB-231 luc cells, OSM did not induce CD44 expression, as basal CD44s levels were already extremely high. Expression of CD44 variant proteins was not observed in MDA-MB-231 luc cells with or without OSM treatment.

CD44 protein is capable of undergoing two sequential cleavages by enzymes that include MMPs and γ-secretase (107, 113). Our lab and others have previously shown that OSM induces the expression of a number of MMPs including MMP-2 and MMP-9 (114, 115). Our studies using the non-specific MMP inhibitor GM6001 suggest that OSM-induced CD44 cleavage may be dependent on MMPs. OSM did not induce the expression of γ-secretase, nor did a γ-secretase inhibitor (DAPT) block OSM-induced CD44 cleavage in MCF-7 cells (data not shown). Together, this data suggests that OSM's ability to cleave CD44 is limited to MMP activity.

The connection between OSM-induced EMT and CD44 expression was examined using a MCF-7 luc cell line with knockdown expression of CD44. Reduced CD44 expression did not prevent an EMT in ER+ MCF-7 cells, though untreated MCF-7 luc-shCD44 cells grew in much tighter colonies than control cells. Furthermore, treatment of MCF-7 luc-shCD44 cells with OSM resulted in small clumps of rounded, epithelial-like cells instead of the classic EMT seen in OSM-treated control cells. These results suggest that with CD44 knockdown expression, MCF-7 cells display tighter cell-cell adhesion.

While our studies indicate that CD44 is not directly involved in breast cancer cell EMT, previous studies have reported a correlation between an increase in CD44 expression and EMT (45, 116).

Metastatic events such as cellular detachment and migration have often been thought to be early steps in the metastatic cascade. The connection between OSM-induced cell detachment and migration and CD44 expression was examined using MCF-7 luc and MDA-MB-231 luc cell lines with knockdown expression of CD44. OSM-induced migration was more prominent in TNBC MDA-MB-231 luc cells than in ER+ MCF-7 luc cells, though CD44 knockdown reduced OSM-induced migration in both cell types. OSM promoted ER+ MCF-7 luc cell detachment, similar to our previously published work on ER+ T47D cells (37). Our studies here show that CD44 expression is necessary for the increase in cell detachment promoted by OSM. While aggressive cells like MDA-MB-231 luc cells do not detach in the presence of OSM, knockdown of CD44 expression promoted OSM-induced detachment. This interesting finding suggests that loss of CD44 may make MDA-MB-231 cells more responsive to OSM. Taken together, our findings demonstrate that CD44 contributes to OSM-induced migration and detachment independently of OSM-induced EMT. These results suggest that regulation of CD44 by OSM contributes to the breast cancer metastatic cascade.

Conclusion

The studies presented here address a role for CD44 in OSM-induced breast cancer metastatic potential. The binding of OSM to its receptor leads to the promotion of EMT, tumor cell migration, and cell detachment (Fig. 6). OSM signaling also increases expression of the transmembrane protein CD44 and its cleavage, most likely through

MMP activity. CD44 contributes to OSM-induced migration and detachment, which is independent of OSM-induced EMT.

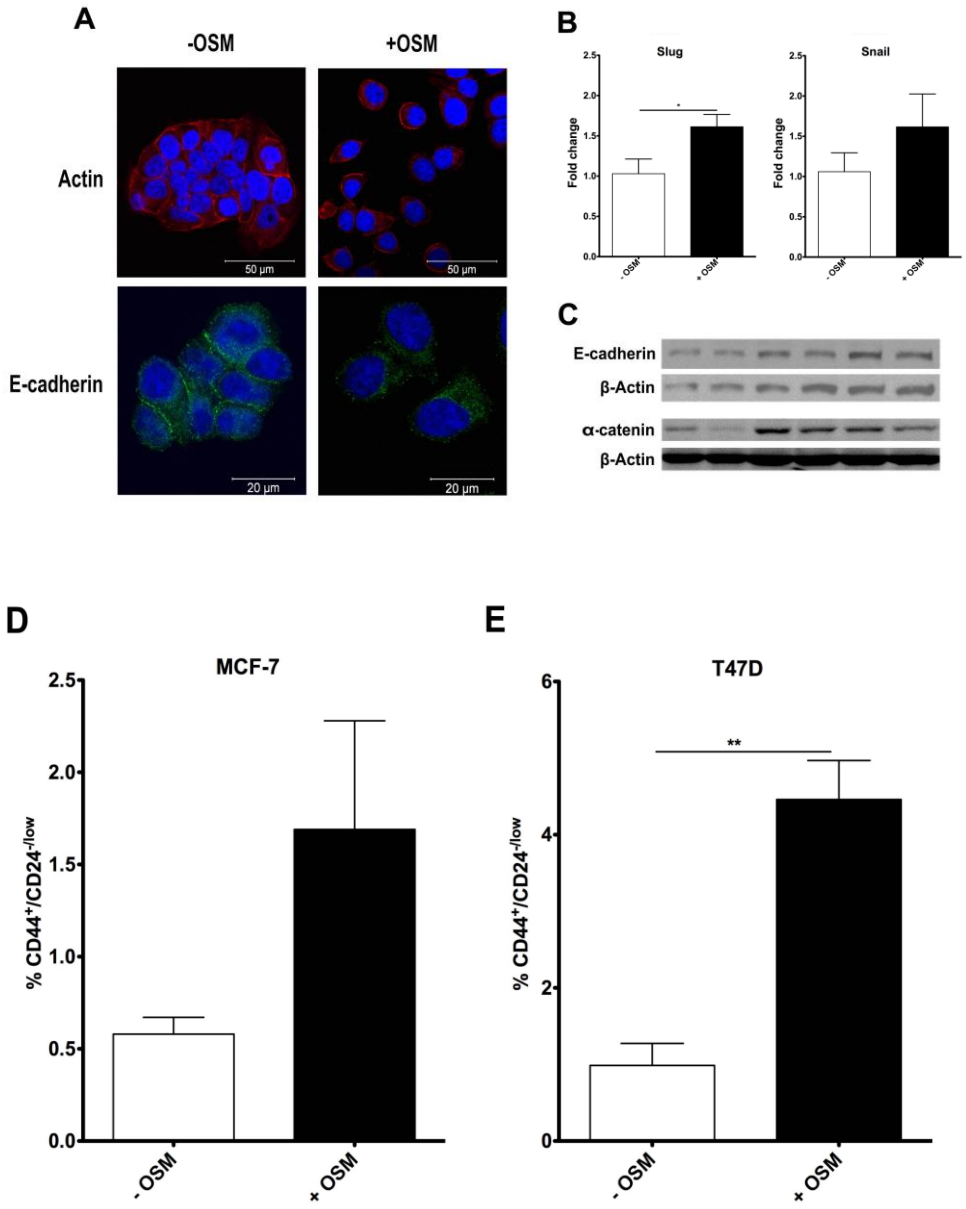


Figure 2.1. OSM induces EMT and a BCSC phenotype in human breast cancer cells. A. MCF-7 luc human breast cancer cells were treated with OSM (25 ng/mL) for 72 hours. OSM induces an increased mesenchymal phenotype and a slight decrease and redistribution in E-cadherin expression, as measured by immunofluorescence using antibodies for E-cadherin (green) and actin (red) (DAPI = blue; 63x magnification). B. MCF-7 cells were treated with OSM (25 ng/mL) for 1 hour, and qPCR was performed with primers for the EMT transcription factors Slug (n = 3, +/- SEM) and Snail (n = 3, +/- SEM) (Slug *p<.05, paired t test). C. MCF-7 cells were treated with OSM (25 ng/mL) for 24, 48, and 72 hours, and cell lysates were assessed by western blot analysis for E-cadherin and α-catenin. D and E. ER+ MCF-7 and T47D cells were treated with OSM (25 ng/mL) for 72 hours and flow cytometry was performed for $CD44^{+}/CD24^{-/low}$. While OSM induces a small non-significant increase

the $CD44^{+}/CD24^{-/low}$ BCSC population in MCF-7 cells, in T47D cells, OSM induces the BCSC population 3-fold (+/- SEM for both) (p<0.005, paired t-test).**

