The role of GPNMB in breast tumor progression

by

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ABSTRACT

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer related deaths among Canadian women. Development of distant metastases is the leading cause of morbidity and mortality from this disease. Breast cancer is a highly heterogeneous disease that is amenable to intervention with targeted therapeutics; however, therapies that are currently available have limited efficacy in the metastatic setting. To identify novel molecular mediators of breast cancer bone metastasis that might also serve as therapeutic targets, we subjected 4T1 mammary carcinoma cells to *in vivo* selection in Balb/c mice and isolated sub-populations with an aggressively bone-metastatic phenotype. Gene expression profiling of these cells revealed Glycoprotein NMB (GPNMB), also known as Osteoactivin, as a gene that was highly expressed in bone metastatic breast cancer cells. GPNMB is a type I transmembrane, cell surface expressed protein with an extracellular RGD and PKD domains and a cytoplasmic hemITAM signaling motif that had not previously been implicated in breast cancer. We demonstrate that ectopic GPNMB expression was sufficient to promote migration and invasion of breast cancer cells *in vitro* and the formation of bone metastases *in vivo*.

Subsequently, we analyzed GPNMB mRNA and protein expression levels in hundreds of breast tumors and found that GPNMB expression positively correlates with increased risk of metastasis and shorter overall survival times. We have also demonstrated that GPNMB is most commonly expressed in breast tumors belonging to the triple negative subtype, for which there are no targeted therapies currently available. We showed for the first time that CDX-011, a GPNMB-targeted monoclonal antibody-drug conjugate, was capable of killing GPNMB-expressing breast cancer cells *in vitro* and inducing tumor regression *in vivo*.

Finally, we investigated the effects of GPNMB on primary tumor progression and found that it inhibits tumor cell apoptosis while enhancing angiogenesis and tumor growth *in vivo*. We demonstrate that the extracellular domain (ECD) of GPNMB can be proteolytically cleaved and shed from the surface of breast cancer cells, which is mediated by ADAM10. We postulated that the shed extracellular domain (ECD) of GPNMB might be responsible for some of its pro-angiogenic effects and showed that this ECD was indeed capable of inducing endothelial cell migration *in vitro*.

The body of work described in this thesis is the first to identify GPNMB as a functional mediator of breast cancer growth and metastasis and to validate it as an important clinical target in human breast cancer.

RÉSUMÉ

Le cancer du sein est le cancer le plus fréquemment diagnostiqué et la seconde cause de mortalité associée au cancer chez les femmes canadiennes. Le développement de métastases est la cause majeure de la morbidité et de la mortalité dûes à cette maladie. Le cancer du sein est une maladie très hétérogène qui peut toutefois être traité par l'utilisation de thérapie ciblée ; toutefois, les thérapies actuellement disponibles ont un effet limité sur la formation des métastases. Dans le but d'identifier de nouveaux médiateurs moléculaires associés à la formation de métastases osseuses dérivées du cancer du sein et qui pourraient être utilisés comme cibles thérapeutiques, nous avons soumis les cellules de carcinome mammaire 4T1 à un processus de sélection *in vivo* dans des souris Balb/c. Nous avons ainsi isolé des sous-populations de cellules caractérisées par leur agressivité à former des métastases osseuses. L'étude de l'expression génique de ces cellules a mis en évidence que le gène codant pour la Glycoprotéine NMB (GPNMB), aussi connu sous le nom de Ostéoactivine, est très fortement exprimé dans les lignées de cancer du sein métastatiques pour l'os.

GPNMB est une protéine de surface transmembranaire de type I qui possède des domaines RGD et PKD extracellulaires ainsi qu'un motif hemITAM de signalisation cytoplasmique et n'avait encore jamais été rapportée comme impliquée dans le cancer du sein.

Nous avons démontré que l'expression ectopique de GPNMB était suffisante pour promouvoir la migration et l'invasion de cellules de cancer du sein *in vitro* ainsi que la formation de métastases *in vivo*.

Par la suite, nous avons analysé les niveaux d'expression des ARNm et de la protéine GPNMB dans des centaines de tumeur du sein humain et avons observé que l'expression

de GPNMB corrèle positivement avec un risque accru de présence de métastases ainsi qu'une réduction du temps moyen de survie. Nous avons également démontré que GPNMB est le plus fréquemment exprimé dans des tumeurs mammaires appartenant au sous-type triple négatif pour lequel il n'y a actuellement aucune thérapie ciblée disponible.

Par ailleurs, nous montrons pour la première fois que CDX-011, une drogue conjuguée à un anticorps monoclonal reconnaissant GPNMB, était capable, *in vitro*, d'éradiquer spécifiquement les cellules de cancer du sein exprimant GPNMB ainsi que d'induire une régression tumorale *in vivo*.

Finalement, nous avons déterminé les effets de GPNMB sur la progression des tumeurs primaires et avons observé que GPNMB inhibait l'apoptose des cellules tumorales tout en augmentant l'angiogenèse et la croissance tumorale *in vivo*. Nous avons démontré que le domaine extracellulaire de GPNMB (ECD) pouvait être clivé de façon protéolytique par ADAM10 et ainsi être libéré de la surface cellulaire des cellules de cancer du sein. Nous avons postulé que la forme extracellulaire clivée (ECD) de GPNMB pourrait être impliquée dans certains des effets pro-angiogénique et avons montré que cet ECD était capable d'induire la migration de cellules endothéliales *in vitro*.

L'ensemble des travaux décrits dans cette thèse implique pour la premier fois est le premier à identifier GPNMB comme médiateur fonctionnel de la croissance du cancer du sein et de ses métastases. Ce travail identifie GPNMB comme une importante cible thérapeutique pour le traitement des patients atteints du cancer du sein.

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PUBLICATIONS ARISING FROM THIS WORK

Portions of the Chapter 1 were published in the following review articles:

1) A.A.N. Rose and P.M. Siegel. (2010) Emerging therapeutic targets in breast cancer bone metastasis. Future Oncol. 6(1): 55-74.

2) A.A.N. Rose and P.M. Siegel. (2006) Breast cancer-derived factors facilitate osteolytic bone metastasis. Bull. Cancer. 93(9): 931-94.

Chapters 2, 3 and 4 were published, respectively, as the following original research articles:

3) A.A.N. Rose, F. Pepin, C. Russo, J.E. Abou Khalil, M. Hallet, P.M. Siegel. (2007) Osteoactivin promotes breast cancer metastasis to bone. Mol. Cancer Res. 5(10): 1001-1014.

4) A.A.N. Rose, A.A. Grosset, Z.F. Dong, C.Russo, P.A. MacDonald, N. Bertos, R. Simantov, M. Hallet, M. Park, L. Gaboury, P.M. Siegel. (2010) GPNMB is an independent prognostic indicator of recurrence and a novel therapeutic target in breast cancer. Clin. Cancer Res. 16(7): 2147-56.

5) A.A.N. Rose, M.G. Annis, Z.F. Dong, F. Pepin, M. Hallet, M. Park, P.M. Siegel. (2010) ADAM10 releases a soluble form of the GPNMB/Osteoactivin extracellular domain with angiogenic properties. PLoS ONE 5(8): e12093.

The following publications arose from work that was performed during my Ph.D. but not presented in this thesis:

6) A. Bemmo, C. Diaz, A.A.N. Rose, C. Russo, P.M. Siegel, J. Majewski. (2010) Exon-level transcriptome profiling in breast cancer reveals alternatively spliced isoforms specific to tumors with different metastatic abilities. PLOS ONE. 5(8): e11981.

7) M. Demers, A.A.N. Rose, A.A. Grosset, L. Gaboury, P.M. Siegel, Y. St-Pierre. (2010) Overexpression of galectin-7, a myoepithelial cell marker, enhances spontaneous metastasis of breast cancer cells. Am. J. Pathol. 176(6): 3023-31.

8) A. B. Dydensborg, A.A.N. Rose, D. Grote, M. Paquet, P. Siegel and M. Bouchard. (2009) GATA3 prevents breast cancer growth and metastasis to the lung. Oncogene. 28: 2634-2642.

9) A.A.N. Rose and P.M. Siegel (2007) Osteoactivin/HGFIN: Is it a tumor suppressor or mediator of metastasis in breast cancer? Breast Cancer Res. 9(6): 403.

CONTRIBUTIONS OF THE AUTHORS

In order to achieve the quality of data presented in this thesis, several collaborations were established with experts outside of my immediate field of study (clinicians, pathologists and bioinformaticians). Author contributions are listed below by manuscript. I performed all other experiments and coordinated those that I did not directly conduct myself. I analysed all of the data generated in these experiments and together with my supervisor, Dr. Peter Siegel, I made all figures and wrote the manuscripts that constitute this thesis.

In chapter 2, F. P. performed gene expression analysis of microarray data described in Figure 4 and M. H. supervised this analysis. C. R. performed the northern blot in Figure 5b, constructed the Osteoactivin expression vectors used in the study and assisted with the generation of Osteoactivin-expressing cell lines. J.E.A.K. assisted with some mouse necropsies and isolation of cancer cells from bones.

In chapter 3, N.B. and A.A.G. constructed databases of patient characteristics associated with each tumor in TMA1 and TMA2, respectively. L.G. Y.S. and M.P. supervised theses activities. Z.D. performed the immunohistochemical staining of TMA1 and TMA2. C.R. constructed the GPNMB expression vectors used in the study, and assisted with the generation of stable GPNMB-expressing cell lines. R.S. provided the CDX-011, unconjugated CR011, and PK16.3-vcMMAE reagents used in this study. M.H. provided gene expression data that was analysed in Table S2. L.G. and I evaluated all tumor cores on TMA1 and TMA2 for stromal or epithelial GPNMB expression.

In chapter 4, M.G.A. performed the migration assays described in Figure 6a. Z.D. performed immunohistochemical staining described in Figure 2. F.P. performed the analysis of gene expression microarray data derived from epithelial human tumor cells, and provided the raw expression data that was analyzed in Figure 3b. M.H. and M.P supervised these analyses.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1) We provided the first evidence that GPNMB is expressed in breast cancer and that it contributes to tumor progression. Specifically, we were the first to identify GPNMB as a gene that is highly expressed in a mouse model of bone metastatic breast cancer. GPNMB had been reported to promote migration, invasion and metastasis in other types of cancer, but we were the first to show that it functioned in a similar fashion in breast cancer.

2) We showed that GPNMB is commonly expressed in human breast cancers at the mRNA and protein level and that its expression can serve as prognostic indicator of recurrence in breast cancer. We made the first observation that GPNMB expression is most commonly associated with breast tumors of the triple negative/basal subtype, and that it serves as a prognostic indicator of metastasis in this sub-population.

3) We provided the first evidence that the novel GPNMB-targeted therapeutic, CDX-011 was capable of killing breast cancer cells *in vitro* and inducing breast tumor regression *in vivo*.

4) Together, this data prompted a collaboration with Curagen Corp. (now Celldex Therapeutics) and provided the scientific rationale for them to initiate a Phase I/II clinical trial with CDX-011 in patients with metastatic breast cancer in June 2008. This study met its primary end point and final, positive results were reported in June, 2010. A randomized, controlled Phase IIb clinical trial investigating CDX-011 in 120 patients with GPNMB-positive metastatic breast cancer is currently underway.

5) We provided the first report that Osteoactivin is capable of promoting primary tumor growth and is associated with increased angiogenesis. We were the first to identify that the protease ADAM10 is responsible for GPNMB ectodomain shedding in breast cancer cells. Finally, we demonstrated that this ectodomain is capable of promoting endothelial migration.

PREFACE

This is a manuscript-based thesis. It contains portions of two published review articles (peer-reviewed) and three published peer-reviewed original research articles. This thesis is divided into six sections:

1. A general introduction and literature review
2-4. Manuscripts, each with their own preface, abstract, introduction, materials and methods, results, discussion, references, figures and tables.
5. A general discussion of all the results and references
6. Appendices

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LIST OF ABBREVIATIONS

ADAM:	a disintegin and metalloprotease
ADC:	antibody drug conjugate
AJCC:	American joint committee on cancer
AI:	apoptosis index
AP1:	activator protein 1
ATP:	adenosine triphosphate
BLAST:	basic local alignment search tool
BMP:	bone morphogenic protein
CD:	cluster of differentiation
CDK:	cyclin dependent kinase
cDNA:	complementary DNA
Clec:	C-type lectin
CLU:	clusterin
CTGF:	connective tissue growth factor
CXCR:	chemokine, CXC motif, receptor
DC-HIL:	dendritic cell heparin integrin ligand
DCIS:	ductal carcinoma in situ
DNA:	deoxyribonucleic acid
ECD:	extracellular domain
ECM:	extracellular matrix
EGF:	epidermal growth factor
EGFR:	epidermal growth factor receptor

EMT:	epithelial to mesenchmal transition
EndoH:	endoglycosidase H
EPC:	endothelial progenitor cell
ER:	estrogen receptor
ErbB2:	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
Erk:	extracellular signal-regulated kinase
ESR:	estrogen receptor
Fc:	Fragment crystallizable
FGF:	fibroblast growth factor
FGFR:	fibroblast growth factor receptor
Fgr:	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
Fos:	FBJ murine osteosarcoma viral oncogene homolog
G-CSF:	granulocyte colony stimulating factor
GM-CSF:	granulocyte, macrophage colony stimulating factor
GPNMB:	glycoprotein non-metastatic B
GSK3:	Glycogen synthase kinase 3
GTP:	guanosine tri-phosphate
Hck:	hemopoietic cell kinase
hemITAM:	hemi-ITAM
Her2:	Human EGF receptor 2
HGFIN:	hematopoietic growth factor inducible, neurokinin-1 type
IFN:	interferon
Ig:	immunoglobulin
IGF:	insulin-like growth factor

IHC:	immunohistochemical
IL:	interleukin
ITAM:	immunoreceptor tyrosine-based activation motif
Jun:	V-jun avian sarcoma virus 17 oncogene homolog
kDa:	kilodalton
LCIS:	lobular carcinoma in situ
LN:	lymph node
LVI:	lymphovascular invasion
Lyn:	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MBC:	metastatic breast cancer
M-CSF:	macrophage colony stimulating factor
MITF:	microphthalemia-associated transcription factor
MMP:	matrix metalloprotease
mRNA:	messenger RNA
MSH:	melanocyte stimulating hormone
MTD:	maximum tolerated dose
MVD:	microvascular density
Myc:	Avian myelocytomatosis
NSBR:	Nottingham modified Scarff, Blume and Richardson
NPI:	Nottingham prognostic index
NRP:	Neuropilin
OA:	Osteoactivin
OPN:	Osteopontin
ORR:	objective response rate

PARP:	poly-ADP (adenosine diphosphate) ribose polymerase
pCR:	pathological complete response
PCR:	polymerase chain reaction
PECAM-1:	platelet endothelial cell adhesion molecule 1
PFS:	progression-free survival
PlGF:	placental growth factor
PKD:	polycystic kidney disease
PMA:	phorbol myristate acetate
Pmel17:	melanocyte protein 17
PNG:	peptide N-glycosidase F
PR:	progesterone receptor
PTHrP:	parathyroid hormone related protein
QNR:	quail neuroretinal cell
RAA:	arginine, alanine, alanine
Rac:	ras-related C3 botulinum toxin substrate
RANKL:	receptor activator for nuclear factor κ B ligand
Ras:	rat sarcoma
RGD:	arginine, glycine, aspartic acid
RGD:	arginine, glycine, aspartic acid, serine
Rho:	ras homolog gene family
RNA:	ribonucleic acid
RT-PCR:	reverse transcriptase - polymerase chain reaction
SBR:	Scarff, Blume and Richardson

SCP: single cell progeny

SD4: syndecan 4

SH2: Src homology 2

siRNA: small interfering RNA

Smad: mothers against decapentaplegic homolog

Src: v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)

SRS: Src related signature

TF: transcription factor

TGF-β: transforming growth factor β

TN: triple negative

TNBC: triple negative breast cancer

TNF-α: tumor necrosis factor α

TMA: tissue microarray

TNM: tumor, lymph node, metastasis

TUNEL: terminal deoxynucleotidyl transferase-mediated UTP nick end label

UICC: international union against cancer

vcMMAE: valine citrulline monomethyl auristatin E

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

ZA: zoledronic acid

ZAP-70: zeta-chain (TCR) associated

CHAPTER 1 - LITERATURE REVIEW & INTRODUCTION

1.1 Cancer Definition & Global Cancer Burden

Cancer is a disease characterized by abnormal and uncontrolled cell growth. It causes tumors that can expand locally and disseminate systemically. It is not a single disease, but rather a collection of diseases that arise in various tissues throughout the body. The severity of and treatments for cancer vary widely depending on the tissue of origin. Even within specific cancer types, sub-groups of tumors can be defined which are characterized by unique clinical manifestations.

Since 1965, the World Health Organization has studied the global burden of cancer. During this time, the pattern of cancer incidence has shifted from occurring predominantly in western, high resource, industrialized nations to becoming a global disease with the bulk of new cases being diagnosed in people living within developing countries. It was estimated that in 2008 there were more than 12 million new cases of cancer diagnosed, 7 million cancer-related deaths and 25 million people living with cancer [1]. In Canada, there were approximately 171, 000 new cased diagnosed and 75 300 deaths from cancer in 2009 alone, with breast cancer being the most commonly diagnosed among Canadian women [2].

1.2 Normal Breast Tissue and Breast Cancer

1.2.1 Anatomy of the breast

Each adult human breast contains a mammary gland, which is a modified sudoriferous (sweat) gland capable of producing milk. Mammary glands are composed of 15-20 lobes that are interspersed with adipose (fat) tissue. Each lobe is comprised of several smaller

compartments called lobules, and each lobule is comprised of grape-like clusters of alveoli. Alveoli are hollow cavities lined by an inner layer of milk-producing cuboidal epithelial cells, which are lined by an outer layer of contractile myoepithelial cells. Milk that is produced in the alveoli passes into secondary tubules, and then into mammary ducts. Proximal to the nipple, these mammary ducts expand into larger cavities called lactiferous sinuses, which serve as temporary storage spaces before the milk is secreted in the lactiferous ducts that open at the nipple [3,4]. Breast cancers generally arise from two of the distinct functional units of the breast: lobules and ducts – these represent distinct histological types of breast cancer are discussed below in section 1.2.5.

1.2.2 Breast cancer statistics

Breast cancer is the most common cancer affecting women worldwide with an estimated 1.38 million new cases reported in 2008. In the same year, this disease accounted for approximately 458 000 deaths [5]. Relative to other cancers, the percentage of breast cancer patients who survive past five years following diagnosis is high: reaching 85-89% in developed countries and 50-60% in undeveloped countries. Among Canadian women, breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer related deaths: approximately 23,300 new cases and 5,400 deaths are expected in 2010. Among Canadian females aged 30-39, breast cancer is the most common cause of cancer death [2]. Notably, while breast cancer incidence rates in Canada are gradually increasing over time, mortality rates are still continuously but gradually decreasing over time. Indeed, breast cancer mortality rates have decreased by 1.8% between 1996 -2005 in Canada [2]. The reasons for this decrease in mortality from

breast cancer are most likely due to improved detection of smaller palpable tumors, and the use of new systemic therapies with increased efficacy in the adjuvant setting [6].