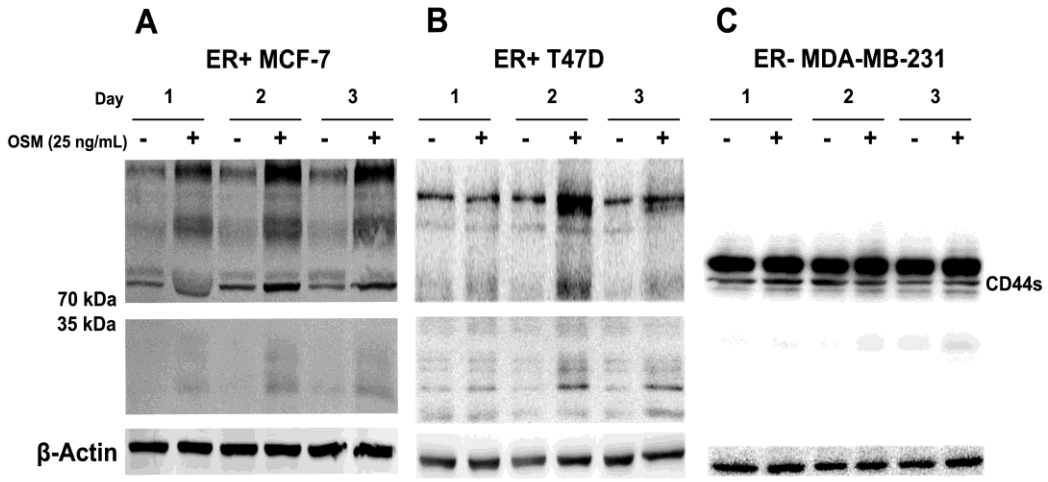


Figure 2.2 OSM induces CD44s (70 – 85 kDa), CD44 isoforms (above 85 kDa), and cleaved products (below 35 kDa) in ER+ cells but not ER- cells. Cells were treated with (25 ng/mL) OSM for 24, 48, and 72 hours, and whole cell lysates were collected for immunoblotting using an anti-CD44 antibody. A. MCF-7 (ER+). B. T47D (ER+). C. MDA-MB-231 (ER-).

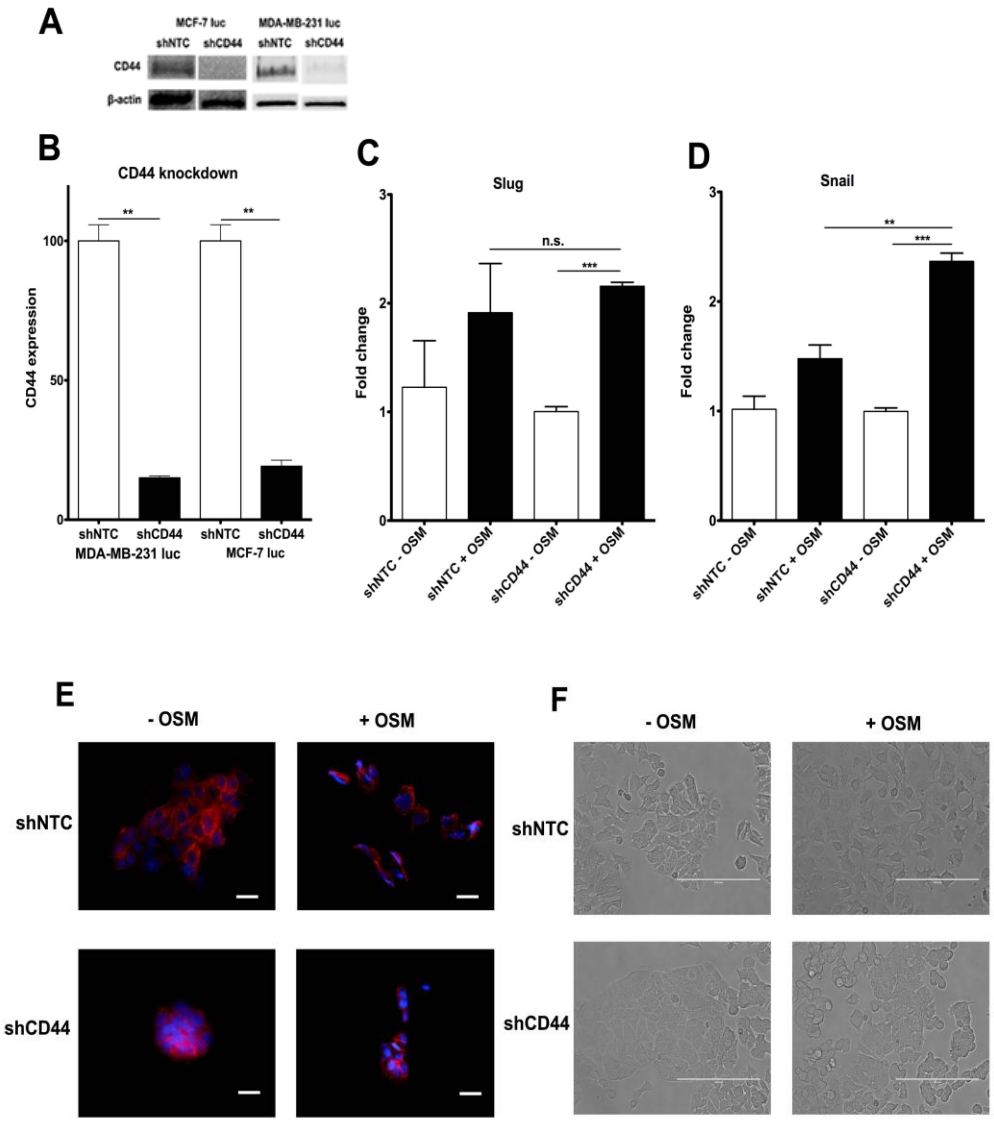


Figure 2.3. Reduced CD44 expression does not abrogate OSM-induced EMT in ER+ cells. A. Both MCF-7 luc-shCD44 and MDA-MB-231 luc-shCD44 cells show knockdown of CD44s protein expression compared to non-targeting control (shNTC) cell lines by western blot analysis. B. Quantification of CD44 knockdown (n = 3, +/- SEM, paired t test $p < 0.005$). C. MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for one hour, and qPCR analysis for Slug and Snail was performed. Knockdown of CD44 expression did not decrease OSM-induced Slug (C), but Snail decreased in shNTC compared to shCD44 cells (D) (n=3, +/- SEM, One Way Anova $p < 0.005$). E. MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for 72 hours, and EMT and E-cadherin was observed via immunofluorescence (20x magnification scale bar in = 30 nm). F. MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for 72 hours to observe EMT morphology by phase contrast images (20x magnification scale bar = 200 μm).

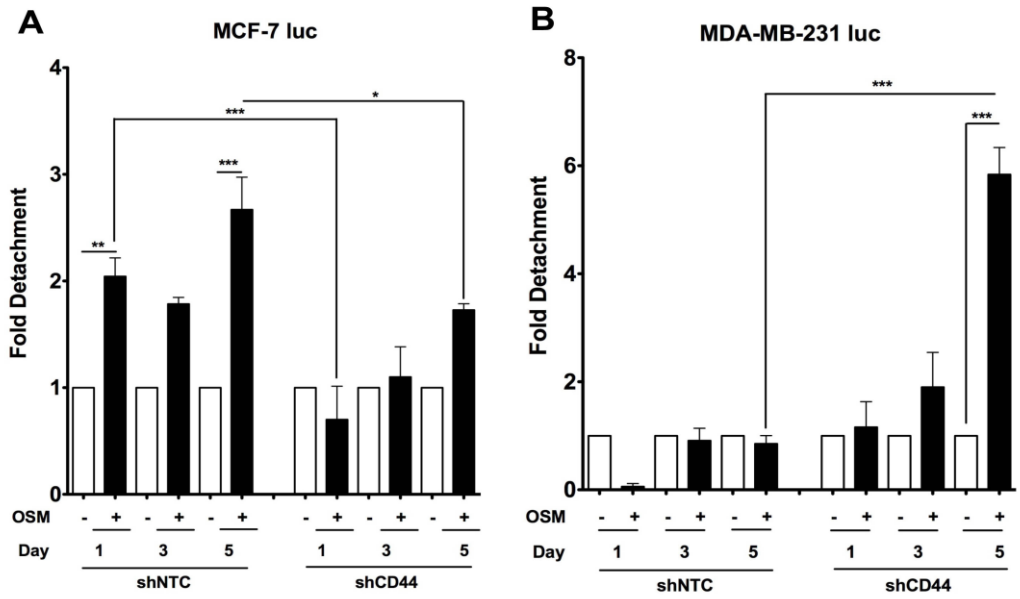


Figure 2.4. Reduced CD44 expression decreases OSM-induced tumor cell detachment in ER+ cells. A. ER+ MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) over a 5-day period, and detached cells were counted on days 1, 3, and 5. Data is represented as fold-change (*p<0.05, **p<0.005, ***p<0.001, one-way ANOVA) B. ER- MDA-MB-231 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) over a 5-day period, and detached cells were counted on days 1, 3, and 5. data is represented as fold change (*p<0.001, one-way ANOVA) (n=3, +/- SEM).**

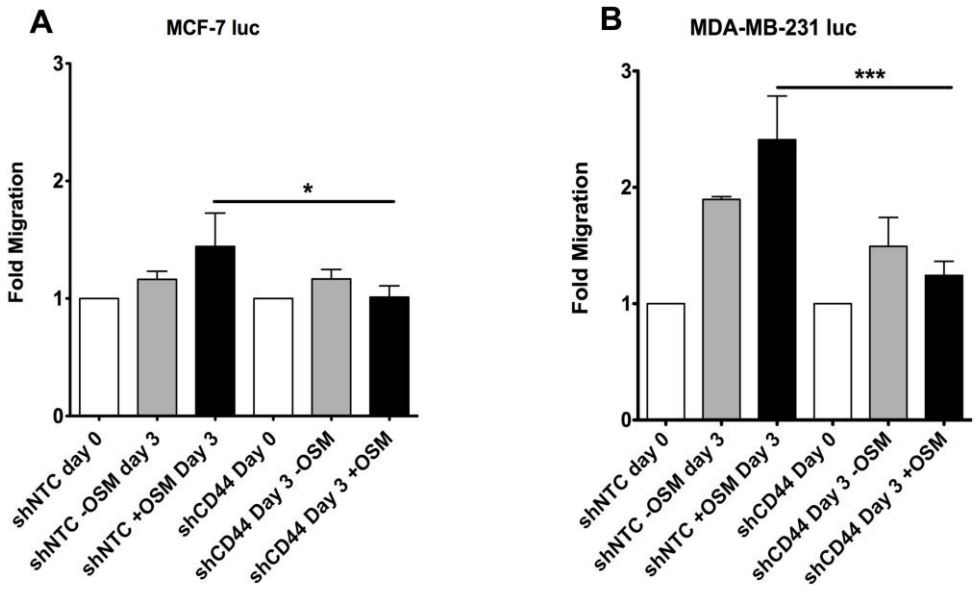


Figure 2.5. Reduced CD44 expression decreases OSM-induced tumor cell migration in ER- cells to a greater extent than in ER+ cells. A. ER+ MCF-7 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for 3 days, and wound healing was analyzed using ImageJ software. Data was interpreted as fold change (*p<0.05, one-way ANOVA) (n=3, +/- SEM). B. ER- MDA-MB-231 luc-shNTC and shCD44 cells were treated with OSM (25 ng/mL) for 3 days, and wound healing was analyzed using Image J software. Data was interpreted as fold change (*p<0.001, one-way ANOVA) (n=3, +/- SEM).**

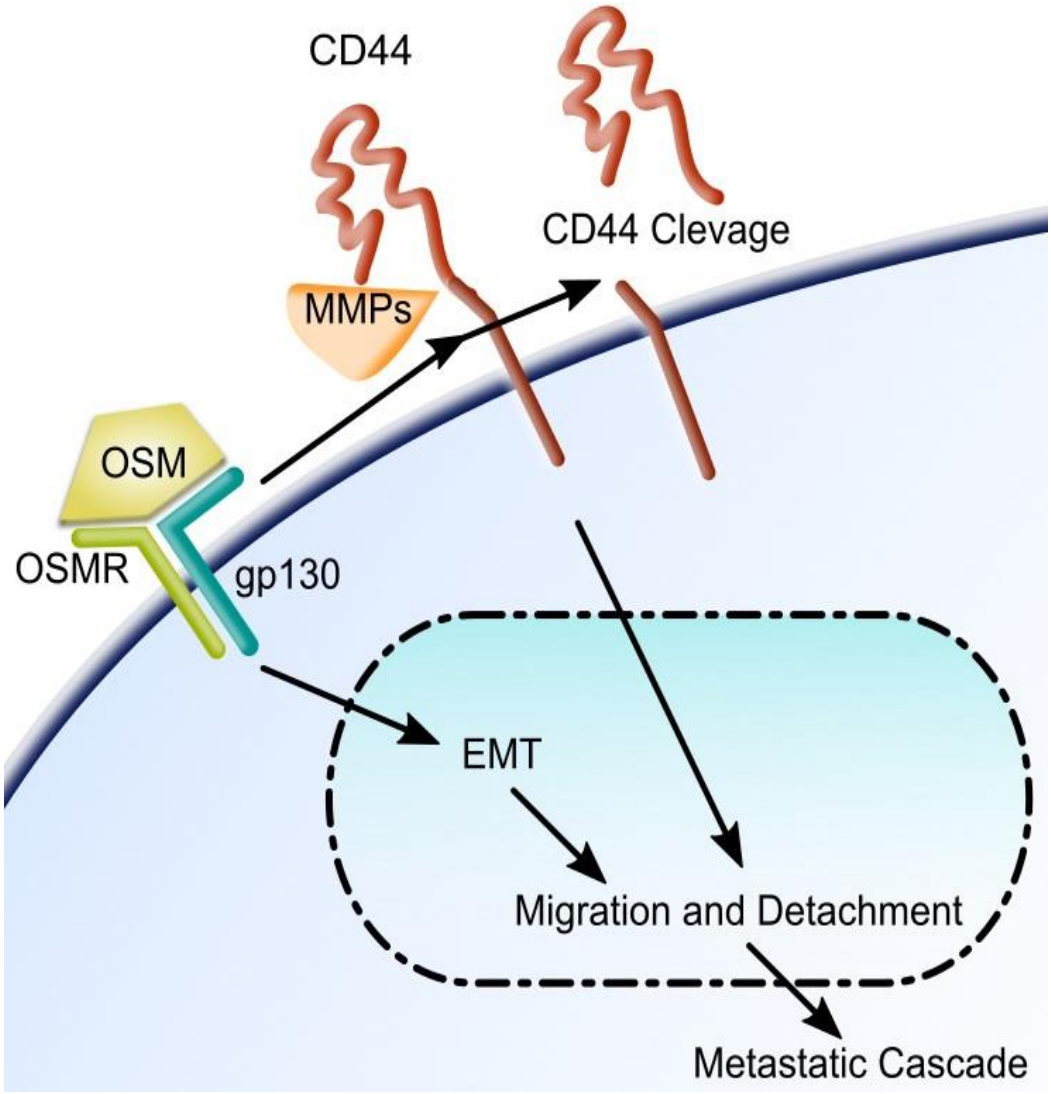
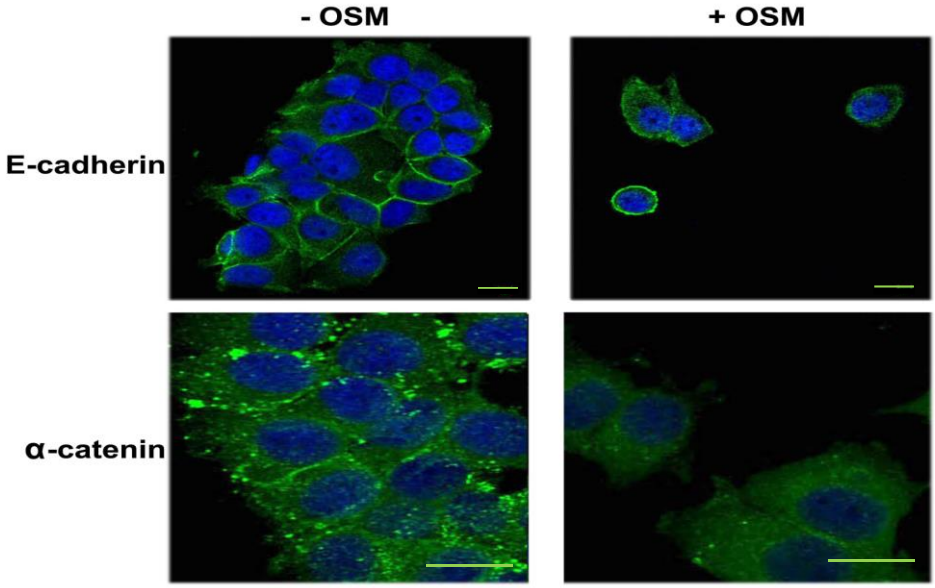
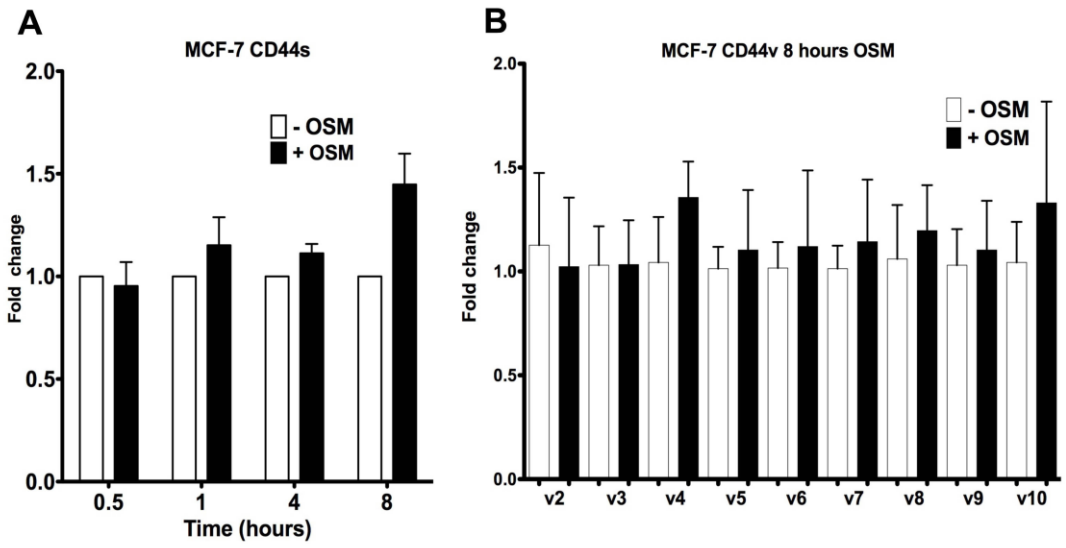