1.2.3 Breast cancer risk factors

As is the case with many cancers, the risk of developing breast cancer increases with age; however, a number of other breast cancer specific risk factors have also been described. People with a family history of breast and ovarian cancer are at higher risk. Risk increases with the number of first-degree family members affected. Indeed, women with three or more affected members are nearly four times more likely to develop breast cancer than the general population [1]. Hereditary breast cancer affecting young women is most often associated with mutations in BRCA1 or BRCA2 genes [7]. The risk of developing breast cancer is directly related to the number of ovarian cycles a woman experiences, thus the risk of breast cancer increases with early age menarche and late onset of menopause. Similarly nulliparous women and women whose first full-term pregnancy occurs after the age of 40 are also at higher risk [1].

1.2.4 Clinicopathological characterization of breast tumors

Breast cancer is a heterogeneous disease. Given the same treatment regimen, two breast cancer patients might have vastly different clinical outcomes, specifically in terms of metastatic progression and response to chemotherapy. In an effort to treat patients effectively, and to avoid over-treatment of those patients who do not require aggressive chemotherapeutic intervention, clinicians and scientists have described a variety of clinical, histological and molecular characteristics of breast tumors that are useful to sub-classifying groups of breast tumors that share similar clinical characteristics.

Staging of breast tumors: Clinical staging of tumors has evolved dramatically over the past 100 years, as patients have gone from regularly presenting with advanced breast cancer in the past to often presenting with early stage breast cancer in the present day. Currently, use of the TNM system is standard practice: it was first conceived by Denoix in 1943, it is applicable to all solid cancers, and was universally accepted by International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC) in 1987 [8]. The TNM system was originally designed as a simple system with binary measures on three different variables: T –tumor size, N-nodal status, M –distant metastases, allowing for a maximum of 8 different outcomes. However, this system is constantly undergoing revision; to improve diagnostic precision, clinicians have expanded the number of possible measures within each variable. For example, the T stage currently accounts for differences in tumor size (T_1 <2cm; 2cm<T_2<5cm; T_3>5cm) as well as tumor type (ie. Tis = ductal or lobular carcinoma *in situ*; T_{4d} = inflammatory carcinoma). Moreover, assessment of N-stage currently incorporates assessment of microscopic disease in the lymph nodes based on immunohistochemistry (IHC) and RT-PCR based measurements [9]. Currently these TNM criteria are used to classify tumors into eight stages (0, I, II_A, II_B, III_A, III_B, III_C, IV). Pre-invasive cancers (DCIS, LCIS) belong to Stage 0, and small tumors that are confined to the breast are classified as Stage I. Patients with Stage 0 and I breast cancer have excellent prognoses. Stage II tumors have regional lymph node involvement, and Stage III tumors are larger and are locally advanced – these groups of patients have worse prognosis. Stage IV patients can have any T or N status but present with distant metastases and have five-year survival rates lower than 20% [9].

1.2.5 Established prognostic factors for breast cancer

Prognostic factors are quantifiable data about the tumor or the patient that can be used to provide information about the expected outcome of a sub-population of patients with similar defining characteristics – in the absence of therapy.

Age: While advanced age is associated with an increased risk of developing breast cancer, the opposite correlation has been observed with respect to survival of patients with breast cancer [10]. Particularly among early breast cancer patients, younger women are more likely to relapse. In a study of 3601 women, compared to women who were older than 50 at the time of diagnosis, women younger than 40 were 1.8 and 1.5 times more likely to experience loco-regional relapse or distant metastases [11]. Although in many studies, young age stands up as an independent prognostic factor, tumors from young breast cancer patients are more likely to have additional characteristics associated with aggressive disease, such as larger tumor size and increased histopathological grade [10]. Breast cancers that arise in young women are more likely to harbor BRCA mutations and/or be triple negative. These types of breast tumors are particularly aggressive and may explain why young women are more likely to experience recurrence and metastases than older women with breast cancer.

Tumor size: Tumor size, defined as the maximal size of the invasive component of the primary tumor [9], has long been directly correlated with relapse-free and overall survival rates. Patients with large invasive breast tumors have poorer metastasis-free and overall survival rates when compared to those who have small tumors at the time of diagnosis [12]. In a study published in 1990, it was reported that women with tumors <10mm, 11-13mm, 14-16mm, and 17-22mm in diameter had 20-year survival rates of 88%, 73%, 65%, and 59%, respectively [13].

Lymph node status: The presence of cancer cells in the axillary lymph nodes (LN) is a prognostic factor for subsequent relapse. Indeed, nodal status has traditionally been defined as the most powerful prognostic indicator of breast cancer recurrence [14]. The average 10-year survival rate is 75% for node-negative patients, but only 25-30% for node-positive patients [15]. The number of involved LN serves as a more precise prognostic factor than simply the presence or absence of LN involvement: It has been reported that breast cancer patients with 1-3 involved LN experience shorter two-year survival rates than node-negative patients (51% vs. 61%, respectively), while women with 4 or more positive nodes had the worst outcome, with only 40% of patients surviving after two years [16]. It should be noted; however, that the accuracy of this prognostic factor is highly dependent on how it was measured. Indeed, clinical detection of LN involvement has been shown to be highly inaccurate, and histopathological analysis of LN is necessary for LN status to be a reliable prognostic factor [17]. It has been suggested that, because the risk of LN involvement increases with larger tumor size, it is a time-dependent variable, meaning that the longer a tumor is present, the greater the likelihood for LN involvement [18]. However, this is now thought to be too simplistic a view; tumor growth and risk of LN involvement will increase with time, but are also dependent on other proliferative, apoptotic, and invasive characteristics of the tumor [12].

Histological grade: Breast cancer grade is based on three histologically evaluated characteristics: mitotic count, nuclear atypia/pleiomorphism and the architectural arrangement of cells (tubule formation). Today, the most commonly used grading scheme is that which was originally devised by Scarff, Blume and Richardson (SBR) and modified by recommendations from the Nottingham/Tenovus study (NSBR) [17,19]. The Nottingham modification improved upon the SBR grading system, which had been

criticized for having low intra-observer reliability, by introducing semi-quantitative measures of these three characteristics [20]. The NSBR grade classifies tumors as Grade I - well differentiated, Grade II – moderately differentiated or Grade III - poorly differentiated. Patients with poorly differentiated, grade III tumors have the worst prognosis with a 45% 10-year survival rate, whereas patients with well differentiated grade I tumors have the best prognosis with an 85% chance of surviving ten years past diagnosis [12].

Histological type: Breast tumors are notoriously heterogeneous and present as a wide variety of histological types; indeed, more than 18 distinct histological types can be applied to breast tumors [17]. Two major classes are ductal and lobular – so named for the architectural compartment of the breast from which they are derived. However, these types behave similarly and are not useful prognostic indicators [17]. One histologically defined distinction that does carry strong prognostic value, occurs between non-invasive (carcinoma in situ) and invasive (infiltrating) carcinomas. This distinction is drawn based on whether malignant cells are confined by, or have invaded the basement membrane. Invasive ductal carcinomas are the most common histological type and they comprise 75-80% of all mammary carcinomas [21].

Lymphovascular invasion: Lymphovascular invasion (LVI) is a term used to describe tumor emboli present in endothelial-lined spaces within the tumor. The endothelial cells lining these spaces may form either lymphatic or blood vessels. Evidence of LVI is controversial as a prognostic factor, as some studies show that it is strongly and independently associated with an increased risk of death [22,23]. However, others have found that, while it may predict recurrence and survival in univariate analyses, LVI does not hold up as an independent prognostic factor in multivariate analyses [24,25,26].

Nottingham prognostic index: None of the prognostic factors listed above are solely sufficient to accurately identify sub groups of patients with zero or 100% likelihood of relapse. Thus, the Nottingham Prognostic Index (NPI), which combines tumor size, nodal status, and histological grade (NSBR), has been designed to more accurately identify those patients who will or won't relapse [14]. The NPI value is calculated using the following formula: NPI = LN stage (1-3) + Histological grade (1-3) + Tumor size (cm)*0.2. Using this formula, six groups, with cut-off values of 2.4, 3.4, 4.4, 5.4, 6.4 and 6.8 were defined. In a set of 2238 patients who were diagnosed between 1990-1999 and had access to adjuvant systemic therapy, the excellent prognosis group had a ten-year survival rate of 96% whereas only 38% of patients categorized as very poor prognosis survived more than ten years after their diagnosis [27]. Despite the strong prognostic value of this index, it has been proposed that its accuracy would be even further improved by including additional prognostic data such as vascular invasion, Her2 and basal phenotype [14,22].

1.2.6 Emerging prognostic indicators

Vascular Density: Vascular density is an imperfect measure of tumor angiogenesis. It is based on a quantitative assessment of the number or percentage of vessels present within a tumor, but does not address the functionality of these vessels. The correlation between high microvascular density (MVD) and poor outcome was first put forth in a study from Judah Folkman's group in 1991: the authors assessed Factor VIII staining in 49 invasive breast tumors, and found that for each increase in 10 vessels per 200X field of view, there was a corresponding 1.17 fold increase in risk of developing metastases [28]. These trends were reiterated in a larger (n=165) follow-up study [29].

Since these initial observations were published, numerous studies using a wide variety of markers of vascular density (ie. Factor VIII, CD31 (PECAM-1), CD34, von Willebrand factor and CD105 (Endoglin)) have substantiated the prognostic utility of MVD for breast cancer recurrence and survival times [30].

Factor VIII, CD31 and CD34 are pan-endothelial markers that may be more readily expressed in larger vessels compared to microvessels [31]. CD105 (endoglin) on the other hand, tends to be more highly expressed in vessels undergoing neoangiogenesis and in tumor vessels relative to inactive vessels or non-tumoral vasculature. Recent evidence suggests that CD105 as a marker for neoangiogenesis may have improved prognostic value over CD31, particularly among lymph node-negative patients [32]. While numerous studies have shown that microvascular density can serve as an independent prognostic marker for recurrence and survival in breast cancer, its clinical utility is limited by a lack of universally recognized standardized techniques for detection and quantitative assessment and interpretation of staining [33].

Proliferation: There is a vast array of methods used to assess proliferation in breast tumors that include, but are not limited to: mitotic index, S-phase fraction, cell cycle associated nuclear antigen IHC, and cyclin E IHC [34]. As was the case with vascular density, numerous groups have shown that proliferation can serve as an independent prognostic indicator of recurrence, but its clinical utility is limited by the lack of standardized techniques, poor reproducibility and labor intensive quantification methods [34]. Of these methods, mitotic index is routinely used clinically in classification of histological grade (see section 1.2.5): higher mitotic indices contribute to higher histological grade [35], and nuclear antigen IHC methods are often used in basic and clinical research [35]. Of the nuclear antigen IHC methods, staining for the nuclear

antigen Ki67 – which is expressed during G1, S, G2, and M-phase but not in senescent G0 cells [36] – tends to have an especially high degree of correlation with other markers of proliferation and in numerous studies. Ki67 is emerging as a potential marker to differentiate luminal A and luminal B molecular subtypes (discussed below in section 1.2.7) based on histological methods [37]. High % Ki67-positivity correlates with poor outcome and stands up as an independent prognostic indicator in multivariate analyses in breast tumors [34,35,37,38]. One recent study of 570 breast tumors suggested using a cut-off value of 15% Ki67-positive tumor cells to predict clinical outcome; however, definitive classification schemes for high, intermediate and low Ki67 groups have not yet been definitively established [35,38].

Apoptosis: The apoptotic index (AI) of a tumor, is a measure of tumor apoptosis, and can be defined by either the percentage of apoptotic cells in a tumor, or the number of apoptotic cells within a given area. Methodological techniques used to quantify AI include: histological light microscopy methods or by employing special staining techniques which are based on *in situ* labeling of fragmented DNA, such as the terminal deoxynucleotidyl transferase-mediated UTP nick end label (TUNEL) assay [39]. Apoptotic index is higher among tumors >2cm than it is among smaller tumors [40] and somewhat counter-intuitively, histological grade III tumors have AI that are nearly twice as high as Grade I tumors [41]. While increased apoptosis was shown to correlate with decreased survival in 3 independent studies, AI did not stand up as a prognostic indicator in multivariate analyses in any of these studies [39]. Thus, while many chemotherapeutic agents work by inducing tumor cell apoptosis, it is questionable whether AI is a useful clinical tool for breast cancer prognosis.

1.2.7 Molecular subtypes as predictive factors for breast cancer

Predictive factors are quantitative measures that can be used to predict response to a given therapy. There are currently 3 receptors that are routinely assessed in the management of breast cancer: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (Her2). Receptor expression is used to determine the molecular subtype of a tumor. The simplest categorization scheme delineates 3 subtypes: ER/PR-positive, Her2-positive, and triple negative. Knowledge of molecular subtype is necessary to determine treatment options and useful in predicting clinical outcome. In addition to immunohistochemically defined subtypes (above), intrinsic molecular subtypes have been defined by gene expression profiling – these intrinsic subtypes, for the most part, overlap with immunohistochemically defined subtypes but are useful for more precisely sub-categorizing breast tumors in clinically meaningful ways.

Estrogen and progesterone receptors: ER and PR are nuclear hormone receptors. There are two ER isoforms: α and ß, encoded by the ESR1 and ESR2 genes, respectively. The receptor exists as either homodimer or a heterodimer. ERα and ERß have opposing actions in response to estrogen in breast cancer: ERα promotes proliferation whereas ERß promotes differentiation and inhibits proliferation [42,43]. ERα is the isoform that is predominantly expressed in breast cancer cells [43]. Ligand binding induces receptor dimerization, nuclear translocation, binding to estrogen-response elements in DNA and subsequent transcription of target genes [44]. The progesterone receptor is one of these transcriptional targets and thus is expressed concomitantly with ER in the vast majority of ER-positive breast tumors. It is rarely expressed in ER-negative tumors, with approximately 1.5% of all breast tumors being characterized as ER-negative/PR-positive

[44,45]. However, when ER-positive tumors lack PR expression (~12.6% of all breast tumors [45]), it is indicative of a defective ER signaling pathway: these tumors are less responsive to endocrine therapy and have worse clinical outcome than tumors that are positive for both ER and PR [44]. ER is expressed in nearly 75% of all breast cancers: it is expressed in 65% of tumors from women younger than 50 and in more than 80% of tumors from women older than 50 at time of diagnosis [46]. ER-positivity is associated with the luminal A and luminal B intrinsic molecular subtypes (discussed below) [44]. ER expression alone has limited long term prognostic value but is strongly predictive of whether patients will respond to estrogen-targeted therapies. There are two main types of estrogen-targeted therapies: 1) anti-estrogens (ie. tamoxifen), which inhibit the interaction of ER with estrogen and 2) aromatase inhibitors (ie. anastrozole, letrozole), which block estrogen hormone synthesis; the latter of these types are particularly effective in post-menopausal women [47]. Adjuvant tamoxifen therapy was associated with a 17.8% decreased risk of breast cancer related death in a 15-year follow-up period [48].

Her2: Her2 and its murine orthologue ErbB2 are oncogenes belonging to the human epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, it is overexpressed in (15-25%) of breast cancers [49,50]. Her2 overexpression is often due to amplification of the Her2 amplicon, which consists of the q12-q21 region on chromosome 17 [51]. More than 20 genes, in addition to Her2 are found within this amplicon, and a number of these genes are thought to contribute to breast tumor progression [51]. Her2 activation in breast cancer promotes angiogenesis, metastasis, proliferation and cell survival [52] and its expression is associated with poor clinical outcome [53]. There are two Her2 targeted therapies currently approved for use in breast cancer: trastuzumab (Herceptin®) and lapatinib, a humanized monoclonal antibody specific for Her2 and a

small molecule Her2/EGFR-targeting kinase inhibitor, respectively and only trastuzumab is approved for use in the adjuvant (following surgical removal of the tumor and or breast) setting [54]. The efficacy of trastuzumab in the adjuvant setting has been validated by several Phase III trial and was shown to significantly decrease the risk of developing distant metastases and increase overall survival from breast cancer [50]. For example, 4-year survival rates were 92.6% for Her2-positive patients who received trastuzumab plus chemotherapy in the adjuvant setting and 89.4% for those who received chemotherapy alone [50].

Triple-Negative status: Triple negative (TN) breast tumors are defined as those that lack expression of ER, PR, and Her2. Epidemiologically, patients with triple negative breast cancer (TNBC) are more likely to be African American, with (26-29% of African American women presenting with TNBC compared to 10.8-16% of Caucasian women [55]. Patients with TNBC tend to be younger than patients with non-TNBC, with a median age of diagnosis of 54 years old for TNBC and 60 years of age for non-TNBC [56]. Currently, this sub-group of tumors is receiving a lot of attention because they are associated with a high propensity to metastasize to distant sites within a few years of initial diagnosis [57,58,59,60,61,62,63]. Moreover, because these tumors lack expression of the estrogen, progesterone and Her2 receptors, they are not suitable to endocrine or Her2 targeted therapies. However, recent data has shown that patients with TNBC are more likely to respond to traditional chemotherapies in the neo-adjuvant setting [64]. Furthermore, TNBC patients who had achieved a pathological complete response (pCR) to chemotherapy in the neo-adjuvant setting were no more likely to relapse then non-TNBC patients who had achieved pCR [64]. However, TNBC who had residual disease following neo-adjuvant chemotherapy were significantly more likely to relapse compared

to non-TNBC patients with residual disease [64]. Thus, TNBC status itself may serve as a prognostic factor for clinical outcome, but in the context of pCR it is emerging as an important predictive factor for response to chemotherapy as well.

Molecular subtype: The first use of gene expression profiling to identify distinct breast cancer subtypes was published a decade ago [65] and ushered in a new age of breast cancer research [66]. This study identified an intrinsic gene list which consisted of 496 genes and identified several breast tumor subtypes including normal-like, luminal/ER+, Erbb2-overexpressing, and basal [65]. The intrinsic gene list and molecular subtypes were later validated but slightly refined in larger datasets [66,67,68,69,70]. These molecular subtypes have distinct clinical outcomes and unique patterns of metastatic spread [67,68,69,71]. While the molecular subtypes categorized by the intrinsic gene list are arguably the most widely accepted, several other gene expression signatures have been described to sub-classify breast tumors and predict clinical outcome [72], including a 70-gene poor prognosis signature [73,74] which has been developed into Mammaprint ®, and is currently being used in the clinic to help guide treatment strategies [72].

Currently there are two commercially available gene expression assays available for clinical use: Oncotype DX® and Mammaprint ®. These assays are designed to provide additional prognostic and predictive information for patients whose tumors are characterized as intermediate-risk based on traditional clinical and histopathological analyses [72]. The Oncotype DX assay assesses expression of 21 genes based on RT-PCR analysis, as such, paraffin embedded tumor tissues are eligible for analyses. The genes tested encode proteins involved in estrogen and Her2 signaling pathways as well as invasion and proliferation. The assay divides tumors into low, intermediate and high risk

groups which are respectively associated with 2.8-6.2%, 10.7-17.8%, and 15.5-19.9% risk of death from breast cancer within 10 years [72]. The Mammaprint assay uses microarray based technology to assess expression levels of 70 genes, and as such requires fresh frozen tissue samples. This assay classifies patients as having a good or poor prognosis, which correspond with an 85.2% or 50.6% chance of remaining distant metastasis free at 10 years post-diagnosis, respectively. In a large meta-analysis comprising 1637 patients, patients classified as poor-prognosis derived benefit from chemotherapy whereas good-prognosis patients did not [72].