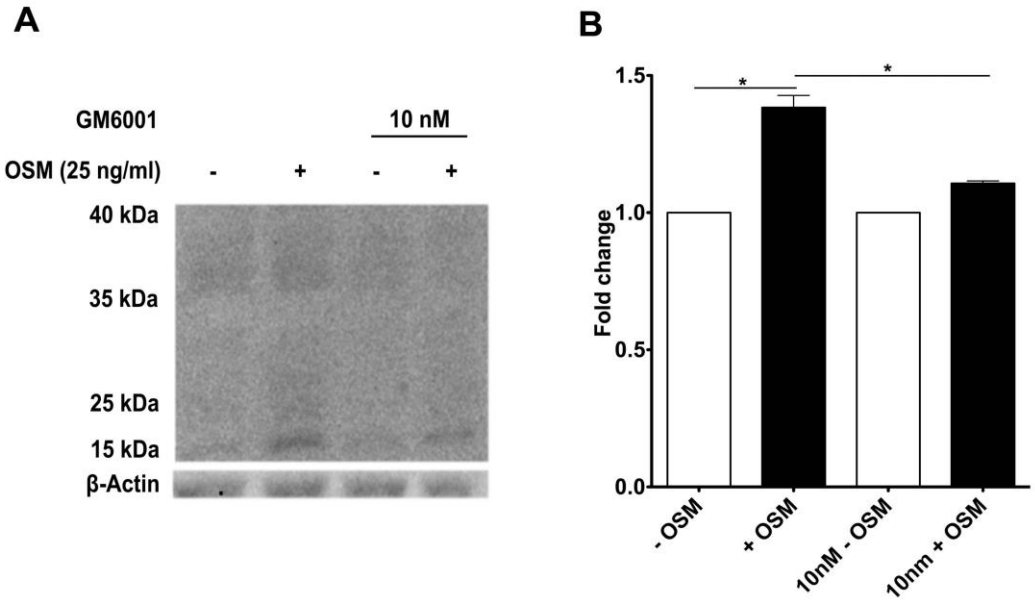
Figure 2.6. Model for OSM-induced migration and detachment. OSM signaling induces an EMT and upregulates CD44 expression and cleavage in ER+ breast tumor cells. Once bound to its receptor, OSM induces cells to undergo EMT and subsequent detachment and migration. OSM signaling also increases expression of the transmembrane protein CD44 and its cleavage, possibly via MMPs. OSM-induced CD44 is involved in migration and detachment as well as other downstream events during the metastatic cascade.



Supplemental Figure 2.1. OSM induces an EMT in MCF-7 cells. MCF-7 breast tumor cells were treated with OSM (25 ng/mL) for 72 hours. OSM induces an increased mesenchymal phenotype, a redistribution in E-cadherin expression (green, upper panels, scale bar = 20 nm), and a slight decrease in α-catenin expression (green, lower panels scale bar = 20 nm), as measured by immunofluorescence (DAPI = blue).



Supplemental Figure 2.2. OSM has no effect on different CD44 variant mRNA levels in breast cancer cells. A. MCF-7 cells were treated with OSM (25 ng/mL) for 0.5, 1, 4, and 8 hours followed by RNA collection and qPCR analysis for CD44s (n = 3, +/- SEM). B. MCF-7 cells were treated with OSM (25 ng/mL) for 8 hours followed by RNA collection and qPCR analysis for different CD44 splice variants. (n = 3, +/- SEM).



Supplemental Figure 2.3. OSM-induced CD44 cleavage is reduced by a general MMP inhibitor in ER+ breast tumor cells. A. T47D cells were treated with 10 nM of GM6001 (global MMP inhibitor) for 48 hours prior to the addition of OSM (25 ng/mL) for an additional 48 hours. Whole cell lysates were collected and CD44 western blot analysis was performed to observe cleaved products. OSM induces CD44 in T47D cells after 48 hours; inhibition of MMP activity reduces OSM-induced CD44 cleavage. B. Quantitation of the 15 kDa – 35 kDa bands by ImageJ demonstrate that GM6001 significantly reduces OSM-induced CD44 cleaved products (n = 3, +/- SEM) (*p<0.05, unpaired t-test).

CHAPTER THREE: CD44 IS IMPORTANT FOR TUMOR CELL EXTRAVASATION DURING THE METASTATIC CASCADE

Title: CD44 is important for tumor cell extravasation during the metastatic cascade

Authors

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Abstract

The transmembrane cluster of differentiation 44 (CD44) protein has been shown to be important in breast cancer tumor progression. Here, our studies extend this knowledge by demonstrating that CD44 is critical for the docking of MDA-MB-231 to endothelial cells, a crucial step in extravasation and the metastatic cascade. These studies were performed using a microfluidic chamber lined with human microvascular endothelial cells (HMECs) over which were flowed MDA-MB-231 luc human breast cancer cells with knockdown expression of CD44 (MDA-MB-231 luc-shCD44). This data was supported using an orthotopic MDA-MB-231 luc-shCD44 mouse model of breast cancer, which showed that reduced CD44 expression resulted in an increased number of circulating tumor cells (CTCs). Here, we also demonstrated that OSM-induced $CD44^{+}/CD24^{-/low}$ breast cancer stem cells (BCSCs) injected orthotopically into the mammary fat pad of mice were capable of initiating tumor formation using just a very small number of cells (500 cells), thus providing beneficial data to the BCSC theory. Finally, based on patient Oncomine^{TM} data, we show that the complex nature of CD44 does not bode well for its use as a therapeutic intervention, as patients with high levels of CD44 mRNA expression actually survive longer than patients with low levels of CD44 when OSM receptor expression is low. In conclusion, these studies contribute new information regarding the role of CD44 in tumor progression and metastasis.

Introduction

Cluster of differentiation 44 (CD44) is a transmembrane protein that has been studied across many fields of cancer biology. As a transmembrane protein, its binding to extracellular hyaluronic acid (HA) occurs through its amino domain (117–120). CD44 is involved in many aspects of tumor progression and metastasis such as cell survival, migration, adhesion, signaling, docking, differentiation, and proliferation, and is a key protein for cytokine signaling (121–123). In order for CD44 to contribute to these events, it must be cleaved just outside the cell surface by matrix metalloproteinases (MMPs) such as MMP-9 to release the extracellular domain from the bound portion of CD44 at the cellular membrane (124, 125). This enables the cell to become migratory after losing CD44 as an anchoring protein (124, 125). The fragment of CD44 embedded into the membrane undergoes a second cleavage event by γ-secretase, which releases the intracellular domain (ICD) into the cytosol where it can then participate in signal transduction (99, 113, 126–128). Transcriptional activity following ICD release and extracellular domain (ECD) cleavage provide many of the key proteins necessary for tumor metastasis, such as migration and invasion (53, 121). While cleaved, CD44 could be a potential target during metastasis due to many events the protein is involved in.

One important step in the metastatic cascade is tumor cell extravasation, and CD44 may play an important role in this process. CD44 and HA are present in many different cellular environments and on many different cell types, including endothelial cells (ECs) (129). The binding of CD44 to HA can create a situation where various blood cell types bind (dock) to ECs by sandwiching HA between each of the cell's CD44. This process may also be required for circulating tumor cells (CTCs), as docking requires a

tumor cell in free flow to come to a stop prior to extravasation, an essential step in the metastatic process.

Breast tumor masses contain a heterogeneous population of cells including breast cancer stem cells (BCSCs). BCSCs present a challenge during treatment because they are able to avoid being targeted by common therapeutics based on their control of self-renewal genes. Following eradication of a large number of rapidly proliferating primary tumor cells, the remaining cells possess tumor-initiating properties and the potential for metastasis and/or recurrence (130). The phenotype widely studied in breast cancer research that identifies this population of BCSCs is $CD44^{+}/CD24^{-/low}$.

In this study, we look at the role of CD44 in breast cancer tumor progression. First, we studied whether CD44 is necessary for breast tumor cell extravasation. Our *in vitro* and *in vivo* findings showed that reduced tumor cell CD44 expression results in decreased tumor cell docking to endothelial cells and increased CTCs that cannot exit the bloodstream. Second, we demonstrated that OSM-induced CD44 contributes to an increased functional population of BCSCs, and we showed that these cells efficiently develop mammary tumors.

Materials and Methods

Cell Culture

MDA-MB-231 luc cells were stably transduced with lentiviral particles containing shCD44 shRNA and shNTC (non-targeting control) shRNA. Western blot analysis was performed to confirm successful knockdown of CD44 (see Chapter 2). Cells were grown to 90% confluency in a T-75 culture flask. Human microvascular endothelial cells (HMEC) were grown directly in microfluidic well plates housing hollow fluidic

channels (Fluxion Biosciences, San Francisco, CA) until the tube displayed an even layer of cellular attachment. HMEC cells were primed by the addition of TNF-α (20 ng/mL) (R & D Systems, Minneapolis, MN) 24 hours prior to adding MDA-MB-231 luc cells. Calcein AM cell permeable dye (1/300, Thermo Fisher Scientific) was added 15 minutes prior to trypsinization of cells.

BioFlux System

BioFlux 200 (Fluxion Biosciences, San Francisco, CA) was used in conjunction with a microfluidic plate lined with HMEC cells. An EVOS inverted microscope (Invitrogen™ EVOS™ FL Auto Imaging System) was used to record video and still frame images of tumor cells as they traveled through a channel while exposed to 0.5, 1.0, 1.5, and 2 dyne/cm^2 shear stress over a period of 1 minute per pulse.

Cd44 Knockdown Mouse Model of Breast Cancer

All animal studies were conducted in accordance with the animal component of research protocol (ACORP) 006-AC15-008 protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the Boise State University Vivarium, Boise, ID. In the first *in vivo* study, 2 x 10^6 MDA-MB-231-luc-shNTC (control) and MDA-MB-231-luc-shCD44 were orthotopically injected into the 4th mammary fat pad of 6 to 8-week old female nude mice (n = 3). Tumor size was measured with calipers bi-weekly once tumors became palpable (after approximately 3 weeks). Bioluminescence (BLI) of live animals was initiated at 21 days after cell line injection and performed weekly.

Bioluminescence *in Vivo* Imaging of Live Animals

Briefly, mice were injected intra-peritoneal (IP) with 150 mg/kg of D-luciferin (Caliper Life Sciences) in PBS, anesthetized with 2.5% isoflurane, and imaged. Mice were imaged using a charge-coupled device camera–based bioluminescence imaging system (IVIS Spectrum, Caliper Life Sciences; exposure time 1–300 sec, binning 4/8/16, field of view 23 cm, f/stop 1, emission filter open). Signal was measured and recorded as total flux (photons/sec). Corresponding grayscale photographs and color luciferase images were automatically superimposed and analyzed with Living Image software (Xenogen Biosciences Corporation, Cranbury, NJ).

Flow Cytometry

A BD FACSCalibur™ (San Jose, CA) was used (see Chapter 2) to observe populations of BCSCs present within a homogenous population. MCF-7 luc cells were treated with OSM (25 ng/mL) for 72 hours before performing flow cytometry with antibodies (see Chapter 2). Software analysis was performed using BD FACSCalibur software: CellQuest Pro, version 6.0 by BD Biosciences.

Cell Sorting

Five T-75 flasks containing MCF-7 luc cells at 80% confluency were treated with OSM (25 ng/mL) for 72 hours prior to the addition of the following antibodies (BD Biosciences, San Jose, CA) CD44 (1:5 FACS buffer), CD24 (1:5 FACS buffer), FITC (1:50 FACS buffer), and PE (1:50 FACS buffer) antibodies after blocking cells for 10 minutes with 1% BSA. Antibodies bound to their appropriate proteins for 30 minutes on ice in the dark. Following incubation, cells were washed 3x with FACS buffer (10% FBS, 0.1% sodium azide in PBS). Cells were resuspended in FACS buffer before sorting was

performed. $CD44^{+}/CD24^{low/-}$ cells were collected in a 6 -well plate containing fresh media. After cell sorting was finished the desired population was placed in an incubator at 5% CO_2, 37 degrees C overnight. Software analysis was performed using BD Influx software: Spigot, version 6.1.4 by Cytopeia (Seattle, Washington)

Estrogen Pellet Implantation in Mice Receiving Mcf-7 Er+ Cells

For the second *in vivo* study, estrogen was supplied to athymic nude female mice. 17β-estradiol pellets (1.7 mg/90-day release) were obtained from Innovative Research of America (Sarasota, FL). Animals were placed in a nose cone for anesthesia prior to surgery. Surgical scissors were used to make a 3-mm opening on the animal's posterior neck and a pocket was made by loosening the skin. A single pellet was implanted into the pocket and gently pressed to secure the pellet. Animals were placed back into their cages for 48 hours to recover before use in the experiment.

Bcsc Mouse Model of Breast Cancer

MCF-7 luc cells sorted for the $CD44^{+}/CD24^{-/low}$ population and MCF-7 luc parental cells (control) were suspended in 10 μL of phosphate buffered saline (PBS) and were injected into the 4th mammary fat pad of 8-week old female nude mice (n=3/group). Two groups of parental cells were injected; 500 cells/mouse and 2×10^6 cells/animal. Three groups of cell-sorted $CD44^{+}/CD24^{-/low}$ MCF-7 luc cells were injected (500, 1,000, and 5,000 BCSCs/animal). Tumor size was measured with calipers bi-weekly once tumors became palpable (after approximately 3 weeks), and BLI was performed weekly.

Oncomine Data

To explore correlations between patient survival and OSM, OSMR, CD44, and CD24 status, we attained the Curtis Breast human mRNA microarray dataset from

Oncomine™ (Compendia Bioscience, Ann Arbor, MI). The constraints used to define the dataset used were "Invasive Ductal Carcinoma" and a detailed survival status of either "Alive" or "Dead of Disease." The resultant dataset was used to calculate quartiles. From these, the upper quartile (>75th percentile) and lower quartile (<25th percentile) were selected for comparison in order to clearly depict survival trends that may have been otherwise muddled by use of all quartile combinations. For co-expression analysis, we calculated survival curves using patients in the upper and lower quartile of both CD44 and OSMR status being ("high/high") and the lower quartile of both ("low/low"). Statistical analyses between survival of two groups was calculated using a log-rank test in GraphPad Prism 5 software (** = $p < 0.01$).

Results

Cd44 Is Necessary for Tumor Cell Docking fo Endothelial Cells

Adaptation and survival is necessary for breast tumor cells once they enter the vascular stream during the metastatic cascade. We wanted to address whether tumor cells use their transmembrane CD44 to help bind and dock to endothelial cells (ECs) during extravasation in a fashion similar to how blood cells utilize their CD44 to bind to hyaluronic acid on ECs prior to their extravasation out of a blood vessel (131). MDA-MB-231 luc-shCD44 and MDA-MB-231 luc-shNTC were suspended in standard growth media, placed in a BioFlux apparatus lined with human microvascular endothelial cells (HMECs), and subjected to 1 dyne/cm^2 of shear stress of force (slightly less than what is found in large veins) (132). Cellular docking was observed via videos and still-image microscopy (Fig 1A). Using still images from 5 fluidic events, chambers were flushed and stationary cells were counted for graphical representation. MDA-MB-231 luc-

shCD44 cells showed a 5-fold reduction in docking to ECs (Fig. 1B; $p = 0.0026$) compared to MDA-MB-231 luc-shNTC control cells. By video, the MDA-MB-231 luc-shCD44 cells showed continuous circulation versus control cells, which were much more likely to dock (video not included). These results suggest that ER- MDA-MB-231 luc human breast cancer cells require CD44 in order to dock and extravasate during the metastatic process.

Decreased Cd44 Expression Leads to an Increase in Breast Circulating Tumor Cells (Ctcs)

Our lab has previously shown that recombinant human OSM (rhOSM) increases circulating tumor cell (CTC) number and subsequent metastasis in an MDA-MB-231 luc orthotopic mouse model of breast cancer (manuscript in preparation). To confirm our *in vitro* results from Figure 1, and to demonstrate that CD44 is needed for CTCs to dock and extravasate, and we used the MDA-MB-231 luc-shCD44 orthotopic mouse model of breast cancer. 2×10^6 MDA-MB-231-luc-shCD44 and shNTC control cells were injected into the 4^{th} mammary fat pad of female nude mice. Once tumors became palpable (roughly 5 mm^2), the animals received peri-tumoral injections of rhOSM (1 μg in PBS) 3x/week for the life of the animal (Fig 2A). BLI images of each animal were recorded 3x/week during the final 8 weeks (12-week total duration of experiment) in order to track prima tumor growth and metastasis. Blood was collected from each mouse at the termination of the study, and qPCR was performed to quantify human Alu repeat sequences (found in human tumor cells but not mouse blood cells), as a measure of relative CTC count. As expected, injection of OSM resulted in an increase in CTC number; ~4-fold for MDA-MB-231 luc-shNTC cells and over 2-fold for MDA-MB-231

luc-shCD44 cells (Fig. 2B). Importantly, knockdown of CD44 expression in the MDA-MB-231 cells led to an increase in CTC number (Fig. 2B), suggesting that without CD44, the tumor cells could not dock and extravasate and remained as CTCs in the blood stream.