1.3 Angiogenesis

Angiogenesis is the process by which new blood vessels are formed from existing vessels by means of increased proliferation and sprouting of new branches. It differs from vasculogenesis - which commonly occurs during embyogenesis – when new blood vessel formation occurs *de novo* via recruitment of endothelial progenitor cells (EPCs) [75]. Both angiogenesis and vasculogenesis are concurrently involved in breast tumor neovascularization [75]; indeed, EPCs may constitute as much as 12% of newly formed vessels in breast tumors [75], but often the process is referred to simply as angiogenesis. Experimental evidence suggests that tumors can grow in an avascular phase up to a diameter of ~1-2mm, at which point existing local vasculature is no longer sufficient to supply the tumor, and it must begin recruiting its own blood supply by forming new blood vessels [76]. This point is referred to as the "angiogenic switch", meaning that the tumor will begin to produce a series of effector molecules that favor the formation of new vessels to initiate angiogenesis [77]. Under physiological conditions, the formation of

new blood vessels is held in check by tightly controlled microenvironmental regulation of pro-angiogenic (ie. VEGF, FGF-2, EGF, MMPs, angiopoietins) and anti-angiogenic (ie. thrombospondin, angiostatin) factors. In the case of a tumor undergoing an angiogenic switch, the balance is shifted in favor of more pro-angiogenic molecules.

Tumor vasculature differs form normal vasculature in that in tends to be more, dilated torturous, irregularly shaped and has more dead, and has more fenestrations, making it more leaky. This "leaky-ness" of tumor vessels not only hinders efficient delivery of systemic chemotherapeutic agents throughout the tumor, but also facilitates access to the circulation by disseminating tumor cells [76]. Angiogenic tumor vessels are formed following detachment of pericytes (supporting cells) and degradation of the surrounding extracellular matrix via proteases (ie. MMPs), this allows endothelial cells to sprout off of the existing vessel and migrate through the degraded matrix towards angiogenic stimuli [76]. Migrating cells can then undergo proliferation to produce endothelial cells, which will anastamose and form lumens to result in functional neovasculature [76,78].

Angiogenic growth factors: Vascular endothelial growth factor A (VEGF) is the best known of pro-angiogenic molecules, its expression is induced by hypoxia, a condition that is commonly found in the tumor microenvironment [76,77]. VEGF belongs to a family of growth factors that also includes VEGF-B, VEGF-C, VEGF-D, VEGF-D and placental growth factor (PlGF) [77]. These function by binding, with varied specificity, to tyrosine kinase VEGF receptors 1, 2, and 3, which in turn activate down stream signaling pathways the culminate in cellular responses such as increased proliferation and migration [77]. Neuropilin-1 (NRP-1) is transmembrane glycoprotein, expressed on endothelial cells that can act as a co-receptor for VEGF to potentiate

VEGFR-2 signaling [79]. Fibroblast growth factors, particularly FGF-2, have also been implicated as key mediators of tumor angiogenesis [80].

1.4 Metastasis

Metastasis is the process by which cancer cells leave the primary tumor and disseminate throughout the body to form secondary tumors in other organs: it is the most common cause of death from breast cancer [81,82]. The metastatic cascade refers to a series of barriers that a cancer cell must overcome in order to produce a metastatic foci. These include: growth in the primary tumor, acquisition of locally invasive characteristics, vascular invasion, survival in circulation, extravasation into secondary metastatic sites, survival in the metastatic environment and responsiveness to local growth signals in order to progressively grow in that site [83]. Current views of metastatic dissemination are challenging the historical view of disease progression to metastasis.

1.4.1 Theories of metastatic progression

There are two main models of metastatic progression that are currently accepted in the literature: the classical model is the linear progression model and the more recent model, which represents somewhat of a paradigm shift, is the parallel progression model [84]. In the linear progression/late dissemination model, a normal epithelial cell acquires mutations that inactivate tumor suppressor genes and/or activate oncogenes, which serve to promote survival and uncontrolled proliferation. Increased proliferation may lead to the acquisition of subsequent mutations in progeny cells, the result of which is a tumor with a variety of mutations heterogeneously distributed throughout discrete sub-populations in the tumor. Some of these mutations are associated with alterations in gene-expression that

facilitate invasion and dissemination, and it is these cells that are capable of distal metastasis [84,85,86]. In this model, acquisition of malignant mutations is linked to large tumor size and suggested to occur at later stages in tumor development [84,87].

The second model has been termed the parallel progression/early dissemination model and has gained favor recently, but dates as far back as the 1950's [88]. This model rejects the notion that tumors must reach a defined size before acquiring the ability to disseminate, and posits that dissemination occurs long before the primary tumor is detected [84,89]. However, both models agree that cancer progression depends on clonal expansion of the "fittest" cells. The idea that tumor cells disseminate early on is supported by the notion that in rare cases patients with very small, early stage primary tumors (T1) will present with metastases at the time of diagnoses [84] and that metastatic cancer of unknown primary origin represents 5-10% of all cancer diagnoses in the United States and Europe [90,91]. This model is further supported by recent research showing that "normal" untransformed mouse mammary epithelial cells can, following tail vein injection, exist in the lung as clusters of epithelial cells for at least 16 weeks: proving that an accumulation of sequential mutations is not necessary for any of the steps in the metastatic cascade beyond vascular invasion, save malignant outgrowth in the metastatic site [92].

Another recent theory known as the "pre-metastatic niche" is based on observations made in the past decade and has radically changed our view of metastatic colonization. This theory holds that secreted factors such as: S100A8, S100A9 and OPN [93,94,95] are produced either by the primary tumor or stromal cells in the metastatic site, which serve to "prime" tissues for later metastatic tumor engraftment. These factors facilitate mobilization and localized recruitment of bone-marrow derived cells that can

liberate pro-inflammatory cytokines such as TNF-α. These inflammatory cytokines "prime the soil" by activating endothelial cells and inducing MMP expression to promote subsequent cancer cell colonization and outgrowth [96].

A key observation in the field of cancer metastasis is that cancers of a particular primary organ tend to disseminate and preferentially colonize specific secondary organs. For example: breast cancer most commonly metastasizes to bone, followed by lung, brain, and liver [97]; whereas colorectal cancer most frequently metastasizes to liver then lung and rarely to bone and brain [98,99]. Stephen Paget first offered an explanation for this observation in 1889. His seminal "seed and soil" hypothesis suggested that in order for a secondary metastasis to grow, the "soil" (ie. metastatic environment) must be sufficiently able to nourish the "seed" (ie. a disseminated cancer cell) [100,101,102]. That is to say, for example, that the bone microenvironment may provide sufficient growth signals to facilitate breast cancer outgrowth but these signals are less effective at promoting colon cancer outgrowth. This hypothesis was challenged nearly 40 years later when James Ewing claimed that differences in metastatic colonization by certain cancers could be explained by the anatomy of the local circulatory system. Thus, cancers will metastasize to those secondary sites to which they have the greatest degree of access via the vasculature and blood flow patterns [83,101]. This is best exemplified by the high propensity of colon cancers to metastasize to liver, which is the first organ they encounter following intravastation into the portal circulation. It was later shown that while local recurrences correlate with increased perfusion rates, the formation of distant metastases could not be completely explained by anatomical vascular access patterns, and Paget's "seed and soil" hypothesis prevailed and remains a guiding principle in the study of organ specific metastasis [100].

Recently, there has been an explosion of research aimed at identifying the genes expressed by breast cancer cells that predispose breast cancers to metastasize to secondary sites, including lung [103,104], liver [105,106], brain [107,108,109,110], and bone [109,111,112,113,114]. The goals of this research approach are to 1) identify gene-subsets expressed by breast cancer cells (ie. seed factors) that predict the likelihood that a cancer will metastasize to a specific organ, 2) characterize molecular mechanisms responsible for promoting organ-specific metastasis and 3) to facilitate the development of targeted therapies for metastatic breast cancer. These studies have made great strides in improving our understanding of the molecular mechanisms governing metastasis and, as discussed below, are yielding results that are beginning to effect clinical practice.

1.4.2. Bone Metastases

Bone is the most common site of cancer metastasis, and breast cancers in particular, metastasize to bone with high frequency; indeed, 65-75% of patients with disseminated breast cancer will suffer from bone metastases [115]. Amongst patients with recurrent breast cancer, those with estrogen responsive tumors are nearly twice as likely to bone metastases when compared to those whose tumors lack expression of the estrogen receptor (ER) [116]. Once breast cancer has spread to bone, it is generally considered to be incurable. While bone metastases contribute significantly to the morbidity of the disease they are rarely the cause of breast cancer deaths. However, serious complications are associated with breast cancer relapse to bone, including chronic bone pain, fracture, spinal cord compression and hypercalcemia, which lead to a dramatic decrease in the quality of life for breast cancer patients [115].

Bone metastases can be divided into two broad categories; osteolytic metastases that

are associated with bone destruction and osteoblastic metastases that are characterized by new bone formation. Bone metastases can be predominantly osteolytic or osteoblastic, or be mixed with features of both types [117,118]. However, breast cancer and multiple myeloma are more frequently associated with osteolytic lesions, whereas prostate cancer predominately induces the formation of osteoblastic metastases [118,119]. The factors that govern the ability of invading cancer cells to disrupt the balance between bone formation and destruction in favor of one extreme are now being identified and characterized.

The two principle cell types responsible for remodeling bone are the osteoclasts, which remove or resorb bone, and the osteoblasts that mediate new bone formation through a process of extracellular matrix deposition and subsequent mineralization [120]. Each cell type arises from distinct lineages; osteoclasts are terminally differentiated multinucleated cells of hematopoietic origin [121] whereas osteoblasts arise from mesenchymal stem cells that can also produce myoblasts, chondrocytes and adipocytes [122]. Although these two cell types arise independently from distinct precursors and regulate opposing functions, the differentiation and activity of osteoclasts are heavily influenced by osteoblast-derived factors. The best example of this is the ability of RANKL, a critical factor produced by immature osteoblasts, to promote osteoclast differentiation and function under both physiological and pathological conditions [123,124]. The arrival of breast cancer cells within the bone microenvironment disrupts the normal regulatory networks that exist between osteoblasts and osteoclasts and tips the homeostatic balance toward increased osteoclast activity, resulting in excessive bone resorption. It is generally accepted that breast cancer cells cannot directly resorb bone, and must exert this effect indirectly through the action of osteoclasts [125,126]. A number

of studies have investigated the ability of breast cancer cells to induce osteoclast differentiation using a variety of *in vitro* systems [127]. The consensus that has emerged from these studies is that osteoblasts are important mediators of breast tumor cell-induced osteoclastogenesis [128]. Breast cancer cells can stimulate immature osteoblasts to produce a variety of factors, including RANKL, prostaglandin E and IL11, which in turn stimulate osteoclast differentiation from monocyte precursors [128,129,130]. Indeed, PTHrP has been demonstrated to be one such tumor-derived factor that is capable of upregulating RANKL expression in osteoblasts [131] and is important for the establishment of osteolytic breast cancer metastases *in vivo* [132]. However, mounting evidence suggests that additional factors can also function to induce osteolysis at metastatic sites in both a PTHrP-dependent and independent manner [133]. In summary, there exists a complex web of interactions among breast cancer cells and host cells resident in the bone that serves to enhance the formation of bone metastases (Fig. 1).

Gene-expression profiling to identify bone metastasis genes: In recent years, gene-expression profiling has become a standard technique used to identify genes that are deregulated in cancer. By coupling gene-expression profiling with pre-clinical mouse models of breast cancer metastasis to bone, a better understanding of the various stages of metastatic progression has been realized. Some of these approaches involve the derivation of sub-populations that preferentially spread to the bone, starting from heterogeneous cultures of breast cancer cells. These cell populations are isolated directly from established bone metastases that have formed following injection of the parental population into the left cardiac ventricle or the mammary fat pads of mice. Successive rounds of *in vivo* selection result in breast cancer cells that aggressively metastasize to the bone when compared to the original cell population. These approaches have facilitated

the identification individual molecular mediators of breast cancer bone metastasis, including interleukin-8 [134]. Sets of genes that work cooperatively to promote bone metastasis, including CXCR4, MMP-1 and the TGF-β regulated genes, CTGF and IL-11, have also been identified and functionally validated in this manner [111,112,135]. The *in vitro* isolation of single cell progeny (SCP) yielded MDA-MB-231 derived populations with vastly variable bone metastatic phenotypes *in vivo* [136]. Importantly, aggressively bone metastatic SCPs had largely overlapping gene-expression profiles with aggressively bone metastatic populations that were derived by *in vivo* selection [112,136]. These studies, in addition to identifying novel mediators of metastasis, provided insights into the nature of the metastatic process.

To extend the findings of these *in vivo* models of bone metastasis, several groups have performed gene-expression profiling on primary human breast tumors to determine whether bone metastasis related signatures could be identified in clinical samples. Minn and colleagues found that their 50-gene bone metastasis signature derived from MDA-MB-231 SCPs was not able to distinguish between tumors that gave rise to bone metastases from those that did not. However, they did find that many of the genes in their signature were up-regulated in primary tumors that formed bone metastases compared to tumors gave rise to metastatic lesions in visceral organs [136]. Smid *et. al.* profiled 107 breast tumors and identified a 31-gene signature that was capable of identifying 100% of the 35 tumors that were derived from patients that went on to develop bone metastases. However, the specificity of this signature was only 50%, meaning that it also incorrectly predicted that an additional 35 breast tumors would produce bone metastases, when they were in fact derived from patients that did not relapse to bone [137]. In the most recent

attempt to identify gene networks present in primary breast tumors that are capable of predicting bone metastasis, Zhang and colleagues examined the gene expression profiles of 615 primary human breast tumors for gene expression signatures that act as surrogate indicators for the activation of specific signaling pathways [111]. A Src-Related Signature (SRS), consisting of a group of genes that are differentially expressed when Src is active, was found to be present in breast cancers that relapsed to bone. In fact, the SRS proved to be more effective than ER status at identifying breast cancer patients who would develop bone metastases [111]. The authors of this study went on to implicate Src as a key causal molecule in the formation of breast cancer bone metastases. The translational relevance of these findings is highlighted by the fact that Dasatinib, a Src inhibitor, is currently being investigated in two Phase II clinical trials (NCT00410813, NCT00566618) designed to assess its efficacy against breast cancer bone metastasis.

Treatments for bone metastasis: Currently, a number of strategies are employed in the clinic to manage bone metastasis from breast cancer. These include surgery, radiation, chemotherapy and treatment with anti-resorptive drugs known as bisphosphonates. Treatment options such as radiotherapy or orthopedic surgery are used to palliate pain and repair metastasis-related fractures and can lead to increased mobility and longer survival times in patients with otherwise good prognoses [138]. However, neither of these strategies are curative as they do not target the underlying process of excessive bone resorption, which drives the formation osteolytic bone metastases and leads to bone pain and fracture [139].

Bisphosphonates are a class of drugs that target the process of bone resorption by inhibiting osteoclast function. Early generation bisphosphonates (i.e. clodronate, etidronate) lack nitrogen and adhere to bone, where they are metabolized by osteoclasts.

Metabolic products include cytotoxic ATP analogues that interfere with mitochondrial membrane potential and lead to osteoclast apoptosis [115,140]. Later generation, nitrogen-containing bisphosphonates (i.e. pamidronate, ibandronate and zoledronate) inhibit osteoclasts by a different mechanism. They inhibit an enzyme called farnesyl diphosphonate synthase that is required for post-translational prenylation of several GTPases; including Ras, Rho and Rac. These GTPases are required for proper cellular vesicle transport, without which, osteoclasts cannot form the tight sealing zones or ruffled borders at the bone surface that are required for resorption [115,140].

In recent years, bisphosphonates - particularly the most potent of the nitrogen-containing bisphosphonates, zoledronic acid (ZA) [140,141,142,143,144] - have been ascribed the ability to inhibit the formation of bone metastasis, independent of their effects on osteoclasts. Results of a recent Phase III clinical trial revealed that addition of ZA to endocrine therapy, in the adjuvant setting, improves disease-free survival but not overall survival in premenopausal patients with estrogen-responsive early breast cancer [145]. In this study, ZA treatment protected against locoregional relapse to a similar extent as it did against bone relapse. In the metastatic setting, ZA is associated with a reduction in the number of skeletal related events when compared to placebo, or earlier generation bisphosphonates [146].

While numerous targeted therapies are currently being developed for the treatment of bone metastases [147], denosumab – a RANKL targeted monoclonal antibody – has progressed the furthest. It has proven superiority over ZA in a Phase III clinical trial where it decreased skeletal-related events by 18% (HR=0.82; P<0.0001)[148]. Identification and characterization novel therapies for bone metastases is an are of active

investigation that is likely to yield numerous additional therapeutic options in the coming years.

1.4.3 Soft tissue and visceral metastases

Excluding lymph nodes, the liver, lung and brain are the most common sites of breast cancer metastases following bone [97]. Prevalence of site-specific metastases varies by molecular subtype [71,149]. In a study of 3726 breast cancer breast cancer patients [149], breast tumors were subdivided into 7 groups based on molecular subtype: luminal A (ER/PR+, low Ki67), luminal B (ER/PR+, high Ki67), Her2+ (ER/PR+), Her2+ (ER/PR-), basal-like (ER-/PR-/Her2-; EGFR+ or CK 5/6+) and non-basal TN (ER-/PR-/Her2-/EGFR-/CK 5/6-). Those patients with TNBC - either basal or non-basal - were significantly more likely to develop brain and lung metastases and less likely to develop bone metastases, when compared to patients with luminal A breast cancer. Interestingly, patients with Luminal B breast cancer had higher incidence of metastases to all sites compared to patients with Luminal A breast cancer, suggesting that higher in proliferation rates do not preferentially favor metastatic out growth in any particular site [149].

Moreover, within a particular subtype the initial site of metastasis is associated with differences in overall survival times. For example, among TNBC patients, those whose first site of metastatic involvement is the lung have the shortest survival time – 18 months versus 26.9, 38.2 and 50.8 months for those whose first site of metastasis is brain, liver and bone, respectively [150]. A number of factors have been implicated in favoring metastasis to these secondary organs, including: cyclooxygenase-2, heparin binding epidermal growth factor, and the α-2,6-sialyltransferase ST6GALNAC5 for brain [108];

claudin-2 for liver [105]; and angiopoietin-like 4, inhibitor of DNA binding 1, vascular cell adhesion molecule 1, epiregulin, MMP-1, and MMP-2 for lung [104,151].