To observe the serological effects of rhOSM and CD44 expression in our mouse model, blood from each animal was collected and complete blood counts (CBCs) were analyzed by West Vet (Garden City, ID). As CD44 contributes to the maturation of white blood cells (WBCs) (133), we investigated neutrophil and lymphocyte values across each experimental group. We observed a 2- to 3-fold decrease in the number of neutrophils and mature lymphocytes in the blood of mice injected with MDA-MB-231 luc-shCD44 cells compared to MDA-MB-231 luc-shNTC control cells (Fig. 2C; $p = <0.001$), suggesting that decreased CD44 expression resulted in a decrease in these blood cell counts. OSM had no significant effect on WBC counts (Fig. 2C). In conclusion, a decrease in CD44 expression, as demonstrated by MDA-MB-231 luc-shCD44 cells, resulted in an increase in CTC number and a decrease in the number of neutrophils and lymphocytes. Therefore, CD44 contributes to events in the metastatic cascade through the promotion of cellular docking as well as through decreasing the number of WBCs that pose a threat to tumor progression.

A Low Number of $Cd44^{+/}Cd^{24-/Low}$ Breast Cancer Cells (BCSCs) Initiate Tumor Formation

To investigate the process of tumor initiation, we observed the effects of BCSCs *in vivo.* Unlike with ER- MDA-MB-231 cells, ER+ parental MCF-7 luc cells were-sorted and these BCSCs require the presence of estrogen for tumor cell proliferation. ER+ MCF-

7 luc cells, cell-sorted for $CD44^{+}/CD24^{-/low}$ expression, were orthotopically injected into athymic nude mice having received 17β-estradiol pellet implantation (Fig. 3A). Commonly used xenograft mouse models of breast cancer require the orthotopic injection of a large number of cells (2×10^6) for tumor formation and metastasis, while injection of BCSCs should require a smaller number of cells (134). To study OSM-induced BCSC tumor initiation and address the issue of BCSC theory, we performed a pilot study using 2×10^6 and 5,000 parental MCF-7 luc cells orthotopically injected into the 4^{th} mammary fat pads of athymic mice, and compared tumor initiation to that of the injection of cell-sorted MCF-7 luc $CD44^{+/}CD24^{-/low}$ cells (Fig. 3B). Injection of 2×10^6 parental MCF-7 luc cells resulted in 3/3 mice developing tumors (mean 8.5 x 6 mm^2) at 5 weeks (Table 1), while 0/3 mice receiving 5,000 cells developed tumors over this same time period (Table 1). On the other hand, as few as 500 $CD44^{+/}CD24^{-/low}$ BCSCs were capable of forming a tumor, as 1/3 mice developed a tumor at 5 weeks (Table 1). Primary tumor initiation precedes all metastatic events and is a first step for tumor progression and potential downstream metastasis. Here we showed that OSM-induced $CD44^{+/}CD24^{-/low}$ MCF-7 BCSCs can form tumors in an animal model with the orthotopic injection of as few as 500 cells.

Cd44 Is Crucial For the Survival Of Human Patients Expressing Low Osmr

To take our metastasis and tumor initiation findings further, we used human survival data from female patients with breast cancer classified into categories of relevance. Oncomine™ gene expression data was obtained and categorized based on female breast tumor OSM, OSMR, and CD44 expression status. The survival of patients with low OSMRβ expression (n=172) was investigated and subsequent CD44 expression

was ranked as high (upper quartile) or low (bottom quartile). Patients with low OSMRβ expression and high CD44 expression had a significantly higher percent survival than patients with low OSMRβ and low CD44 expression (Fig. 4; p=0.0046). Other categories that were addressed but were not found significant were: i) OSM low patients who had either CD44 high or CD44 low (p=0.0586); ii) OSM high patients with either CD44 high or CD44 low (p=.2719), and iii) OSMRβ high patients with either CD44 high or CD44 low (p=0.293). The data in Figure 4 suggests that in patients whose breast tumors express little OSMR, and therefore have a reduced ability to respond to OSM, high CD44 expression may play a role in delaying tumor progression.

Discussion

In these studies, we show that CD44 expression in breast cancer cells is necessary for tumor cell docking in a simulated vasculature environment, which correlates with CTCs numbers seen *in vivo*. Furthermore, OSM-induced $CD44^{+}/CD24^{-/low}$ MCF-7 luc BCSCs can form tumors when as few as 500 cells were injected into athymic mice.

CD44 has been shown to be critical for memory T cells homing and exiting blood vessels during the process of fighting infection (135–137). This suggests that a potential role for CD44 on breast tumor cells could be as a location-specific protein during the process of extravasation. In our studies, we demonstrated that MDA-MB-231 luc-shNTC and shCD44 cells behaved quite differently from each other in a orthotopic xenograft mouse of breast cancer. As expected, rhOSM increased the number of CTCs (manuscript in preparation) in both mice injected with MDA-MB-231 luc-NTC control and shCD44 cells, but we observed that the number of CTCs from mice injected with MDA-MB-231 luc-shCD44 cells was greater than the number found in mice injected with control cells.

This data suggests that CD44 is necessary for tumor cells to dock to ECs, which is essential for the initiation of extravasation. Intravasation appeared to be unaffected by CD44 levels as both shCD44 and shNTC cells entered the blood and became CTCs. These important *in vivo* findings were supported by findings from our microfluidics *in vitro* model, which showed that there was a significant decrease in the number of MDA-MB-231 luc that docked on ECs when CD44 expression was reduced.

Tumor initiation is necessary for a single cell to proliferate into a primary tumor. During metastasis, BCSCs are capable of homing to a secondary location as an individual cell, or a small number of cells that have controlled cell proliferation to establish a secondary metastatic tumor. Human breast cancer cells display more metastatic behavior *in vivo* when introduced into the animal at high cell numbers or using various models that bypass tumor initiation, such as through intracardiac injection (105). However, our *in vivo* model focused on the capacity of tumor formation using $CD44^{+}/CD24^{-/low}$ MCF-7 luc cells orthotopically injected in the murine fat pad and not directly injected into the circulating blood stream. Here we show as few as 500 BCSCs were capable of tumor formation even when 5,000 parental cells were not. As this was a pilot study, statistical analysis could not be performed. Nevertheless, our results suggest the need for our laboratory to further investigate the role of OSM-induced BCSCs tumor initiation *in vivo*.

We also compared the expression levels of OSM, OSMRβ, and CD44 on female breast cancer patient survival. We found that patients with low levels of OSMR and high levels of CD44 had a higher survival rate compared to low OSMR and low CD44. As puzzling as this data seemed, it is suspected that CD44 contributes to both positive and

negative outcomes during the multi-step process of breast tumor metastasis as shown by our docking, *in vivo*, and human patient data.

Conclusion

MDA-MB-231 luc breast tumor cells dock to HMEC cells with CD44 in a fluidic environment similar to the bloodstream. The absence of CD44 in MDA-MB-231 luc cells increases the number of CTCs *in vivo* as they are unable to extravasate during metastasis. MCF-7 luc cells treated with OSM for 72 hours showed an increase in $CD44^{+}/CD24^{-/low}$ BCSCs. 500 BCSCs orthotopically injected into mammary fat pads of nude mice developed tumors compared to parental MCF-7 luc cells that needed large numbers of cells to develop a palpable tumor.