Treatments for metastatic breast cancer (MBC): Approximately 6% of newly diagnosed breast cancer patients will present with metastatic disease and 40% of those who initially present with localized disease will eventually progress to metastatic disease [152]. Metastatic breast cancer is considered to be incurable [152]. The good news is that survival times of patients with MBC are slowly but steadily improving [153] - the risk of death from MBC is decreasing by 1%–2% each year [154]. This is due, in large part, to improvements in systemic therapies given in the metastatic setting [155], and while surgical intervention for MBC is also gaining credibility as a treatment option, only 1-3% of patients meet the criteria (ie. very limited metastatic disease) necessary to warrant surgical resection [155]. In terms of chemotherapeutic options, many of the regimens that are employed in the adjuvant setting are also used in the metastatic setting, but with limited efficacy. Partially because previous exposure to chemotherapy in the adjuvant setting is often associated with acquired resistance to these therapies in the metastatic setting [152]. Another explanation for the relative ineffectiveness of targeted therapeutics in the metastatic setting arises from the fact that receptor status (ie. Her2, ER, PR) is not necessarily static throughout tumor progression: differences in receptor status between primary tumors and metastatic lesions are present in 20-40% of patients [156]. Thus, treatment decisions based on receptor status in the primary tumor may prove ineffective for metastatic breast.

Thus, there is much interest in developing novel therapeutics that are effective in the metastatic setting. Indeed, there are a number of novel therapeutics that have recently been approved for the treatment of metastatic breast cancer, including ixabepilone, a

microtubule stabilizing agent, which when used in combination with capecitabine (a pro-drug that is converted to 5-flurouracil by tumor cells) increases overall survival times and improved median progression-free survival from 4.2 months (capecitabine alone) to 6.2 months [157]. The practice of combining two or more therapies, for which there is evidence of synergistic activity, is becoming more popular, but depending on the combination, can be plagued by increases in toxicity that dramatically reduce quality of life [152]. Nonetheless, there are a number novel combination regimens and single-agent therapies in Phase II and Phase III clinical trials, (ie. PARP inhibitors, histone deacetylase inhibitors CDX-011 (discussed in section 1.5.8), some of which hold great promise for patients with TNBC.

1.4.2 Mouse models of breast cancer metastasis

The complex nature of cancer progression necessitates the use of multiple model systems to fully understand this process. *In vitro*, cell culture based assays can provide a wealth of information regarding the molecular underpinnings of metastatic processes such as migration, invasion and adhesion; however, the full complexity of the tumor microenvironment, which involves dynamic interactions between numerous cell types, cannot effectively be re-capitulated *in vitro*. And while the analysis of primary human tumors allows us to assess the relevance of our findings from our *in vitro* models, they represent only a static snapshot of the disease. Mouse models afford us an opportunity to validate results from *in vitro* assays and allow us to model the dynamic nature of cancer progression *in vivo*.

Experimental and spontaneous models of metastasis: *In vivo* metastasis assays can be classified as "experimental" or "spontaneous". In experimental metastasis assays,

cancer cells are injected directly into the arterial or venous circulatory systems, thereby circumventing the earliest requirements of metastasis. The site of secondary metastasis is highly dependent upon the route of injection [158], thus experimental metastasis assays are useful tools to study organ-specific metastases. Cancer cells injected into the lateral tail vein of mice arrive, and due to size restriction of pulmonary capillaries, most become trapped in the lung [159]. To produce liver metastases, cancer cells can be injected intra-splenically, many of which will drain through the portal vein into the liver [160]. Cells injected into the arterial circulation via the left cardiac ventricle are disseminated throughout the body before the blood returns to the lung – this is particularly advantageous model for the development of bone metastases. Finally, injecting cells directly into the carotid artery, which delivers blood to the brain and the face, is the most efficient method to produce brain metastases [158]. Experimental metastasis assays are effective due to the fact that a large bolus cells are introduced to a metastatic organ, increasing the likelihood of lesion formation. However this model does not fully recapitulate the entirety of the metastatic cascade.

Spontaneous metastasis models involve injection of breast cancer cells into the orthotopic site (mammary fat pad) or subcutaneously, where they first form a primary tumor prior to dissemination. These tumors will grow, become invasive and shed individual cancers cells, which can then intravasate into the lymphatic or hematogenous circulation. These cells will arrive at different organs and, depending on the metastatic capacity of the cells, grow as secondary lesions. Spontaneous metastasis assays tend to be more stringent, as many cancer cell lines that have the capacity to metastasize in an experimental metastasis assay will not form spontaneous metastases. Employment of both

of these models can be helpful to characterize metastasis-modifying proteins and identify the stage(s) of metastasis that are affected by their expression.

1.5 GPNMB

1.5.1 Identification of GPNMB and its orthologues

In a search for novel molecular mediators of breast cancer metastasis to bone, we identified Osteoactivin (GPNMB) as a protein that was highly expressed in aggressively bone-metastatic breast cancer cells (Chapter 2) [114]. Glycoprotein non-metastatic melanoma protein B (GPNMB) was first cloned and described in 1995 [161]. The authors of this study used a cDNA subtraction library generated from two melanoma cell lines with high and low metastatic capacities, and identified a cDNA, encoding GPNMB, that was more highly expressed in the low-metastatic cell line. Hence, it was initially coined NMB for "non-metastatic gene B", a name that is now understood to be a misnomer (discussed in section 1.5.7). GPNMB, also known as hematopoietic growth factor inducible, neurokinin-1 type (HGFIN) [162], is located on the small arm of chromosome 7 (7p15). In 1996, a quail orthologue, with 50% identity to GPNMB, was identified in a screen for myc-inducible genes specifically expressed in pigmented neuroretinal cells [163]. The mouse and rat orthologues of GPNMB were subsequently identified in 2001. The rat orthologue, termed Osteoactivin, is expressed in the long bones of mice bearing a mutation associated with osteopetrosis and shares 65% protein identity with human GPNMB [164] (Fig. 2). The mouse orthologue of GPNMB was identified using a subtractive cDNA screen to identify markers for a particular subset of dendritic cells and was coined DC-HIL for dendritic cell heparin integrin ligand and shares 71% protein

identity with human GPNMB [165] (Fig. 2). For the remainder of this introduction, I will use GPNMB to refer to all orthologues, unless otherwise specified.

Relation to Pmel17: GPNMB belongs to the vertebrate Pmel17/NMB family, [163] which encompasses GPNMB, Pmel17 (melanocyte protein 17) and their orthologues; thus, the human protein with the closest identity (33%) to GPNMB is Pmel17 (Fig. 3)[161,166]. Pmel17 is a melanosome specific protein, where it constitutes the main structural component of melanosomes, and plays a key role in the pigment biogenesis of melanocytes [166,167].

1.5.2 Structure and function of GPNMB

GPNMB is a type I transmembrane protein that contains an N-terminal signal peptide, an integrin-binding motif (RGD) and a polycystic kidney disease (PKD) domain in its extracellular domain (ECD), a single pass transmembrane domain, and a 53 amino acid (aa) cytoplasmic tail [165,168]. The cytoplasmic tail harbors a half immunoreceptor tyrosine-based activation motif (hemITAM) and a dileucine-based sorting signal [169]. Each of these domains is discussed in detail below and illustrated schematically in (Fig. 2). There are two known splice isoforms of GPNMB: short and long, consisting of 560aa and 572aa, respectively (Fig. 2) [170]. The long isoform contains a 12aa insertion, into a poorly conserved region, just c-terminal to the PKD-domain (Fig. 2) [170]. To date, there has been no evidence that the short and long isoforms have disparate functions. However, in one study it was reported that the short isoform (560aa) was more abundantly expressed in glioma specimens and was significantly correlated with poor survival times whereas the correlation between the long isoform and survival times failed to achieve statistical significance [170]. In our studies, described in chapters 3 and 4 of this thesis,

the long isoform was employed in experiments where human GPNMB was ectopically expressed [171,172].

RGD domain: This motif, comprised of only 3 amino acids, arginine (R), glycine (G), and aspartic acid (D), is found near the N-terminus of the GPNMB ECD (Fig. 2) and is well characterized in numerous proteins as an integrin-binding motif [173]. Integrins are heterodimeric transmembrane proteins expressed on a wide variety of cells, which regulate cell spreading, adhesion, migration, proliferation and apoptosis [174]. The following integrins recognize RGD peptides: α5β1, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α8β1and αIIbβ3 [173,174], specifically it is the αmonomer that physically interacts with the RGD domain [174]. In GPNMB, it appears that the RGD domain does indeed confer adhesive properties: in the presence of an RGDS competitive inhibitor peptide, endothelial cells and keratinocytes were unable to adhere to an immobilized recombinant protein consisting of the ECD of GPNMB fused to the Fc portion of human IgG1 (Fc-GPNMB) [165,175]. Moreover when the RGD region of this Fc-GPNMB was mutated to RAA (arginine, alanine, alanine), the immobilized, mutant protein was incapable of promoting endothelial or keratinocyte adhesion whereas the wild type Fc-GPNMB induced robust adhesion [165,175].

PKD domain: The PKD domain was first identified in the PKD1 gene, encoding the human protein, polycystin-1, in which this domain is tandemly reiterated 16 times [176]. This domain is commonly found in proteins with protease, chitinase or hydrolase functions [177]. The PKD domain belongs to the Ig-like fold superfamily (E-set), which also includes: cadherins, several families of bacterial Ig-like domains and several fibronectin type III domain-containing families. While the function of the PKD domain is still unclear, based on its structure, it has been proposed to mediate protein-protein or

protein-carbohydrate interactions [176], and has been shown to mediate cell-cell adhesion [178]. In a recent study, the PKD-domain of the bacterial protein deseasin-1 was responsible for binding to and promoting the swelling of collagen [177]. GPNMB, like Pmel17, is one of a few human proteins that contain a single PKD domain. In one study, the PKD of Fc-GPNMB was deleted, and this abrogated its ability to bind T-cells and inhibit their activation [179]. While this effect of GPNMB on T-cell activation is reported to occur as a consequence of its interactions with syndecan-4 on activated T-cells, it is still unclear whether the PKD domain is required for GPNMB-syndecan-4 interactions.

hemITAM: An ITAM is a motif that is commonly found in the cytoplasmic domains of receptors expressed by cells of the immune and hematopoietic systems. It consists of the sequence YxxL/I with six to twelve intervening residues before the YxxL/I sequence is repeated (Y is tyrosine, L is leucine, I is isoleuine and x is any residue] [180]. ITAM motifs are found in cytokine receptors, tumor necrosis factor receptor family members and toll-like receptors [181]. ITAM signaling usually occurs subsequent to ligand binding, via phosphorylation of the ITAM resident tyrosines, primarily by Src-family kinases (ie. Src, Hck, Fgr, Lyn) [182]. These phosphorylated residues then serve as docking sites for the SH2-domains of Syk-family kinases (Syk, ZAP-70), which then self-activate by autophosphorylation [183]. Syk/Zap-70 kinases activate various down-stream signaling molecules that ultimately result in the regulation of cellular responses such as: cytoskeletal rearrangement, differentiation, proliferation, survival and cytokine production [183]. GPNMB is one of several proteins (ie. Clec2, Clec7a, Clec9a, NKp65 [180,183,184]) whose cytoplasmic tail contains a highly conserved, single YxxI sequence (Fig. 2), which has been referred to as a hemi-ITAM or hemITAM [180]. Proteins with hemITAMs still exhibit robust "ITAM" signaling capacity, but thus far have only been

shown to mediate signals through Syk, and not Zap-70 [183]. The current prevailing view is that ligand binding stimulates dimerization of hemITAM-bearing receptors. Syk forms a bridge between the two monomers, with each engaging one of Syk's two SH2 domains. It remains to be seen whether GPNMB is capable of forming homodimers.

Another possibility is that this YxxI/L motif may constitute a tyrosine-based sorting motif of the type Yxxϕ (ϕ: any amino acid with a bulky hydrophobic sidechain, including isoleucine) and thus may be responsible for sub-cellular localization [185]. Sorting motifs of this type are traditionally associated with facilitating rapid internalization from the plasma membrane or targeting to endosomes or lysosomes [185]. However, it is unlikely that the YNPI sequence in GPNMB constitutes a lysosomal-targeting Yxxϕ- motif, as these usually have a glycine residue preceding the critical tyrosine and acidic "x" residues: neither is the case for GPNMB or its orthologues (Fig. 2) [185]. Indeed, mutation of this tyrosine to alanine in the quail orthologue (QNR-71) of GPNMB had no effect on its sub-cellular localization when expressed in HeLa cells [169]. In GPNMB expressed by bone marrow-derived dendritic cells, the hemITAM tyrosine of GPNMB becomes phosphorylated upon antibody cross-linking with its ECD, binding to its ligand syndecan-4, or exposure to dermatophytic fungi, for which GPNMB is a pattern recognition receptor [186]. Moreover, these same treatments that caused GPNMB tyrosine phosphorylation also induced widespread changes in gene and protein expression- including increased cytokine secretion (TNFα, Il-1β) [186]. Pmel17, which thus far has not been ascribed any signaling functions, does not contain this hemITAM motif [166]. While these findings are strongly suggestive of functional hemITAM-based

signaling in GPNMB, more research is needed to definitively characterize the role of this motif when GPNMB is expressed in immune and other cell types.

Dileucine sorting motif: GPNMB contains a dileucine motif in its cytoplasmic tail, near the carboxy-terminus, with the sequence EKDPLL (E: glutamic acid, K: lysine, D: aspartic acid, P: proline, L, leucine) (Fig. 2). Dileucine-based motifs of this type (D/ExxxLL) are often implicated in rapid receptor internalization from the plasma membrane and lysosomal/endosomal targeting [185]. Indeed, when either of these leucine residues is mutated to glycine in quail GPNMB, it is retained at the plasma membrane of HeLa or pigmented quail cells, and not routed to endosomes and lysosomes, as is the case for wild type GPNMB [169]. Interestingly, sequences of this type are associated with basolateral targeting in polarized epithelial cells [185].

1.5.3 Post-translational modification of GPNMB

Glycosylation: Glycosylation is one of the most frequent post-translational modifications of proteins and GPNMB has 12 putative N-glycosylation sites within its extracellular domain, 6 of which reside in the PKD domain (Fig. 2) [161]. Glycosylation of GPNMB has been confirmed by treatment of cell lysates with N-Glycosidase F (PNGaseF), which removes N-linked glycosides, and results in a molecular weight shift of a recombinant GPNMB ECD protein from 70kDa to 54kDa [170]. In immunoblot analyses, human GPNMB is detected as two broad bands; one at ~90kDa (P1 – precursor) and another at ~115kDa (M – mature) [168,171]. The relative abundance of these bands varies based on the cell type in which GPNMB is expressed [168,172]. Interestingly, the P1 form is largely sensitive to EndoH – which only removes ER-modified glycosylation sites – but the M form is EndoH resistant but PNGase sensitive (PNGase removes all N-

glycans) [168]. This suggests that GPNMB is first N-glycosylated in the ER, and these N-glycans are further modified during processing in the golgi to produce the M-form. Furthermore, the shed form of GPNMB (discussed below) is EndoH sensitive and PNGase sensitive [168], suggesting that only the M form is subject to further post-translational processing (shedding), yet both forms are susceptible to tyrosine phosphorylation [186]. Interestingly, in some cells, PNGase treatment does not fully reduce the molecular weight of GPNMB to the 63kDa that is predicted by its primary structure [168]. Indeed, PNGase-treated GPNMB can still exist as multiple bands, suggesting that further post-translational modifications are present [168]. Treatment of murine GPNMB with an O-glycosidase in addition to PNGase, further decreases its molecular weight [187]. To date, the importance of these glycosylation events for GPNMB function has not been assessed; but, given that only the mature form of GPNMB is proteolytically processed, and that altered glycosylation patterns promote aberrant protein-protein interactions [188], we can presume that these modifications are required for at least some of its functions.

Phosphorylation: As discussed above, it has been observed that GPNMB is subject to tyrosine phosphorylation upon engagement of its ECD [186]. It has also been proposed that serine 542 (Fig. 2), through a purely informatics based analysis, may be subject to phosphorylation by GSK3, phosphorylase kinase, or cyclin/CDK complexes, but this has yet to be determined experimentally [189].

Proteolytic cleavage: GPNMB is also subject to proteolytic processing, which was first uncovered by the detection of two heavily glycosylated, high molecular weight forms of murine GPNMB (97kDa, 116kDa; discussed above) and a stable c-terminal fragment of ~20kDa [190]. Furochi *et. al.* noted that a band with a molecular weight

intermediate between the two high molecular weight forms (97kDa and 116kDa) could be detected in media conditioned by GPNMB expressing cells. They postulated that the ECD of GPNMB was being shed from the surface of cells, leaving behind a relatively stable c-terminal fragment, which was further subject to lysosomal and proteasome-mediated degradation [190]. They postulated that GPNMB was susceptible to shedding by members of the matrix metalloprotease (MMP) family, such as ADAMs (a disintegrin and metalloprotease), because treatment with a broad spectrum inhibitor of MMPs (GM6001) reduced the degree to which GPNMB was shed [190]. Two additional groups have confirmed that human GPNMB is susceptible to shedding and that proteolytic processing could be abrogated through MMP inhibition [168,191]. Hoashi *et. al.* confirmed that GPNMB detected in the cell media was not a full-length secreted form, as it was only detectable with an antibody that recognized its N-terminus but not its C-terminus [168]. Treatment with a calmodulin inhibitor (W7) or a protein kinase C activator (phorbol myristate acetate (PMA)), enhanced GPNMB shedding, further implicating the ADAMs, as these compounds have both been reported to enhance ADAM-10 and ADAM-17 activity, respectively [168]. Finally, in our most recent study (Chapter 4), we have observed constitutive GPNMB shedding in breast cancer cells and definitively characterized ADAM10 as the sheddase responsible for this cleavage event in breast cancer cells [172]. Finally, we showed that this shed ECD was functionally relevant as it was capable of inducing endothelial migration [172].

1.5.4 Putative GPNMB ligands

Syndecan-4 (SD4): The extracellular domain of murine GPNMB interacts with SD4; this was first shown in a co-immunoprecipitation experiment employing Fc-

GPNMB (a recombinant protein encoding the extracellular domain of GPNMB fused to the Fc region of IgG) and a V5-tagged SD4 [192]. Moreover, incubation of SD4 expressing T-cells with Fc-GPNMB induced serine and tyrosine phosphorylation of SD4 [193]. Thus, interactions between SD4 and GPNMB result in reciprocal phosphorylation of both proteins by unknown kinases. The interaction between GPNMB and SD4 could be inhibited with heparinase treatment of SD4, suggesting that GPNMB recognizes both carbohydrate and peptide regions on SD4. This is consistent with the observation that its PKD domain - which is postulated to mediate protein-carbohydrate and protein-protein interactions – is required for GPNMB binding to T-cells [179]. Interestingly, GPNMB binds to a form of SD4 possessing heparin sulfate modifications, which is specifically expressed by T-cells [193]. In agreement with these observations, we have been unable to replicate this interaction in our GPNMB-expressing breast cancer cells (MacDonald and Siegel, unpublished observations); however, it is of interest that SD4, like GPNMB, is over expressed in ER-negative breast cancers [194].

Integrins: As discussed above, GPNMB contains a motif capable of binding integrins and this RGD domain has been functionally implicated in the GPNMB-mediated adhesion [165,175]. However, at present the only evidence indicating interactions with specific integrins was provided by experiments demonstrating that β1 and β3 integrins from murine osteoclasts could immunoprecipiate with osteoactivin. Interestingly, only the immature, non-glycosylated, 60kDa isoform of Osteoactivin could be detected in complexes with integrins [195]. This study did not identify physical interactions between GPNMB and RGD binding α-integrins.

Fungal antigens: GPNMB functions as a pattern recognition receptor in dendritic cells by binding to the dermatophytic fungi, *Trichophyton rubrum*, which in turn enhances their antigen-presenting capacity. While this interaction was inhibited by first treating the fungi with glycosidase or by incubating the fungi with Fc-GPNMB in the presence of heparin, the specific GPNMB-binding antigen expressed by *T. rubrum* has not been identified. However, it is postulated to be a protein(s) modified with saccharide-group similar to the heparin sulfate molecules on SD4 expressed by T-cells [186].

Substance P: Substance P is a peptide tachykinin derived from the preprotachykinin I gene, which has been proposed to interact with GPNMB based on *in silico* analyses, but to date, there has been no convincing biological evidence to substantiate this observation [162].

1.5.5 GPNMB expression and function in normal tissue types

Several groups have set out to determine which normal tissues express GPNMB: based on northern and RT-PCR analyses of rat, mouse and human tissues, GPNMB mRNA has been identified in: long bones, calvaria, bone marrow, adipose, thymus, skin, placenta, heart, kidney, pancreas, lung, liver and skeletal muscle [164] [165] [162]**,** but there was some conflict among these studies with respect to GPNMB expression in some tissues. There is also debate as to the cell types predominantly responsible for GPNMB expression within a given tissue (ie. osteoblasts [164] vs. osteoclasts [195]in bone), yet still we can safely conclude from these studies that GPNMB is expressed in many tissue-types throughout the body.

Bone: The first link between GPNMB expression and bone physiology was made when it was identified as highly expressed in osteopetrotic bones relative to normal bones

in rats. This study showed that, in bones, GPNMB is predominantly expressed by mature, matrix producing osteoblasts [164]. Subsequent studies have shown that inhibition of GPNMB, using neutralizing antibodies or siRNA, in developing osteoblasts inhibits their differentiation and their ability to produce bone matrix [196,197]. These results suggest that GPNMB-targeted therapies for bone metastasis may, by inhibiting osteoblast function, indirectly promote osteoclast differentiation and enhanced osteolysis. In addition to these studies, it was recently reported that GPNMB is abundantly expressed in differentiated osteoclasts [198] and plays an important role in mediating cell fusion to produce multi-nucleated osteoclasts. The role of GPNMB in cell-fusion may be mediated by its ability to interact with integrins β1 and β3, both of which are expressed on developing osteoclasts [198]. Neutralizing antibodies against Osteoactivin resulted in fewer osteoclasts that were smaller, possessed fewer nuclei and were less able to resorb bone [195]. This observation is consistent with reports that GPNMB is induced by the transcriptional activator MITF (micropthalmia-associated transcription factor) [198,199], which plays a critical role in regulating osteoclastogenesis and melanocyte biology [200].

Immune system: The molecular functions of GPNMB are just beginning to be elucidated and perhaps have been best characterized in cells of the immune system. Leukocytes and antigen presenting cells such as macrophages and dendritic cells express GPNMB, which can promote adhesion to endothelial cells in an RGD-dependent manner [165,186,201,202,203]. In addition, the extracellular domain of GPNMB is capable of suppressing T-cell activation and proliferation, by binding to syndecan-4 on the surface of activated T-cells and inducing its auto-phosphorylation [179,192,193]. Mutation of the RGD-domain of GPNMB had no effect on its ability to inhibit T-cell activation, whereas

deletion of the PKD domain abrogated this inhibition all together [179]. GPNMB has conflicting roles in the immune system: when it specifically binds to SD4 on T-cells it inhibits their activation [179,192,193]. In contrast, dendritic cells expressing GPNMB are activated by ligand binding to GPNMB or antibody cross-linking and this enhances their ability to activate T-cells [186].

1.5.6 Regulation of GPNMB expression

Growth factors and cytokines: Depending on the cell type in which it is expressed, GPNMB expression is enhanced by numerous cytokines and growth factors, these include: G-CSF [162], M-CSF [162,204], GM-CSF [162], TGF-β [193], IL-3 [162], FGF-2 [205], PDGF [205], BMP-2 [196], α-MSH [175]. Ultraviolet light (type A) has also been shown to induce GPNMB expression [175]. Moreover, while they had no effect on overall GPNMB levels, IFN-γ and TNF-α were reported to promote mobilization of GPNMB from intracellular stores to the plasma membrane [175].

Transcription factors (TFs): In the promoter region of GPNMB, there are two highly conserved consensus sequences for microphthalemia-associated transcription factor (Mitf)[198,199]. Mitf is a master regulator of osteoclastogenesis [200] and melanocyte differentiation [206], and enhances the expression of GPNMB in developing osteoclasts [198] or its quail orthologue, QNR-71 in neural retinal cells [163]. The GPNMB promoter region also contains a consensus sequence for the AP1 transcription factor [198,199,207]. AP1 is a heterodimeric TF, comprised of proteins from the Fos and Jun TF families [208]; this is of particular interest because AP1 transcription factors have been implicated in regulating osteoblastogenesis [208] and tumor cell invasion [209],

both of which are processes that depend on GPNMB function. Its promoter region also contains consensus sites for p53, and in T47D but not HCC70 breast cancer cells, p53 was capable of binding to these sites [207], but it has yet to be determined if GPNMB mRNA expression is indeed regulated by p53. Finally, in osteoblasts, GPNMB protein expression is reduced when the smad-1 transcription factor (which is induced by BMP-2) is knocked down [196].

1.5.7 GPNMB expression and function in cancer

GPNMB was first characterized as a gene that was associated with non-metastatic melanoma, after a partial GPNMB cDNA, which encoded a protein that lacked the first 90 amino acids - including the signal peptide - was transfected into a highly invasive melanoma cell line and suppressed spontaneous metastasis in one of three transfected clones [161]. The role of GPNMB in cancer progression was next re-visited in 2003 when it was reported to promote the invasion of glioma cells [210]. It was subsequently identified as being highly expressed in a wide array of tumors, including those that are benign, such as subependymal giant cell astrocytomas [211] and several that are malignant, including: hepatocellular carcinoma [212], uveal melanoma [213], glioma [170,191] and - contrary to its initial characterization – in malignant cutaneous melanoma [168,175,191]. By contrast, GPNMB is highly methylated (ie. transcriptionally silenced) in the vast majority of colorectal carcinomas [214].

In addition to being expressed in tumor epithelium, GPNMB is also highly expressed in tumor stroma [171]. Specifically, it was overexpressed in including endothelium derived from ovarian carcinoma [215], and in a subset of CD10-positive cancer associated fibroblasts [216]. In macrophages treatment with tumor cell conditioned

media induced an 83-fold increase in GPNMB expression [217]. Interestingly, these tumor-conditioned macrophages were phenotypically similar to the M2-type macrophages [217], which are known for their role in promoting tumor progression [218]. Despite these findings the role of stromal GPNMB in tumor progression has not yet been addressed.

We have identified GPNMB as a gene that is highly expressed in breast cancer [114,171] - particularly in tumors of the triple negative subtype (discussed in chapters 2 and 3). It should be noted however, that in an independent study of GPNMB expression in breast cancer, where the authors employed *in situ* mRNA hybridization for GPNMB in human breast tumors, it was reported to be expressed at lower levels in tumor tissues than in normal tissues [219]; however the specificity of the 3 probe sequences used to detect GPNMB was not assessed; indeed BLAST analyses revealed that each of these sequences showed a high degree of homology with known protein coding sequences. They also identified GPNMB to be expressed at high levels in immortalized cell lines derived from normal breast epithelium and at low levels in breast cancer cell lines, which was in direct opposition to our findings and those of others [220,221].

In addition to being highly expressed in many cancers, GPNMB has been implicated as a functional mediator of tumor progression. GPNMB, or its murine orthologue Osteoactivin, in glioma, melanoma, hepatocellular carcinoma and breast cancer cells is both necessary and sufficient to enhance migratory and invasive phenotypes *in vitro* and metastasis capabilities *in vivo* [114,171,210,212,222]. In cancer cells and fibroblasts, GPNMB induces expression of pro-invasive matrix metalloproteases, such as MMP-3 and MMP-9, via Erk-dependent signaling; this may represent one mechanism by which it promotes metastasis [114,205,210]. Interestingly, GPNMB-mediated upregulation of

MMP-3 in fibroblasts was inhibited in the presence of heparin, but not an RGDS peptide inhibitor, suggesting that this induction does not occur in an integrin-mediated fashion [205].

In one recent study, knockdown of GPNMB in B16F10 murine melanoma cells had no effect on cell growth *in vitro*, nor did it have any effect on the ability of these cells to grow in athymic, nude mice; In contrast, GPNMB knockdown significantly delayed the growth rate of tumors from B16F10 cells that were injected into fully immunocompetent, C57BL/6 mice [222], suggesting that the tumor growth dependent effect of GPNMB was entirely dependent on T-cells. The authors further demonstrate that knockdown of GPNMB in melanoma cells that were implanted subcutaneously into mice caused an increase in the number of T-cells capable of responding to melanoma-associated tumor antigens. The ability of B16F10 melanoma cells to promote T-cell activation was enhanced when the T-cell ligand for GPNMB, SD4 was blocked. Interestingly, they also reported that GPNMB could be released from melanoma cells in the form of exosomes, and that this dissemination of GPNMB might facilitate systemic immunosuppression of anti-tumor responses [222].

In terms of the ability of GPNMB to promote metastasis, Tomihari *et. al.* found that control and knockdown cells elicited a similar number of metastatic foci in the lungs of C57BL/6 mouse following tail vein injection, but those foci developing from GPNMB knockdown cells contained less melanin [222]. The authors presumed that less melanin was indicative of fewer cells per focus, and concluded that GPNMB has no effect on the initial establishment of lung foci, but is important for promoting tumor growth within established foci [222]. An alternative explanation is that the increase in melanin observed in foci from control B16F10 cells, is not due to the presence of more cells per focus, but

an enhanced ability of GPNMB-expressing cells to produce melanin. Indeed, due to its structural similarity to Pmel17 [161] and its high degree of expression in stage IV melanosomes [168], GPNMB has been suggested – though not yet proven – to be involved in melanin biogenesis.

In our recent study we ectopically expressed GPNMB in 66cl4 murine mammary carcinoma cells, and in agreement with Tomihari *et. al.*, we found that it was sufficient to promote primary tumor growth [172]. Conversely, we found that while the effect of GPNMB on tumor growth was more dramatic when cells were injected into immunocompetent Balb/c mice, it was also capable of promoting tumor growth when cells were injected into athymic nude mice, suggesting alternative tumor-promoting functions of GPNMB in addition to its ability to mediate suppression of anti-tumor immunity [172]. Indeed, in this study we identified a role for GPNMB in promoting tumor angiogenesis [172], which is detailed in chapter 4 of this thesis.

Given the increasing association of GPNMB expression and function with multiple cancers, there has been growing interest in the development of GPNMB-targeted therapies. Beyond these considerations, the pattern of GPNMB expression makes it an intriguing target for cancer therapy. It is highly expressed at the surface of cancer cells [171,191,223] but is predominantly expressed intracellularly in normal cells, such as macrophages or melanocytes [175,203,224]. Therefore, GPNMB is particularly attractive for antibody based therapies because, as a target, it would be more readily accessible in cancer cells than in normal cells, thereby reducing potential complications due to side effects associated with targeting and killing normal cells.

1.5.8 CDX-011

CDX-011, also known as CR011-vcMMAE (CR011) or glembatumumab vedotin, is a GPNMB-targeted therapeutic agent that belongs to a class of drugs known as antibody drug conjugates [223]. These drugs consist of antibodies that bind to cell surface molecules, which are linked to highly potent cytotoxins. In the case of CDX-011, the cytotoxin is auristatin E, a tubulin destabilizer. Upon internalization, the drug is released and induces cell cycle arrest and apoptosis of the cancer cell [225].

Pre-clinical data: At concentrations as low as 2.5mg/kg, CDX-011 was capable of inducing complete regression in 100% of GPNMB-expressing xenografted SK-Mel-2 and SK-Mel-5 melanoma cells [226]. In breast cancer, a single dose of 20mg/kg CDX-011 was sufficient to induce sustained MDA-MB-468 tumor regression *in vivo* [171]. We and others have reported that cell killing efficacy of CDX-011 is directly proportional to cell surface GPNMB expression [171,191,223,226]. Interestingly, treatment with imatinib and inhibitors of the Erk pathway enhance cell surface expression of GPNMB in cancer cells, which in turn increases sensitivity to CDX-011 [191]. These findings suggest combination with additional targeted therapies - that are capable of enhancing cell surface GPNMB expression - could further enhance the efficacy of CDX-011.

Clinical trials: CDX-011 is currently being investigated in two multi-centre Phase I/II clinical trials; one for patients with unresectable melanoma [227] and the other for patients with locally advanced or metastatic breast cancer [228]. Mature results from these trials were presented at the 2010 annual meeting of the American Society for Clinical Oncology and were very promising [229,230]. To date, 117 melanoma and 42 breast cancer patients have been treated with varying doses of CDX-011. Tumor shrinkage was reported in 56% of melanoma patients and 62% of breast cancer patients who were treated with a maximum tolerated dose (MTD) of 1.88 mg/kg once every 3

weeks [229,230]. Development of a rash was one of the most common side-effect experienced by treated breast cancer (48%) and melanoma (57%) patients. This finding was of great interest, given that GPNMB is expressed in the skin (Rose and Siegel unpublished observations): EGFR is another molecule that is expressed in skin, and the use of EGFR inhibitors for the treatment of cancer are also associated with rash [231]. Interestingly, melanoma patients who experienced rash within their first cycle of treatment also had significantly longer progression free survival (PFS) than CDX-011-treated patients who didn't develop rash (4.8 vs. 1.2 months; $p<0.001$), suggesting that rash may be an early indicator of a patients' ability to tolerate and respond to the drug [229]. Despite its usefulness as a clinical indicator, rash can reduce quality of life for patients receiving therapy. Importantly pre-emptive rash therapy in the context of EGFR inhibitors has significantly diminished the appearance of rash without affecting treatment efficacy, and this approach may also be beneficial for patients treated with CDX-011 [231]. Another biomarker that appears to have predictive value is GPNMB expression: small subset of melanoma patients with the highest levels of tumoral GPNMB expression ($n =7$) had longer median PFS times (4.9 months) compared to the median PFS for all patients ($n= 34$; including those with high tumoral GPNMB) treated with MTD, which ranged from 1-3.9 months depending on the dose frequency [223]. This observation was recapitulated in a subset of breast cancer patients treated with CDX-011: the median PFS for GPNMB-positive patients ($n=9$) was 17.3 weeks compared to 9.1 weeks for all 34 patients – including those with high GPNMB expression levels - treated with the MTD [230]. Interestingly patients with strong GPNMB expression in stromal cells responded to CDX-011 just as well, if not better, than patients with strong GPNMB expression in the tumor epithelium [230]. These preliminary data suggest that tumoral GPNMB expression

and incidence of rash will serve as important predictors for response to CDX-011 therapy in the future. Such insights will be useful for sparing patients who are unlikely to benefit from treatment, and selecting a group of patients who are most likely to respond for CDX-011 therapy. Importantly, only a small subset of patients in the Phase I/II trials had tumors available for assessment of GPNMB expression [230], but proof of tumoral GPNMB expression is a requirement for enrollment in the Phase IIB, placebo-controlled clinical trial, which is now beginning to enroll 120 patients with metastatic breast cancer [232].

1.6 Summary

Breast cancer is a disease with devastating consequences on morbidity and mortality in Canada and across the world. Breast cancer related deaths are most commonly attributed to distant metastases. While survival from breast cancer is increasing, partly due to the use of molecularly targeted therapies, the success of personalized medicine depends on the identification of molecules that can be associated with clinical outcomes and are amenable to targeted interventions. The work described in this thesis resulted from our initial desire to identify novel molecules that are associated with development of bone metastases from breast cancer. From these mouse-model based studies, we identified GPNMB as protein that is highly expressed in bone metastases and functionally implicated in this process (Chapter 2) [114]. We next characterized the importance of GPNMB expression in human breast cancers using large-scale gene expression datasets and tissue microarrays. We found that GPNMB expression is associated with increased risk of metastasis and shorter survival times, it is most commonly expressed in triple

negative breast cancers and that GPNMB-expressing breast tumors regressed in response to the GPNMB-targeted therapeutic, CDX-011 (Chapter 3) [171]. In the work described in the fourth chapter of this thesis, we returned to mouse model and *in vitro* cell-based approaches to better characterize the effects of GPNMB on tumor progression and the domains of GPNMB protein that are functionally important for its pro-tumorigenic and pro-invasive activities. We found that GPNMB enhances tumor angiogenesis, and that the shed extracellular domain of GPNMB was functionally implicated in this process (Chapter 4) [172]. In summary, we have identified GPNMB as a novel molecular mediator and prognostic indicator of breast cancer progression. Our work demonstrated that GPNMB serves as a viable target for CDX-011 in breast cancer, which, while still in clinical development, may soon be added to the growing arsenal of targeted therapeutics available for breast cancer patients, and be of particular benefit to those with GPNMB-positive and/or and triple negative breast cancer.

1.7 References

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1.8 Figures and Legends

1.8.1 Figure Legends

Figure 1. Soluble factors secreted by breast cancer cells establish a complex communication network amongst the host cells and the bone matrix. Breast cancer cells impair osteoblast differentiation and promote osteoblast apoptosis (**A**) while indirectly inducing expression of RANKL on immature osteoblasts through the production and secretion of PTHrP (**B**). Increased RANKL expression by osteoblasts promotes the differentiation of osteoclasts and leads to increased bone resorption (**C**). Growth factors stored in the bone matrix are released during the process of bone resorption (**D**) and act as mitogens for breast cancer cells (**E**). Secreted proteins from breast cancer cells such as OPN can mediate cell adhesion to the bone matrix (**F**). Breast cancer cells secrete proteins such as IL8 and IL11, that can directly affect osteoclast differentiation (**G**).

Figure 2: Alignment of mammalian GPNMB orthologues and schematic representation of human isoforms. The following domains of human GPNMB are highlighted as indicated: RGD domain in yellow, PKD domain in green, 12aa insertion of the long isoform in blue, transmembrane domain in grey, hemITAM in pink, putative phospho-serine in maroon, the dileucine sorting motif in olive, and N-linked glycosylation sites in purple. * = conserved amino acid; : = conserved amino acid property. Human GPNMB shares 77%, 75%, 69% and 68% identity with cow, pig, mouse and rat, respectively.

Figure 3: Protein alignment of human GPNMB and human Pmel17. The following domains of human GPNMB are highlighted as indicated: Signal peptide in red, RGD domain in yellow, PKD domain in green, 12aa insertion of the long isoform in blue,

transmembrane domain in grey, hemITAM in purple, and the dileucine sorting motif in olive green.