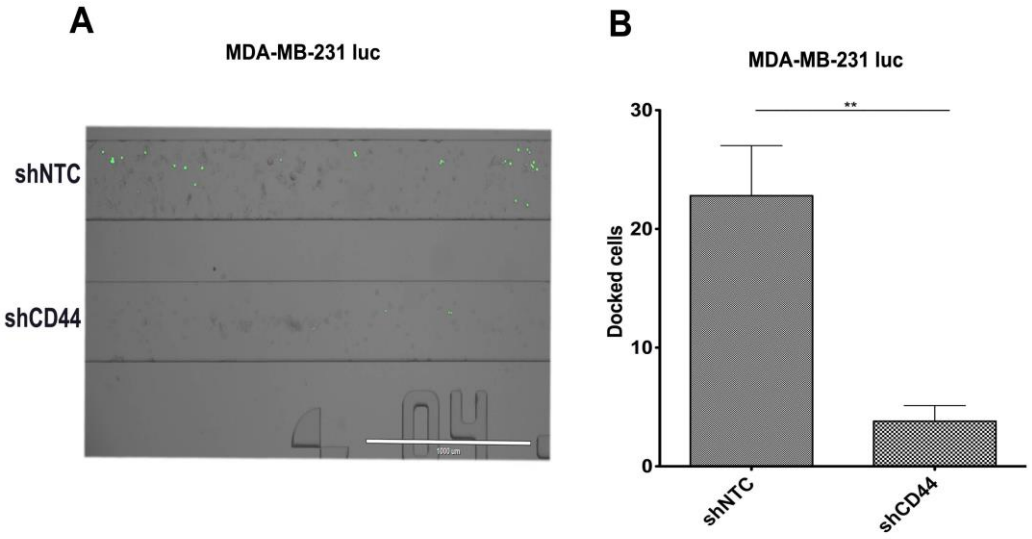

Figure 3.1: CD44 increases tumor cell docking in MDA-MB-231 luc cells in an *in vitro* model. A. MDA-MB-231 luc-shNTC and shCD44 cells were stained with Calcein AM for 15 minutes in a cell culture flask in the incubator. Cells were removed from the flask via trypsin and placed in a conical on ice. 250,000 cells suspended in 0.5 mL media was slowly added to the BioFlux apparatus where a single layer of HMEC endothelial was present. Using 1 dyne of shear stress, cells were pumped through the hollow tube containing endothelial cells. Flow rate was paused followed by a wash cycle to remove any undocked cells and fluorescent cell counts were performed. B. MDA-MB-231 luc-shNTC and shCD44 docked cells counted and statistically analyzed. (n=5, +/- SEM) ($p<0.005$) $p = 0.0026$

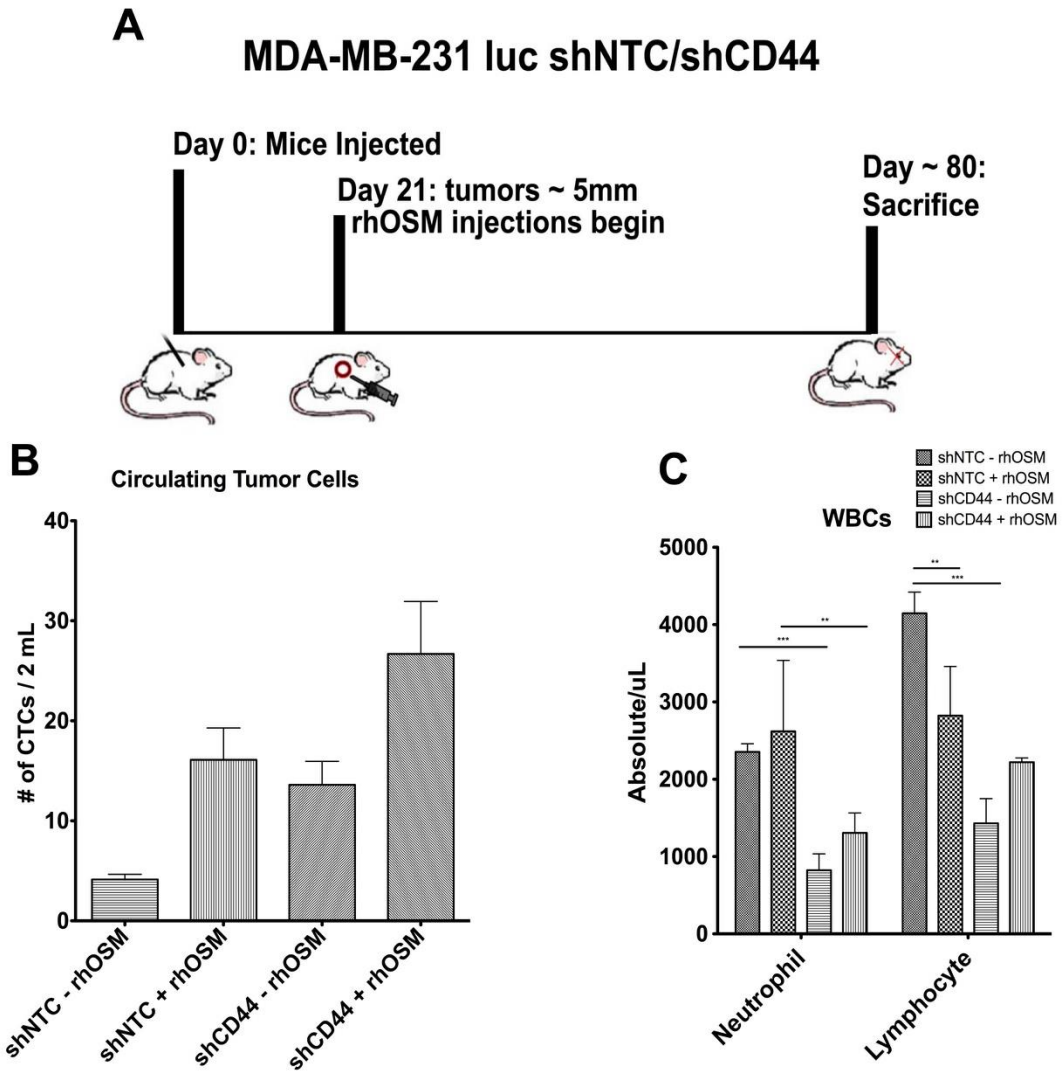

Figure 3.2: Orthotopic injection of breast cancer cells with decreased CD44 expression leads to increased CTC numbers and decreased WBC maturation. A. Nude mice were injected with either MDA-MB-231 luc-shCD44 or shNTC control cells, and tumor development was observed over a 12-week period. shCD44 and shNTC groups were split into two groups, and half received rhOSM (1 μg) peri-tumorally 3x/week. B. Following the termination of the experiment, blood was drawn for each animal for CTC analysis using qPCR for Alu amplification. C. White blood cell counts for each murine group at the termination of the experiment. A significant difference of p = <0.001 when comparing shNTC –rhOSM to shCD44 –rhOSM. (n=3, +/- SEM).

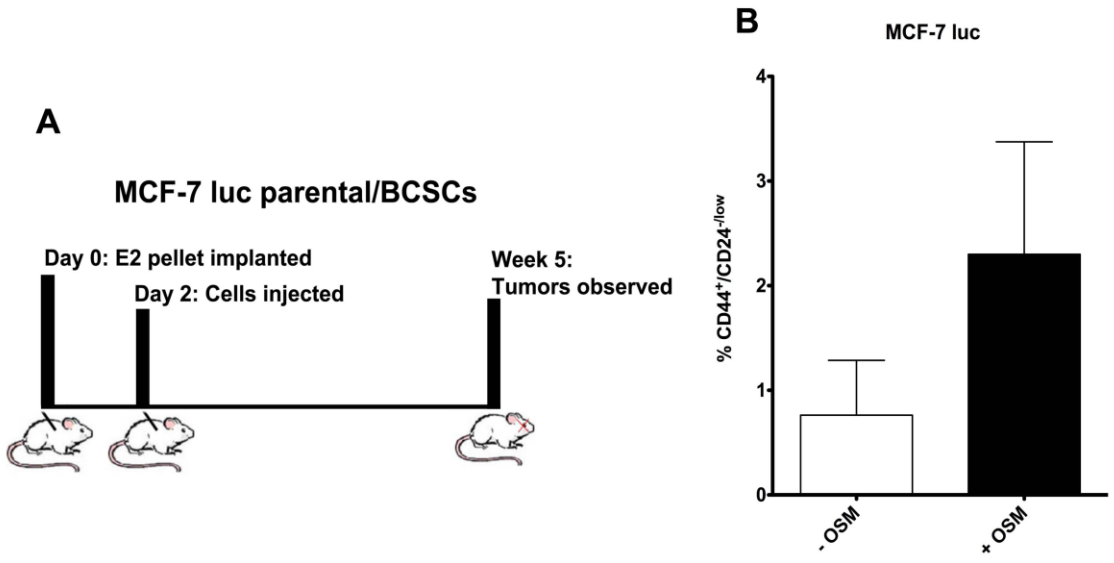

Figure 3.3: BCSCs promote tumor initiation at low numbers. A. BCSCs and parental MCF-7 luc cells were collected and orthotopically injected into mammary fat pads of nude mice to observe tumor initiation. MCF-7 luc cells were treated with OSM (25 ng/mL) for 72 hours followed by performing flow cytometry analysis B. OSM increased BCSC phenotype, which was subsequently used for the second animal experiment and data is shown in Table 1. (n = 3, +/- SEM).