1.8.2 Figures

Figure 1:

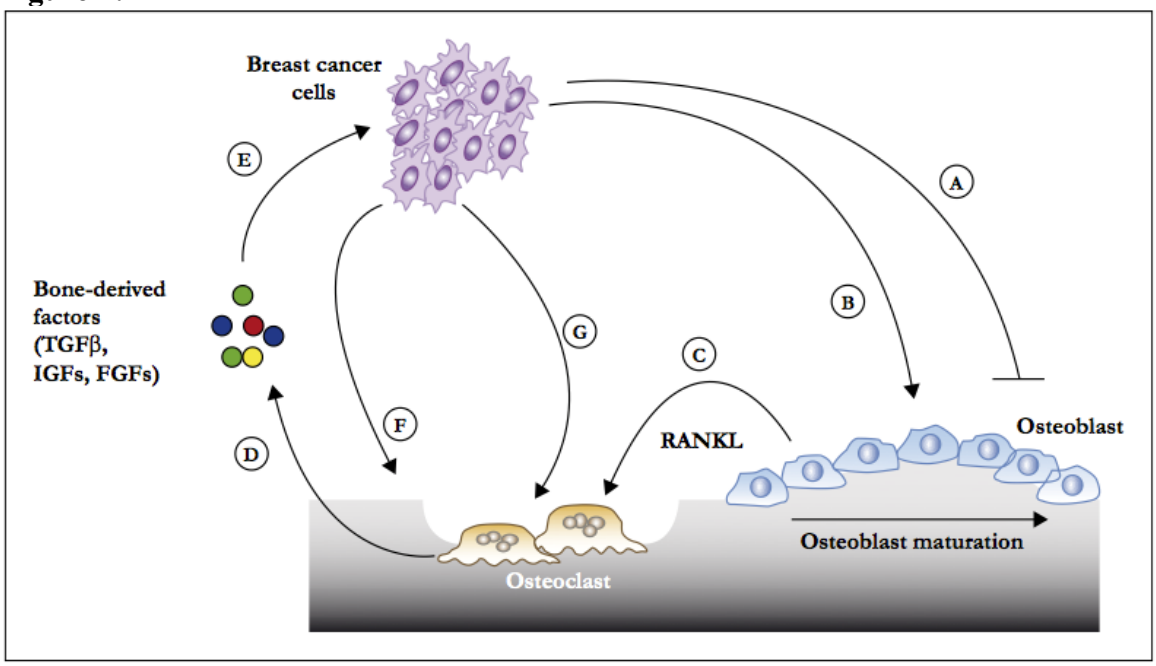

Figure2:

```
cow             MECLYCFLGFLLLAAGLPLDAAKRFHDVLSNERPSGYMREHNQLSGWSSDENDWNEKLYP
pig             MECLYCFLGFLLLAAILPLDAAKRFQDVLSNERPSSYMREHNQLNGWSSDENDWNEKLYP
human           MECLYYFLGFLLLAARLPLDAAKRFHDVLGNERPSAYMREHNQLNGWSSDENDWNEKLYP
mouse           MESLCGVLGFLLLAAGLPLQAAKRFRDVLGHEQYPNHMRENNQLRGWSSDENEWDEHLYP
rat             MESLCGVLVFLLLAAGLPLQAAKRFRDVLGHEQYPDHMRENNQLRGWSSDENEWDEQLYP
                **.*  .* ****** ***:*****:***.:*: . :***:*** ********:*:*:***

cow             VWKRGDSRWKSSWKGGRVQAVLTSDSPALVGSTITFAVNLVFPRCQKEDASGNIVYEKNC
pig             VWKRGDSRWKNSWKGGRVRAVLTSDSPALVGSTITFAVNLVFPRCQKEDADGNIVYEKNC
human           VWKRGDMRWKNSWKGGRVQAVLTSDSPALVGSNITFAVNLIFPRCQKEDANGNIVYEKNC
mouse           VWRRGDGRWKDSWEGGRVQAVLTSDSPALVGSNITFVVNLVFPRCQKEDANGNIVYEKNC
rat             VWRRGEGRWKDSWEGGRVQAALTSDSPALVGSNITFVVNLVFPRCQKEDANGNIVYERNC
                **:**: ***.**:****:*.**********.***.***:*********.******:**

cow             RNDTGASPDLYVYNWTAGTEDSDWGNDTSEGHHNVFPDGKPFPRP---WKKNFVYVFHTL
pig             RNDSGPSPDPYVYNWTAWTEDNDWRDGTSQGHHNVFPDGKPFPHP---WKRNFVYVFHTL
human           RNEAGLSADPYVYNWTAWSEDSDGENGTGQSHHNVFPDGKPFPHHPGWRRWNFIYVFHTL
mouse           RNDLGLTSDLHVYNWTAGADDGDWEDGTSRSQHLRFPDRRPFPRPHGWKKWSFVYVFHTL
rat             RSDLELASDPYVYNWTTGADDEDWEDSTSQGQHLRFPDGKPFPRPHGRKKWNFVYVFHTL
                *.:   :.* :******: ::* *  :.*...:*  *** :***:     : .*:*******

cow             GQYFQKLGQCSVTISINTANVSLGPQIMEVTVYRRHRRAYVPIAKVKDVYVVTDQIPVFV
pig             GQYFQKLGRCSVSVSINTANVTLGPQVMEVTVYRRHGRTYVPIAKVKDVYVVTDQIPIFV
human           GQYFQKLGRCSVRVSVNTANVTLGPQLMEVTVYRRHGRAYVPIAQVKDVYVVTDQIPVFV
mouse           GQYFQKLGRCSARVSINTVNLTAGPQVMEVTVFRRYGRAYIPISKVKDVYVITDQIPVFV
rat             GQYFQKLGRCSARVSINTVNLTVGPQVMEVIVFRRHGRAYIPISKVKDVYVITDQIPIFV
                ********:**. :*:**.*:: ***:*** *:**: *:*:**::*******:******:**

cow             TMSQKNNRNSSDETFLRDLPITFSVLIHDPSHFLNESAIYYKWNFGDNTGLFVSNNHTLN
pig             TMSQKNNRNSSDEIFLKDLPIIFNVLIHDPSHFLNESAIYYKWNFGDNTGLFVSNNPIMN
human           TMFQKNDRNSSDETFLKDLPIMFDVLIHDPSHFLNYSTINYKWSFGDNTGLFVSTNHTVN
mouse           TMSQKNDRNLSDEIFLRDLPIVFDVLIHDPSHFLNDSAISYKWNFGDNTGLFVSNNHTLN
rat             TMYQKNDRNSSDETFLRDLPIFFDVLIHDPSHFLNYSAISYKWNFGDNTGLFVSNNHTLN
                ** ***:** *** **:**** *.********** *:* ***.**********.*  :*

cow             HTYVLNGTFSLNLTVQAEVPGPCPLPSPRPPKTTPP-----------------LVTAGD
pig             HTYVLNGTFSFNLTVQATVPGLCSSPSPRPPKTTPS-----------------LFLAGG
human           HTYVLNGTFSLNLTVKAAAPGPCPPPPPPPRPSKPTP----SLATTLKSYDSNTPGPAGD
mouse           HTYVLNGTFNLNLTVQTAVPGPCPPPSPSTPPPPSTPPSPPPSPLPTLSTPSPSLMPTGY
rat             HTYVLNGTFNFNLTVQTAVPGPCPSPTP--SPSSSTSPSPASSPSPTLSTPSPSLMPTGH
                **********.:*****:: .** *. *.*     . ..                  :*

cow             STLELREIPDESCHITRYGYFKATITIVEGILEVNIIQVTDVPMPRPQPDNSLVDFVVTC
pig             KPLELREIPVENCKINRYGYFMATITIVAGILQVNILQVTDVLTPGPQPDDSLVDFVLTC
human           NPLELSRIPDENCQINRYGHFQATITIVEGILEVNIIQMTDVLMPVPWPESSLIDFVVTC
mouse           KSMELSDISNENCRINRYGYFRATITIVEGILEVSIMQIADVPMPTPQPANSLMDFTVTC
rat             KSMELSDISNENCRINRYGYFRATITIVDGILEVNIIQVADVPIPTPQPDNSLMDFIVTC
                ..:**  *. *.*:*.***:* ****** ***:*.*:*::**  * * * .**:** :**

cow             HGSIPTEVCTIISDPSCQITQNPVCDPVAMELGDTCLLTVRRAFSGSGTYCMNLTLGNDA
pig             QGSIPTEICTIISDPTCQVTQDTVCNTVDEDLGDTCLLTVRRAFSSPGTYCMNLTLGDDA
human           QGSIPTEVCTIISDPTCEITQNTVCS--PVDVDEMCLLTVRRTFNGSGTYCVNLTLGDDT
mouse           KGATPMEACTIISDPTCQIAQNRVCS--PVAVDGLCLLSVRRAFNGSGTYCVNFTLGDDA
rat             KGATPTEACTIISDPTCQIAQNRVCS--PVAVDELCLLSVRRAFNGSGTYCVNFTLGDDA
                :*: * * *******:*::::*: **.     :.  ***:***:*...****:*:***:*:

cow             SLALTSTLVSINSRDPASLLRTANGILVSLGCLAILVTVIAFLMYKKHKEYKPIENSPGI
pig             SLALTSTLVSIPGRDPAFLLRMTNGILVFVGFLTIFVTVIAILVSKKYRKYKAIESNTGI
human           SLALTSTLISVPDRDPASPLRMANSALISVGCLAIFVTVISLLVYKKHKEYNPIENSPGN
mouse           SLALTSTLISIPGKDPDSPLRAVNGVLISIGCLAVLVTMVTILLYKKHKAYKPIGNCPRN
rat             SLALTSALISIPGKDLGSPLRTVNGVLISIGCLAMFVTMVTILLYKKHKTYKPIGNCTRN
                ******:*:*:* .:*    ** .*. *: :* *:::**::::*: **:: *:.* . .

cow             VIRGKGLNVFLNHAKTLFFPGNQEKDPLLKNQPGIL--
pig             VVKDKGLNVFLSRAKTMFFPGNQEKDPLLKN-------
human           VVRSKGLSVFLNRAKAVFFPGNQEKDPLLKNQEFKGVS
mouse           TVKGKGLSVLLSHAKAPFFRGDQEKDPLLQDKPRTL--
rat             VVKGKGLSVFLSHAKAPFSRGDREKDPLLQDKPWML--
                .::.***.*:*.:**: *  *::********::
```

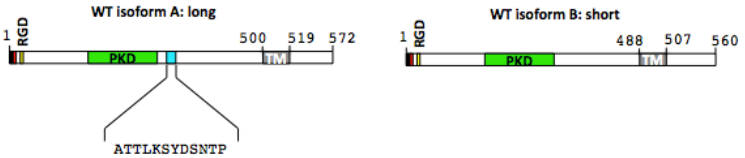

Figure 3:


GPNMB MECLYY--FLGFLLLAARLPLDAAK--RFHDVLGNERPSAYMREHNQLNGWSSDENDWNE
PMEL17 MDLVLKRCLLHLAVIGALLAVGATKVPRNQDWLGVSR---------QLR-----TKAWNR
GPNMB KLYPVWKRGDMRWKNSWKGGRVQAVLTSDSPALVGSNITFAVNLIFPRCQKEDANGNIVY
PMEL17 QLYPEWTEAQRL--DCWRGGQVSLKVSNDGPTLIGANASFSIALNFPGSQKVLPDGQVIW
GPNMB EKNCRNEAGLSADPYVYNWTAWSEDSDGENGTGQSHHNVFPDGKPFPHHPGWRRWNFIYV
PMEL17 VNNT----------IINGSQVWGGQPVYPQETDDAC--IFPDGGPCPSGSWSQKRSFVYV
GPNMB FHTLGQYFQKLGRCSVRVSVNTANVTLGPQLMEVTVYRRHG-RAYVPIAQVKDVYVVTDQ
PMEL17 WKTWGQYWQVLGGPVSGLSIGTGRAMLGTHTMEVTVYHRRGSRSYVPLAHSSSAFTITDQ
GPNMB IPVFVTMFQKNDRNSSDETFLKDLPIMFDVLIHDPSHFLNYSTINYKWSFGDNTGLFVST
PMEL17 VPFSVSVSQLRALDGGNKHFLRNQPLTFALQLHDPSGYLAEADLSYTWDFGDSSGTLISR
GPNMB NHTVNHTYVLNGTFSLNLTVKAAAP----------
PMEL17 ALVVTHTYLEPGPVTAQVVLQAAIPLTSCGSSPVPGTTDGHRPTAEAPNTTAGQVPTTEV
GPNMB -GPCPPPPPPPRPS----------
PMEL17 VGTTPGQAPTAEPSGTTSVQVPTTEVISTAPVQMPTAESTGMTPEKVPVSEVMGTTLAEM
GPNMB ----------KPTPSLATTLKSYDSNTP
PMEL17 STPEATGMTPAEVSIVVLSGTTAAQVTTTEWVETTARELPIPEPEGPDASSIMSTESITG
GPNMB --GPAGDNPLELSRIPDE---NCQINRYGHFQATITIVEGILEVNIIQMTDVLMPVPWPE
PMEL17 SLGPLLDGTATLRLVKRQVPLDCVLYRYGSFSVTLDIVQGI------ESAEILQAVPSGE
GPNMB SSLIDFVVTCQGSIPTEVCTIISDPTCEITQNTVCSPVDVDEMCLLTVRRTFNG-SGTYC
PMEL17 GDAFELTVSCQGGLPKEACMEISSPGCQPPAQRLCQPVLPSPACQLVLHQILKGGSGTYC
GPNMB VNLTLGDDTSLALTSTLISVPDRDPASPLRMANSALISVGCLAIFVTVISLLVYKKH--K
PMEL17 LNVSLADTNSLAVVSTQLIMPGQEAG----LGQVPLIVGILLVLMAVVLASLIYRRRLMK
GPNMB EYNPIENSPGNVVRSKGLSVFLNRAKAVFFPGNQEKDPLLKNQEFKGVS
PMEL17 QDFSVPQLPHSS------SHWLRLPRIFCSCPIGENSPLLSGQQV----

CHAPTER 2 - Osteoactivin promotes breast cancer metastasis to bone

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Preface

Bone is the most common site of breast cancer metastasis, and to date there are no curative therapies available for bone metastases. With this in mind, we set out to identify novel molecules that contributed to the pathogenesis of breast cancer bone metastases, which could potentially serve as targets for therapeutic intervention. Previous studies with similar goals had employed an *in vivo* selection method that involved the isolation of breast cancer cells that had metastasized to the bones of athymic mice following intracardiac injection. These metastatic sub-populations were then subjected to gene expression profiling to identify differentially expressed genes. We felt we could identify previously unidentified mediators of bone metastasis by modifying this approach to use breast cancer cells that would metastasize to bones from the primary tumor through the use of a cell-based breast cancer model capable of recapitulating the entire metastatic cascade in fully immunocompetent mice.

Abstract

The skeleton is a preferred site of metastasis in patients with disseminated breast cancer. We have utilized 4T1 mouse mammary carcinoma cells, which metastasize to bone from the mammary fat pads of immunocompetent mice, to identify novel genes involved in this process. *In vivo* selection of parental cells resulted in the isolation of independent, aggressively bone metastatic breast cancer populations with reduced lung metastatatic capacity. Gene expression profiling identified Osteoactivin as a candidate that is highly and selectively expressed in the aggressively bone metastatic breast cancer cells. These cells displayed enhanced migratory and invasive characteristics *in vitro,* the latter requiring sustained Osteoactivin expression. Osteoactivin depletion in these cells, by siRNA, also lead to a loss of Matrix Metalloproteinase -3 (MMP-3) expression, whereas forced Osteoactivin expression in parental 4T1 cells was sufficient to elevate MMP-3 levels suggesting that this matrix metalloproteinase may be an important mediator of Osteoactivin function. Overexpression of Osteoactivin in an independent, weakly bone metastatic breast cancer cell model significantly enhanced the formation of osteolytic bone metastases *in vivo*. Finally, analysis of publicly available gene expression datasets reveals significant correlations between high *OSTEOACTIVIN* expression in human breast cancers and aggressive tumor features such as estrogen negative status and increased tumor grade. Thus, we have identified Osteoactivin, a protein that is expressed in aggressive human breast cancers and is capable of promoting breast cancer metastasis to bone.

Introduction

It is estimated that 65 - 75% of breast cancer patients with advanced disease develop skeletal metastases, making the bone a preferred site for metastatic dissemination of breast cancer [233]. Numerous complications are associated with the development of osteolytic bone metastases, including pain, hypercalcaemia, fracture and spinal cord compressions, resulting in a significant reduction in the patient's quality of life [234]. While a better understanding of the processes controlling breast cancer metastasis to bone is emerging [113,127], the identification of novel molecular mediators that can potentially be exploited as therapeutic targets for treating osteolytic bone metastases is needed.

Primary breast tumors are heterogeneous in nature, and cancer cells with vastly distinct metastatic capacities exist within a single tumor [235]. Isolation of tumor cells from the metastatic site allows selection of sub-populations that are pre-disposed to metastasize to a particular secondary organ [101,104,112]. This method has allowed the identification of genes that promote cancer metastasis to lung [104,236], brain [237] and bone [112,237]. Indeed, breast cancer cells that preferentially metastasize either to bone or lung express distinct and largely non-overlapping gene expression signatures [104,112], providing insights into the mechanisms controlling organ-specific metastasis [101,238,239]. While these xenograft models have been very useful, they incompletely approximate the metastatic cascade, recapitulating only the final stages of metastasis - including dissemination of tumor cells through the circulation, extravasation into the secondary organ and growth of the nascent lesion. In addition, human-derived cancer cells require the use of immunocompromised mice, which precludes study of cancer

cell/immune cell interactions that facilitate cancer spread, including the formation of osteolytic bone metastases [240].

4T1 mouse mammary carcinoma cells possess the ability to form tumors that spontaneously metastasize from the mammary fat pad to distinct sites such as the bone, brain, liver, lung and lymph node, and thus represents an excellent model of aggressive stage IV breast cancer [241,242]. Importantly, the 4T1 model has proven useful in the identification and characterization of metastatic mediators, such as Twist, that are relevant to human breast cancer [243]. We have employed this cell model to enrich for breast cancer cells that aggressively metastasize to bone, by *in vivo* passage, using both spontaneous and experimental metastasis approaches. Using Agilent genomic profiling, we have identified Osteoactivin, a cell surface glycoprotein, which is overexpressed in all *in vivo* selected bone metastatic populations.

Osteoactivin has previously been shown to be overexpressed in patients with glioblastoma multiforme, which correlated with poor outcome [170]. Moreover, forced expression of Osteoactivin in transformed human astrocytes enhanced their motility and invasion *in vitro* and promoted local invasion following intracranial injection [210]. However, the importance of Osteoactivin in promoting breast cancer metastasis is unknown. We demonstrate that high levels of Osteoactivin expression in the *in vivo* selected breast cancer cells are necessary for their enhanced invasiveness. Furthermore, forced Osteoactivin overexpression in weakly bone metastatic cell lines is sufficient to increase their migratory and invasive characteristics *in vitro* and also significantly increases the formation of osteolytic bone metastases *in vivo.*

Results

In vivo selection of bone metastatic 4T1 breast cancer cells

To better approximate the entire metastatic cascade in an immunocompetent host, we have employed the 4T1 murine mammary carcinoma cell line, which was isolated from a spontaneously arising mammary tumor in a Balb/c mouse [241]. These cells form mammary tumors when injected into the mammary fat pads of Balb/c mice and spontaneously metastasize to the lung, liver, brain and bone, matching the most common metastatic sites in breast cancer patients [241,242,244].

The parental 4T1 cell population was subjected to two rounds of *in vivo* selection following mammary fat pad (Fig. 1A, *upper*) or left cardiac ventricle (Fig. 1A, *lower*) injection. After resection of the primary tumor, 47% of mice injected with the parental 4T1 cells developed osteolytic bone metastases as determined by x-ray imaging (Fig. 1B, *left*). In contrast, after two rounds of *in vivo* selection, three cell populations were identified that produced osteolytic metastases in 71% (590 BM2), 68% (592 BM2) and 80% (593 BM2) of the mice (Fig. 1B, *left*). In comparison, 55% of mice injected intra-cardially with the parental 4T1 cells developed bone metastases, which increased to 75% following two rounds of selection (606 BM2) (Fig 1a, *lower; data not shown*). Interestingly, injection of the cardiac injection-derived 606 BM2 cells into the mammary fat pad also increased the frequency of bone metastasis relative to parental cells (Fig. 1B, *left*). Notably, another mammary fat pad-derived population (511 BM2) was carried through two rounds of *in vivo* selection but did not display the more aggressive bone metastatic phenotype exhibited by the 590, 592, 593 or 606 BM2 populations (Fig. 1B, *left*). The higher percentage of mice developing bone metastases was accompanied by

significantly increased numbers of osteolytic lesions per mouse in those animals injected with the 590, 592, 593 and 606 BM2 cell populations. The 511 BM2 population behaved like parental 4T1 cells with respect to the severity of the bone metastatic phenotype (Fig. 1B, *right*; Fig. 1C). The animals in each cohort were sacrificed within the same timeframe post-injection (44 – 49 days, on average). This precludes the possibility that the enhanced bone metastatic phenotype of the *in vivo* selected populations is the result of these cells residing in the animal for a prolonged period of time.

Bone metastatic 4T1 subpopulations do not display enhanced growth characteristics in vivo

To ensure that the enhanced bone metastatic phenotype did not result from elevated growth rates of the *in vivo* selected populations, we examined their primary mammary tumor outgrowth following mammary fat pad injection. These analyses did not reveal any significant differences in the outgrowth between any of the parental or *in vivo* selected cell populations (Fig. 2A). Moreover, the bone metastatic populations displayed a decreased propensity for local spread to the axillary lymph nodes and comparable levels of metastasis to the liver relative to 4T1p or 511 BM2 cells (Fig. 2B). Although all cell populations are lung metastatic, we observed a substantial decrease in the percentage of mice with lung metastases in the cardiac-selected population (606 BM2) (Fig. 2B). Moreover, the overall tumor burden was decreased in the lungs of the bone metastatic populations (590, 592, 593 or 606 BM2) compared to mice injected with 4T1p or 511 BM2 cells (Fig. 2C and D). This reduction in the burden of lung metastases reached significance in the 592 and 606 BM2 populations ($P < 0.0109$ and $P < 0.0053$, respectively; Fig. 2C). Thus, the enhanced bone metastatic ability displayed by the *in vivo*

selected 4T1 cells does not reflect a general increase in their overall aggressiveness with respect to tumor outgrowth or metastasis.

Bone metastatic 4T1 subpopulations are highly motile and invasive in vitro

To better understand the underlying biological properties that confer a bone metastatic phenotype, we assessed the migratory and invasive behavior of the *in vivo* selected subpopulations using modified Boyden chamber assays. In addition to the parental 4T1 cells (4T1p), we also assessed the migration and invasion of 67NR and 66cl4 cells, which were isolated from the same spontaneous tumor as 4T1 cells, but display different metastatic abilities [241,244]. The 67NR population readily forms mammary tumors but fails to metastasize from the primary site. The 66cl4 population, in contrast, readily forms tumors that spontaneously metastasize to lung, but not the bone [244]. The aggressively bone metastatic populations (592 and 606 BM2) were 2-3.5 times more motile (Fig. 3A and B) and 2-4 times more invasive (Fig. 3C and D) than the weakly (4T1p and 511 BM2) and non-bone metastatic (67NR and 66cl4) populations. This suggests that the bone metastatic potential of the *in vivo* selected populations may reflect, in part, their increased migratory and invasive potentials.

Gene expression profiling reveals a small subset of genes common to mammary fat pad and cardiac selected bone metastatic 4T1 cell populations

To identify mediators that are responsible for the increased bone metastatic potential of the *in vivo* selected populations, we performed gene expression profiling experiments using Agilent whole mouse microarrays. Two independent isolates of the parental 4T1 population (4T1_A and 4T1_B) and 511 BM2 cells represented the weakly

bone metastatic 4T1 populations. The mammary fat pad-derived (590, 592 and 593 BM2) and cardiac selected populations (44 BM1 and 606 BM2) represented the aggressively bone metastatic populations. When these populations were clustered using all 43,790 features on the Agilent microarrays, the parental 4T1 replicates segregated into a distinct subgroup separate from both the mammary fat pad- and cardiac-selected populations (Fig. 4A). Three two-way comparisons were performed between distinct 4T1 populations and differentially expressed genes were characterized by a greater than two fold change and a Holm-adjusted $P < 0.05$ (Fig. 4B). Comparison of the mammary fat pad-selected bone metastatic cells (590, 592 and 593 BM2) with parental cells (4T1_A and 4T1_B) revealed 180 differentially expressed genes (123 genes with elevated expression and 57 genes with reduced expression). To control for changes in gene expression associated with mammary tumor outgrowth, the bone metastatic cells originating from the mammary fat pad (590, 592 and 593 BM2) were compared to the mammary fat pad-derived population that did not display an aggressive bone metastatic phenotype (511 BM2). As expected, this comparison resulted in fewer genes that were differentially expressed between these groups (38 genes with elevated expression and 31 genes with reduced expression). Finally, the cardiac-selected bone metastatic cell populations (44 BM1 and 606 BM2) were compared to parental 4T1 cells (4T1_A and 4T1_B), producing 152 differentially expressed genes (100 genes with elevated expression and 52 genes with reduced expression). To identify genes whose expression strictly correlated with a bone metastatic phenotype, we restricted our focus to those candidates found at the intersection of the three comparisons. Interestingly, only 12 genes were found in this intersection, with 8 displaying elevated expression and 4 expressed at lower levels in aggressive versus weakly bone metastatic 4T1 cells (Fig. 4C).

Osteoactivin overexpression correlates with a bone metastatic phenotype and is required for the invasive phenotype of bone metastatic 4T1 cells

One of the genes overexpressed in both the mammary fat pad and cardiac selected 4T1 populations that aggressively metastasize to bone is Osteoactivin (Gpnmb, DC-HIL, HGFIN). This gene was of immediate interest to us since it was recently shown to promote the motility and invasion of glioma cells [210]. To verify that *OSTEOACTIVIN* message is indeed elevated in our *in vivo*-selected bone metastatic 4T1 populations in the manner indicated by the Agilent expression data (Fig. 5A), we performed northern blot analysis on several non (67NR and 66cl4), weakly (4T1p and 511 BM2) and aggressively (590, 592, 593 and 606 BM2) bone metastatic populations. These results confirmed that *OSTEOACTIVIN* transcripts are significantly overexpressed in those populations possessing a strong bone metastatic phenotype (Fig. 5B). Osteoactivin protein levels are elevated in all bone metastatic populations but not in weakly or non-bone metastatic cells (Fig. 5C). Finally, we performed immunoblot analysis on explants derived from primary mammary tumors originating from parental 4T1 cells to assess when high levels of Osteoactivin expression are first selected. A range of Osteoactivin expression was observed in these 4T1 tumor cell explants when compared to the levels of Osteoactivin observed in the parental 4T1 cells. Of the six tumor explants examined, only one (149 BT) displayed Osteoactivin levels that were similar to those observed in bone metastatic 592 BM2 cells (Fig. 5D). In the remaining samples, one possessed a strong increase (148 BT), three displayed modest increases (151 BT, 152 BT and 154 BT) and one revealed no discernable change in Osteoactivin expression (156 BT) when compared to the parental 4T1 cells (Fig. 5D). Thus, while selection for Osteoactivin expression may occur during

growth of the primary tumor, there appears to be additional pressure for higher Osteoactivin expression in osteolytic bone lesions. This conclusion is further supported by the fact that 4T1 cells isolated from bone metastases that developed following cardiac injection, in which no primary tumors were formed, also display high levels of Osteoactivin expression (Fig. 5C)

To determine if Osteoactivin expression is required for the enhanced migratory and invasive phenotypes displayed by the *in vivo* selected bone metastatic 4T1 cells, we performed transient Osteoactivin knock-down experiments. Osteoactivin protein levels were ablated 72 hours post-transfection in *OSTEOACTIVIN* siRNA-transfected 592 and 593 BM2 cells relative to control siRNA-transfected cells (Fig. 6A and data not shown). While 592 and 593 BM2 cells transfected with control or *OSTEOACTIVIN* siRNAs did not exhibit any changes in motility (Fig. 6B and C, *upper panels*), transient knockdown of Osteoactivin resulted in a clear and statistically significant reduction in invasion compared to control siRNA-transfected cells (Fig. 6B and C, *lower panels*). Previous studies have suggested that Osteoactivin expression is capable of inducing the expression of matrix metalloproteinases, including MMP-3 and MMP-9 [210]. Using quantitative real-time PCR, we discovered that *MMP*-3 transcripts were indeed three-fold higher in the *in vivo* selected bone metastatic populations compared to the parental 4T1 cells (Fig. 6D). The elevated *MMP*-3 expression was not reflected in the Agilent microarray data due the fact *MMP*-3 levels were not uniformly differentially expressed by greater than two fold in all of the aggressively bone metastatic populations; the criteria we used for the comparisons that were performed (Fig. 4). Moreover, we observed that MMP-3 protein levels are low in parental 4T1 cells and are increased in 592 BM2 *in vivo*-selected bone metastatic cells (Fig. 6A, *middle panel*). Interestingly, MMP-3 levels were clearly

diminished in cells with a siRNA-induced loss of Osteoactivin expression, relative to control siRNA-transfected 592 BM2 cells (Fig. 6A, *middle panel*). Thus, our results indicate that Osteoactivin expression is necessary for the enhanced invasiveness of the *in vivo* selected, bone metastatic 4T1 cells.

Overexpression of Osteoactivin is sufficient to induce enhanced migration of parental 4T1 cells

To determine whether Osteoactivin is sufficient to confer enhanced migratory and invasive phenotypes to parental 4T1 cells, we established pooled populations and clonal cell lines expressing Osteoactivin to levels observed in 592 BM2 cells (Fig. 7A*, upper panel*). Exogenous Osteoactivin expression is sufficient to increase MMP-3 expression to levels at or above those observed in the bone metastatic 592 BM2 population. Moreover, progressively higher Osteoactivin levels in the clonal 4T1 stable cell lines resulted in correspondingly elevated MMP-3 expression levels (Fig. 7A, *middle panel*). Together with the results obtained from the Osteoactivin knockdown experiments (Fig. 6), we demonstrate that Osteoactivin expression is both necessary and sufficient for MMP-3 expression in breast cancer cells.

Small but statistically significant increases in cell migration were observed in pooled cell populations, as well as three individual clones expressing OA, when compared to 4T1 empty vector control cells (Fig. 7B and C). The observation that loss of Osteoactivin expression does not affect the baseline motility of *in vivo* selected 4T1 cells that are metastatic to bone (Fig. 6) but is sufficient to enhance motility in parental 4T1 cells, suggests that redundant mechanisms capable of promoting cell migration have been selected for in the explanted populations that absent in the parental 4T1 cells. In contrast,

Osteoactivin expression alone was not sufficient to further promote invasion of 4T1 cells. (Fig. 7B and C). The fact that Osteoactivin expression is necessary (Fig. 6) but not sufficient to induce 4T1 breast cancer cell invasion argues that it must function in conjunction with other mediators present in the *in vivo* selected bone metastatic 4T1 populations, to exert these effects. Indeed, the invasiveness of breast cancer cells requires additional capabilities that extend beyond their migratory characteristics. Thus, these results clearly indicate that the ability of Osteoactivin to modulate cell motility and invasion is influenced by additional changes that have occurred during the *in vivo* selection process, a cellular context that is distinct from the parental 4T1 cells.

Osteoactivin expression promotes breast cancer metastasis to bone

To determine whether Osteoactivin can promote the ability of breast cancer cells to metastasize to bone *in vivo*, we derived pooled stables overexpressing Osteoactivin in the 66cl4 breast cancer cell line, along with empty vector controls (Fig. 8A). These cells were chosen because they do not express endogenous Osteoactivin (Fig. 5B and C; Fig. 8A) and have not previously been demonstrated to metastasize to bone [241,244], providing a rigorous test for the ability of Osteoactivin to promote bone metastasis. Interestingly, Osteoactivin expression was sufficient to significantly induce both the motility (Fig. 8B, *upper panel*) and invasion (Fig. 8B, *lower panel*) of 66cl4 cells compared to empty vector controls. To determine if Osteoactivin could promote bone metastasis *in vivo*, we injected both the vector control and Osteoactivin-expressing 66cl4 pooled cell populations into the left cardiac ventricle of Balb/c mice. Examination of blinded x-rays revealed that 81% (n=13) of mice injected with Osteoactivin-expressing 66cl4 cells developed osteolytic bone metastases compared to only 27% (n=15) of mice

injected with vector control cells (Fig. 8C, *upper left panel*). Moreover, mice injected with Osteoactivin-expressing 66cl4 cells developed, on average, 2.5 times the number of osteolytic lesion per mouse compared to animals injected with the vector control cells (Fig. 8C, *lower left panel*). Breast cancer cells were flushed from osteolytic lesions that formed in mice injected with 66cl4 OA pool cells, and the expression of *OSTEOACTIVIN* was confirmed by quantitative real-time PCR (Fig. 8D, *left panel*). These same bone metastases explant cultures also displayed elevated levels of *MMP-3* expression, suggesting a potential role for this matrix metalloproteinase in promoting the outgrowth of osteolytic lesions in the bone (Fig. 8D, *right panel*). These results confirm that Osteoactivin is capable of enhancing the bone metastatic ability of weakly bone metastatic breast cancer cells.

To determine its potential relevance to human breast cancer, we examined *OSTEOACTIVIN* expression in several publicly available gene expression datasets. Interrogating the recently published dataset from Gray and colleagues [221], we found that *OSTEOACTIVIN* is expressed in many human-derived breast cancer cells at levels much higher than observed in MCF10A cells, an immortalized but non-transformed human breast epithelial cell line (Fig. 9A). *OSTEOACTIVIN* was expressed in breast cancer cell lines that are characterized as belonging to either the luminal or basal phenotype. We selected a subset of these cell lines and confirmed that *OSTEOACTIVIN* was indeed expressed using quantitative real-time PCR and that these results were in good agreement with the microarray data (Fig. 9B). Furthermore, *OSTEOACTIVIN* was also found to be expressed at higher levels in primary breast tumor samples compared to normal breast tissue [245] (Fig. 9C). Intriguingly, high *OSTEOACTIVIN* expression is also frequently associated with ERα negative breast tumors [74] (Fig. 9D); a statistically

significant correlation that is present in several independent microarray data sets [69,246,247,248]. Moreover, increased *OSTEOACTIVIN* expression also correlates with increasing breast tumor grade [67] (Fig. 9E), which is reinforced by independent microarray studies [249,250]. Together, these data argue that Osteoactivin, which we have identified through a metastasis screen in mice, represents a target of interest in the progression of human breast cancer.

Discussion

We have utilized the 4T1 breast cancer cell line, which is capable of spreading to multiple organs and tissues from the primary site, in a fully immunocompetent host [241,242,244], to identify genes associated with the bone metastatic phenotype. By employing both mammary fat pad and cardiac selection protocols, we have identified a common set of 12 genes that were differentially expressed (8 overexpressed and 4 underexpressed) in the aggressive versus weakly bone metastatic 4T1 cells. We have demonstrated that one of these candidates, Osteoactivin, is selectively expressed in aggressively bone metastatic breast cancer cells, promotes breast cancer cell motility and invasion, and significantly enhances bone metastasis of breast cancer cells that normally do not form osteolytic lesions in this site.

Our *in vivo* selection strategy is the first to isolate aggressively bone metastatic breast cancer cell populations from the orthotopic site in an immunocompetent host. Two previous studies have compared 4T1 breast cancer cells to sister populations that were isolated from the same primary tumor but display weaker metastatic abilities relative to 4T1 cells [243,244]. While these experiments were designed to identify genes that are

associated with the overall metastatic behavior of 4T1 cells, we have identified a novel set of genes that are specifically associated with a bone metastatic phenotype.

Bone metastatic MDA-MB-231 human breast cancer cells have previously been isolated using an *in vivo* selection protocol in athymic mice; however, these cells cannot metastasize to bone from the orthotopic site and require the use of an immunocompromised host [112]. Therefore, it may not be surprising, considering our stringent *in vivo* selection criteria, that we have identified novel candidate genes not observed in the *in vivo*-selected MDA-MB-231 breast cancer cells. This raises the possibility that the novel genes identified in our study may contribute to tumor/host interactions that govern breast cancer cell metastasis to bone.

A recently published gene expression dataset has been generated by comparing primary breast tumors taken from patients with known relapse to bone with breast cancer patients lacking bone involvement [251]. While no overlap exists between our 12 genes and those identified in the human samples, it is interesting that only one gene was common between bone metastatic MDA-MB-231 human breast cancer cells and primary breast tumors obtained from patients with known bone metastases [251]. Two important differences between studies employing breast cancer cell lines versus primary breast cancers are likely to account for the lack of overlapping candidate genes. First, gene expression profiles identified using cell based models were derived from breast cancer cells flushed directly from bone metastases compared to those generated from primary breast tumors, in which only a small fraction of the cells represents the bone metastatic population. Second, the use of cell models permits the profiling of pure breast cancer cell populations in the absence of contaminating host cell types, which is not the case with

primary breast tumor material. Therefore, the novel candidates that we have identified warrant further investigation into their role in promoting breast cancer metastasis to bone.

The identification of *OSTEOACTIVIN* as a gene whose expression is associated with a bone metastatic phenotype was of immediate interest to us. *OSTEOACTIVIN* was identified as a gene normally expressed in differentiating osteoblasts and has subsequently been implicated in osteoblast function [164,196,197] Osteomimicry is a term used to describe the observation that certain types of cancer that preferentially metastasize to bone acquire the expression of genes normally associated with osteoblasts. These osteomimetic characteristics were first ascribed to prostate cancer [252] but this phenomenon has also been observed with breast cancer cells [135]. Examples of osteoblast genes expressed by breast cancer cells, which have been functionally implicated in breast cancer metastasis to bone, include Bone Sialoprotein, Osteopontin and Cbfa1 [112,253,254]. Thus, Osteoactivin may confer osteomimetic properties to breast cancer cells, which promotes their metastatic outgrowth in the bone microenvironment.