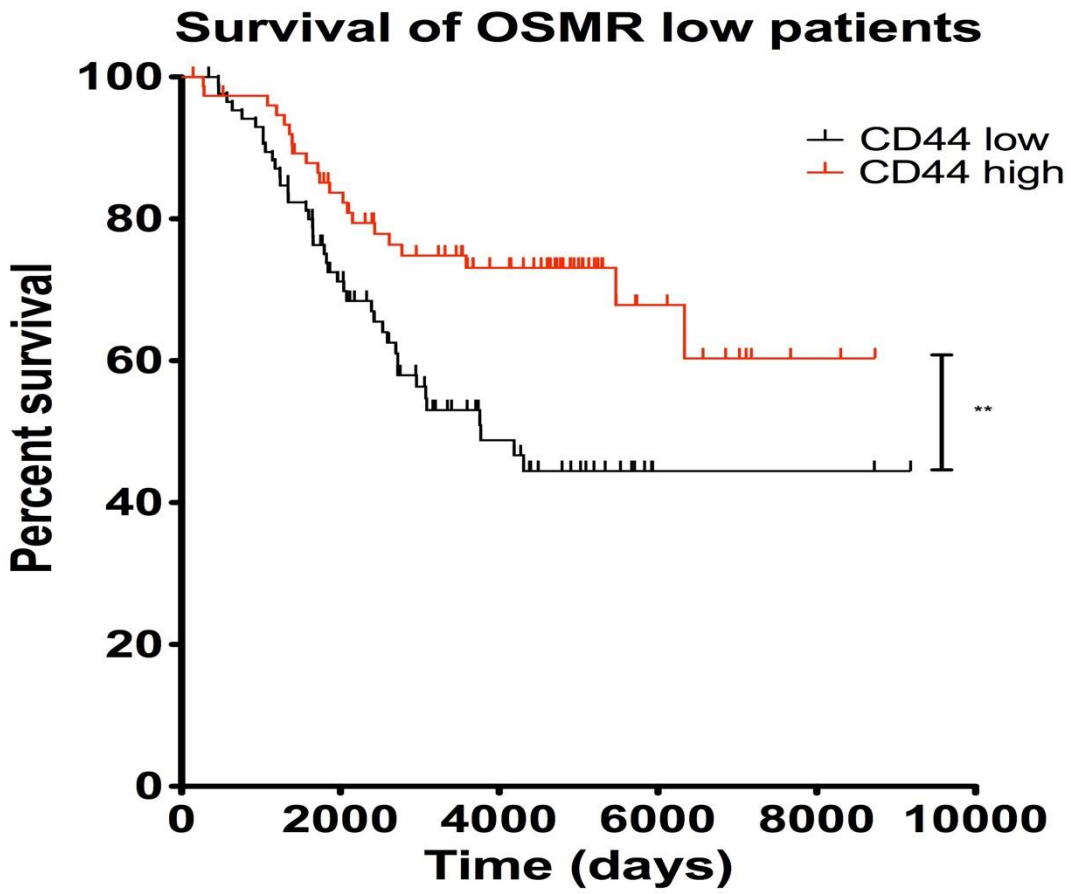

Figure 3.4: Patients who express low levels of OSMR have a better survival rate if CD44 levels are high. An Oncomine™ microarray data set from invasive ductal carcinoma patients (n = 172) was sorted to analyze only those who had low levels of OSMR (n = 86). After isolating OSMR low data, it was then broken into categories of CD44 low and CD44 high. Statistical analysis between the two groups was performed by using a log rank test, p = 0.0046.

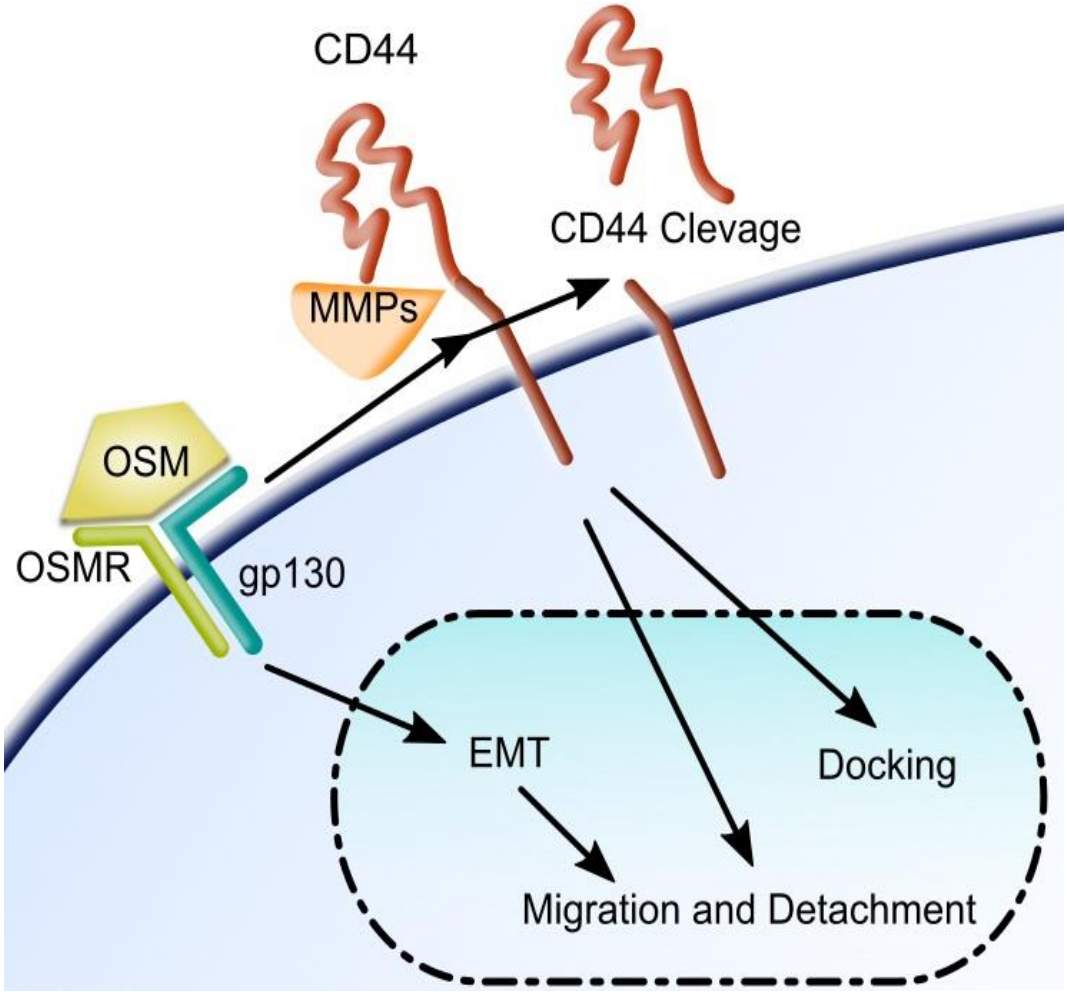

Figure 3.5: OSM and CD44 promote metastasis in different ways. OSM binds to its receptor, signaling downstream events such as EMT, migration, and detachment. OSM also activates the cleavage of CD44 through MMPs leading to increased migration and detachment (see Chapter 2). CD44 also leads to breast tumor cell docking and extravasation.

Table 1: CD44^{+}/CD24$^{-/low}$ MCF-7 luc BCSCs are capable of tumor initiation *in vivo*. Cells were collected after cell sorting was performed. Following cell sorting, CD44^{+}/CD24$^{-/low}$ cells were compared to MCF-7 luc parental cells.

CELLS RECEIVED	TUMOR INTITATION	TUMOR AT 5 WEEKS (mean in mm)
MCF-7 luc (Parental) 2×10^6	3/3	8.5 x 6
MCF-7 luc (Parental) 5,000	0/3	0
MCF-7 luc CD44+/CD24- (BCSCs) 500	1/3	5 x 5

Table 2 Primers

NAME	5’	3’
CD44s	AGTCACAGACCTGCCCAATGCCTTT	TTTGCTCCACCTTCTTGACTCCCATG
CD44v2	GACAGCAACCAAGAGGCAAG	TTTGTGTTGTTGTGTGAAGATGATT
CD44v3	CGTCTTCAAATACCATCTCAGCA	ATCTTCATCATCAATGCCTGA
CD44v4	AACCACACCACGGGCTTT	CATCCTTGTGGTTGTCTGAAGTA
CD44v5	ATGTAGACAGAAATGGCACCAC	GTGCTTGTAGAATGTGGGGTC
CD44v6	GGCAACTCCTAGTAGTACAACG	GTCTTCTCTGGGTGTTTGGC
CD44v7	CTCATACCAGCCATCCAATGC	CTTCTTCCTGCTTGATGACCTC
CD44v8	CTCCAGTCATAGTATAACGCTTCA	GTTGTCATTGAAAGAGGTCCTGT
CD44v9	AGCAGAGTAATTCTCAGAGCTTC	CAGAGTAGAAGTTGTTGGATGGT
CD44v10	ATCATTCTGAAGGCTCAACTACTT	TAAGGAACGATTGACATTAGAGTTG
Slug	AGAGACATGACGGTGGTTTGT	GTTTTGCACCCAGAAAGTGTGA
Snail	GTAAGGGAGGAGGGGACAGG	CCCAGGACTCCAAAGCCCTA
β-Actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

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APPENDIX A

Research on this project was conducted under the approval of the Institutional Animal Care and Use Committee at Boise State University, protocol #006-AC15-008.