Osteoactivin may function to promote metastasis of cancer cells through various mechanisms. For instance, Osteoactivin expression in mouse dendritic cells enhances endothelial adhesion and transendothelial migration, two important steps for tumor cell extravasation [165]. More recent studies have demonstrated that forced Osteoactivin expression in transformed human astrocytes or rat hepatoma cells results in enhanced invasiveness, both *in vitro* and *in vivo* [210,212]. Moreover, Osteoactivin expression has been linked to the upregulation of MMPs, such as MMP-3 and MMP-9, in transformed astrocytes and fibroblasts [205,210]. Our data illustrate that Osteoactivin expression contributes to the migratory and invasive properties of 4T1 breast cancer cell populations

and is both necessary and sufficient for MMP-3 expression. Moreover, Osteoactivin-expressing 66cl4 cells, which have been flushed directly from osteolytic bone lesions, retain high levels of *OSTEOACTIVIN* and *MMP*-3 transcripts. Interestingly, studies have linked increased MMP-3 expression in osteoblasts under conditions associated with enhanced bone resorption, such as cytokine stimulation [255], estrogen withdrawal [256] or mechanical loading [257]. The ability of Osteoactivin to induce MMP-3 expression in breast cancer cells may be particularly relevant with respect to their ability to metastasize to bone. Recently, purified MMP-3 has been shown to cleave and solubilize RANKL [258], a key mediator of osteoclastogenesis. Therefore, Osteoactivin-mediated MMP-3 expression in bone metastatic breast cancer cells may contribute to RANKL-induced osteoclast differentiation and bone destruction. The importance of MMP-3 in mediating breast cancer metastasis to bone *in vivo* remains an open question. Finally, Osteoactivin/DC-HIL has recently been characterized as a negative regulator of T-cell activation [259]. Osteoactivin/DC-HIL binds to an unknown ligand that is expressed on activated T-cells, which results in a dramatic attenuation of IL-2 production and subsequent T-cell proliferation in response to anti-CD3 stimulation. Moreover, Osteoactivin/DC-HIL can block reactivation of T lymphocytes that have previously been stimulated by antigen presenting cells [259]. Thus, Osteoactivin/DC-HIL expression by breast cancer cells may facilitate the suppression of anti-tumor immunity and facilitate metastasis. This may explain why Osteoactivin was identified in our screen using immunocompetent Balb/c mice and not when similar experiments were performed using human breast cancer-derived cell populations in athymic animals [112].

Although we have identified Osteoactivin in a screen for molecules which promote breast cancer metastasis to bone, it is possible that it may play a more general

role in metastasis to multiple organs. Indeed, high Osteoactivin expression has been associated with malignant glioblastoma multiforme and poor patient prognosis [170]. Although originally associated with weakly metastatic melanoma cells [161], a recent survey of metastatic melanoma cell lines and clinical specimens has revealed that Osteoactivin is expressed in the majority of these samples and that an Osteoactivin-specific antibody linked to a cytotoxic agent resulted in the regression of subcutaneous melanoma formation in xenograft models [223]. Interestingly, a recent report examining mutations in breast and colon cancers identified *OSTEOACTIVIN* (*GPNMB*) mis-sense mutations specifically in breast tumor samples at a higher frequency than expected from the background mutation rate [260].

The importance of Osteoactivin in human breast cancer is also supported by numerous gene expression microarray datasets generated from primary breast tumors. *OSTEOACTIVIN* is expressed in many breast cancer derived cell lines at levels higher than observed in normal mammary epithelial cells (MCF10A) [221] and more highly expressed in breast tumor samples compared to normal breast epithelium [245]. Interestingly, statistically significant correlations exist between high *OSTEOACTIVIN* expression and ERα negative status [69,74,246,247,248], increasing grade [67,249,250] and p53 mutational status [67,261]. Thus, *OSTEOACTIVIN* expression correlates with several features that are associated with an aggressive breast cancer phenotype. Together with these observations, our discovery that Osteoactivin is selectively overexpressed in aggressively bone metastatic breast cancer cells and that Osteoactivin expression is sufficient to confer a bone metastatic phenotype to weakly bone-metastatic cells suggests an important role for this molecule in the progression to metastatic breast cancer.

Materials and methods

Cell culture and transfections

The 4T1 murine mammary carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Non-metastatic 67NR and lung-metastatic 66cl4 murine mammary carcinoma cell lines were kindly provided by Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, Michigan). All cell lines were grown in DMEM supplemented with 10% FBS, 10mM HEPES, 1mM sodium pyruvate, 1.5g/l sodium bicarbonate, penicillin/streptomycin and fungizone. The pEF1/OA vector was constructed by ligating the full-length murine *OSTEOACTIVIN* cDNA (Open Biosystems; clone ID: 4164706) into a pEF1/V5-His expression vector (Invitrogen) using 5' *Eco*R1 and 3' *Not*1 restriction enzyme sites. 4T1 and 66cl4 cell lines were engineered to express *OSTEOACTIVIN* by lipofectamine 2000 (Invitrogen) mediated transfection of the pEF1/OA vector. Stable cell lines were maintained under 1mg/ml G418 antibiotic selection.

Experimental and spontaneous metastasis assays

Female Balb/c mice (4-6 weeks) were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed in facilities managed by the McGill University Animal Resources Centre and all animal experiments were conducted under a McGill University approved Animal Use Protocol in accordance with guidelines established by the Canadian Council on Animal Care. Experimental metastasis assays were preformed by injecting the 4T1 mammary carcinoma cells (10^5 cells) into the left cardiac ventricle of 4-5 week old Balb/c mice as previously described [112]. For the spontaneous metastasis

studies, 4T1 mammary carcinoma cells were harvested from sub-confluent plates, washed once with PBS, and resuspended (10^4 cells) in 50µl of a 50:50 solution of matrigel (BD Biosciences) and PBS. This cell suspension was injected into the right abdominal mammary fat pad of Balb/c mice and measurements were taken beginning on day 7 post injection for the time periods indicated. Tumor volumes were calculated using the following formula: $\pi LW^2/6$, where L is the length and W is the width of the tumor. Tumors were surgically removed, using a cautery unit, once they reached a volume between 100-125 mm^3.

Radiographic analysis of bone metastases

Immediately prior to sacrifice, mice were anesthetized and digital x-rays were obtained with a Faxitron Specimen Radiography System (model: MX-20 digital). At the termination of these experiments, all digital x-rays were blinded and scored by two independent researchers. Each x-ray was examined for the presence of osteolytic lesions in the following 6 sites: proximal humerus, distal femur and proximal tibia (left and right side). Each x-ray was given a score between 0-6 depending on the number of affected sites. The number of mice possessing at least one osteolytic lesion was divided by the total number of animals in each cohort to deterimine the percentage of mice developing bone metastases. The number of metastatic lesions produced by the injection of each 4T1 *in vivo* selected population is the average of the 6 point scoring system for all animals in the cohort. At the time of necropsy, lungs and hindlimbs were removed and fixed in 4% paraformaldehyde. Fixed tissues were paraffin embedded, sectioned, stained with Hematoxylin and Eosin (H&E), and examined by light microscopy. Routine histological

services were provided by the Centre for Bone and Periodontal Research (McGill University) histology platform.

RNA amplification, labeling and hybridization to Agilent microarrays

4T1 parental and individual *in vivo* selected bone metastatic sub-populations were plated (10^6 cells) in 10cm tissue culture dishes and RNA was extracted 48 hours later using RNeasy Mini Kits and QIAshredder columns (Qiagen). One µg of purified total RNA was subjected to T7-based amplification using the Amino Allyl MessageAmp II kit (Ambion), and the resulting aRNA was conjugated to Cy3 and Cy5 dyes (Amersham). RNA concentration and dye incorporation was measured using a UV-VIS spectrophotometer (Nanodrop ND-1000). RNA quality was assessed by electrophoresis through a 1% agarose gel (1X MOPS, 0.67% formaldehyde) followed by staining with ethidium bromide. The same labeling procedure was utilized for universal mouse reference RNA (Stratagene). Hybridization solutions were prepared with the In Situ Hybridization Kit Plus (Agilent Technologies) and dye swaps (Cy3 and Cy5) were preformed for RNA extracted from each population. Labeled RNA was hybridized to 44K whole mouse genome microarray gene expression chips (Catalog Number G4122A; Agilent Technologies) for 17 hours at 60˚C. Microarray chips were then washed, dried with gaseous N_2 and immediately scanned using a DNA Microarray Scanner (Model G2565BA, Agilent Technologies).

Gene expression analysis

Microarray data were feature extracted using Feature Extraction Software (v. 7.11) available from Agilent using the default parameters. Outlier features on the arrays were

flagged by the same software package. Data preprocessing and normalization was automated using the BIAS system [262]. Raw feature intensities were background corrected using the RMA background correction algorithm [263,264] and the resulting expression estimates were converted to log2-ratios. Within array normalization was performed using spatial and intensity-dependent loess [265]. Median absolute deviation (MAD) scale normalization was used to normalize between arrays [266].

The hierarchical clustering was performed using Ward's minimum variance method with a correlation distance metric. The significance of the clusters was done using 1,000 permutations with the pvclust package in R [267]. Heatmaps are generated by scaling each row (gene) by its mean and dividing by its standard deviation. Above-average expression is colored in red, while below-average expression is colored in blue. The dendograms are generated as defined for hierarchical clustering.

Differential expression was done using Linear Models for Microarray Analysis (LIMMA) [268,269](R Development Core Team, 2006: http://www.R-project.org). If a gene is represented by several probes, only the probe with the largest interquartile range is used. Probes which could not be mapped to any gene were ignored. A gene is considered differentially expressed if it displays fold change of 2 or greater and a Holm-adjusted *P*-value of 0.05 or below between the two categories [270].

Northern blotting

RNA was extracted and purified as described above. 10µg of purified RNA was separated on a 1% agarose gel and northern blots were performed as previously described [271] with the following modifications. The membranes were hybridized in Express Hyb (BD Biosciences) at 65^{o}C with a ^{32}P labeled probe to *OSTEOACTIVIN* (full length mouse

cDNA, GenBank® accession number NM_053110), stripped with boiling 0.5% SDS and subsequently reprobed for *GAPDH* (full length rat cDNA, GenBank® accession number X02231) as a loading control. Following exposure to x-ray films, the membranes were exposed to phosphor-imager screens and signal intensity was quantified with a Storm® imaging system (GE Healthcare) and ImageQuant software.

Quantitative real-time RT-PCR

Total RNA was extracted from cell lines and purified as described above. One (1) µg of total RNA was converted to DNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Following the RT reaction, all samples were diluted 1:333.3 in ddH_2O and 1 µL (mouse cell lines) or 10 µL (human cell lines) was subjected to real time PCR analysis with SYBR Green PCR Master Mix (Applied Biosystems). Primers were used at a concentration 200 fMol/ µL in a total reaction volume of 25 µL. For mouse cell lines, the cycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles each consisting of 95°C for 15 seconds and 58°C for 1 minute. For human-derived cell lines, cycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, followed by 45 cycles each consisting of 95°C for 15 seconds and 60°C for 1 minute. Incorporation of SYBR Green dye into the PCR products was monitored with a 7500 Real time PCR system (Applied Biosystems). The integrity and specificity of the amplified PCR products were confirmed by dissociation curve analysis (SDS 2.0 software, Applied Biosystems) and by agarose gel electrophoresis. To normalize the degradation of total RNA used in cDNA synthesis, the threshold cycle (CT) values were determined for target genes (mouse *OSTEOACTIVIN*, mouse *MMP*-3, human *OSTEOACTIVIN*) and corresponding housekeeping genes (mouse *GAPDH*, human *β*-

actin) in each sample, and the target gene/housekeeping gene ratio was calculated from the following formula:

Target Gene/Housekeeping Gene ratio = $2^{(Ct\ [housekeeping\ gene]-Ct\ [target\ gene])}$

Relative *OSTEOACTIVIN* or *MMP*-3 mRNA levels were expressed in terms of fold induction rate over control cell lines (4T1p, 66cl4VC or MCF10A). All measurements were done in triplicate and three independent experiments were performed. **Human primer sequences:** OSTEOACTIVIN (sense) 5'-CACTTCCTCAATTATTCTAC-3', OSTEOACTIVIN (antisense) 5'-TAAAGAAGGGGTGGGTTTTG-3', *β*-actin (sense) 5'-CCAACCGCGAGAAGATGACC CAGATCATGT-3', *β*-actin (antisense) 5'-GTGAGGATCTTCATGAGGTAGTCAGTCAGG-3'

Mouse primer sequences: GAPDH (sense) 5'-CAAGTATGATGACATCAAGAAGGTGG-3' GAPDH (antisense) 5'-GGAAGAGTGGGAGTTGCTGTTG-3', OSTEOACTIVIN (sense) 5'-TCCCTGGCAAAGACCCAGA-3', OSTEOACTIVIN (antisense) 5'-TTTGTACAGCAAGAT GGTA ACCATG-3', MMP-3 (sense) 5'-CTTTGAAGCATTTGGGTTTCTCTAC-3', MMP-3 (antisense) 5'-AGCTATTGCTCTTCAATATGTGGGT-3'.

Immunoblotting

Sub-confluent cells were lysed for 20 minutes on ice in TNE lysis buffer. Protein concentrations were determined by Bradford Assay (Bio-Rad) and 45µg of total protein was subjected to immunoblot using the following antibodies: Osteoactivin (1:10 000

dilution; Cat # AF2330, R&D Systems), α-Tubulin (1: 10 000 dilution; Cat #: T9026, Sigma) and MMP-3 (1: 1000 dilution; Cat #: MAB4581 R&D Systems). The appropriate HRP conjugated secondary antibodies (Jackson Laboratories) were used at a dilution of 1:50,000 and membranes were visualized with Chemiluminescent HRP Substrate (Immobilon) on Bioflex scientific imaging film (Clonex Corp.).

Motility and invasion assays

Motility and invasion assays were performed as previously described [272] with minor modifications. 10^5 cells, resuspended in serum free media, were added to the top chamber of the transwell inserts (catalogue #353097, Falcon) and allowed to migrate through 8μm pores toward complete media over a 24 hour period. For invasion assays, the transwell inserts were pre-coated with a 5% matrigel solution. At the termination of each experiment, cells were fixed in formalin and stained with crystal violet (Sigma). Five images were taken for each insert and the cells were quantified using Scion Image software (Scion Corporation). Data for each insert is represented as the average pixel count from the five images. All experiments were performed a minimum of three times.

siRNA-mediated depletion of Osteoactivin

Transient knockdown of Osteoactivin in 592 and 593 BM2 cells was accomplished by 2 sequential transfections (Lipofectamine 2000) using 1nM of the following dicer substrate RNAi duplex: 5'-GGCUUAGGGAGUGUGGUUAAAUAGC-3' and 5'-GCUAUUUAACCACACUCCCUAAG CCAC-3' (Integrated DNA Technologies, Inc.) at t=0hr and t=24hr. An *HPRT* RNAi duplex (5'-GCCAGACUUUGUUGGAUUUGAAATT-3' and 5'-

UUCGGUCUGAAACAACCUAAACUU UAA-3') (592 BM2 cells) or a scrambled RNAi duplex (5'-CUUCCUCUCUUUCUCUCCCUUGUGA-3' and 5'-AGGAAGGAGAGAAAGA GAGGGAACACU-3' (593 BM2 cells) were used as a controls. Protein lysates were taken at the beginning (t=48hr) and end (t=72hr) of the migration/invasion assays to confirm efficient Osteoactivin knockdown over the duration of the experiment.

Statistical analysis

Statistical significance (*P* values) for bone metastasis severity and lung wet weight were assessed with Mann Whitney rank sum test. Statistical significance values for motility and invasion assays were obtained by using a two-tailed, heteroscedastic Student's t-Test.

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Figures Legends

Figure 1. *In vivo* selection of 4T1 breast cancer cell sub-populations with increased metastatic ability to bone. **A.** Two rounds of selection were performed following mammary fat pad *(upper)* and left cardiac ventricle *(lower)* injection of parental 4T1 cells (grey). The *in vivo* selected cell populations include 511 BM2 (green), 590, 592, 593 BM2 (blue) and 606 BM2 (purple). *P*, parental; *BM1*, bone metastatic 1; *BM2*, bone metastatic 2. **B.** X-rays were obtained from mice injected with the indicated cell populations, blinded and scored for the percentage of mice with bone metastases *(left)* and average number of lesions per animal *(right)*. *Sample sizes*, 4T1p (n=31); 511 BM2 (n=17); 590 BM2 (n=12); 592 BM2 (n=14); 593 BM2 (n=15); 606 BM2 (n=14). *, $P < 0.0163$; **, $P < 0.0150$; ***, $P < 0.0184$. **C.** Representative x-ray images showing areas of osteolytic bone destruction (white arrows).

Figure 2. Bone metastatic 4T1 sub-populations do not display a generalized increase in primary tumor growth or metastasis to other organs. **A.** Primary mammary tumor outgrowth following mammary fat pad injection of the indicated populations. *Sample sizes*, 4T1p (n=37); 511 BM2 (n=19); 593 BM2 (n=21); 606 BM2 (n=23). **B.** The percentage of mice with soft tissue metastases at the time of necropsy for the indicated cell populations. *Sample sizes*, 4T1p (n=36); 511 BM2 (n=10); 590 BM2 (n=12); 592 BM2 (n=13); 593 BM2 (n=12); 606 BM2 (n=13). **C.** Lung wet weights were determined from cohorts of mice that were uninjected (Uninject.), or injected with the indicated populations into the mammary fat pad. The primary tumor was removed once it reached a volume of 125 – 150 mm^3. *Sample sizes*, Uninject. (n=12); 4T1p (n=31); 511 BM2

(n=12); 590 BM2 (n=10); 592 BM2 (n=11); 593 BM2 (n=10); 606 BM2 (n=13). *, $P < 0.0109$; **, $P < 0.0053$. **D.** Representative images of lungs at necropsy illustrating surface lesions *(upper)* and corresponding H&E sections *(lower)*. *Scale bar*, 200 μm.

Figure 3. Bone metastatic 4T1 populations are more migratory and invasive compared to non- or weakly metastatic breast cancer cells. Motility (**A**, **B**) and invasion (**C**, **D**) of weakly and *in vivo* selected bone metastatic 4T1 populations. For each cell line, 4 digital images/well (triplicate wells per experiment) were quantified using Image J software. The data for both motility and invasion assays represent results from at least 3 independent experiments for each cell population **B.** Representative images for each cell population are shown for both motility (**B**) and invasion (**D**) assays. (**A**) *, 592 BM2 versus 4T1p, $P < 1.2527E-05$; **, 606 BM2 versus 4T1p, $P < 0.0048$ (**C**) *, 592 BM2 versus 4T1p, $P < 0.0013$; **, 606 BM2 versus 4T1p, $P < 0.0070$.

Figure 4. Gene expression analyses reveal a small set of genes that are differentially expressed in both mammary fat pad- and cardiac-selected populations that are aggressively metastatic to bone. **A.** Hierarchical clustering of samples using all features on the Agilent whole mouse genome arrays segregates the parental and strongly bone metastatic mammary fat pad-injected samples. **B.** Venn diagram illustrating three separate comparisons performed between aggressively and weakly bone metastatic 4T1 populations. The number of genes that are overexpressed (red) and underexpressed (blue) in each of these contrasts are indicated. **C.** A heatmap depicting the 12 genes that are differentially expressed among all comparisons and lie at the intersection of the contrasts shown in (**D**).

Figure 5. Osteoactivin is overexpressed in aggressively bone metastatic 4T1 sub-populations versus weakly bone metastatic breast cancer populations. **A.** The relative expression of *OSTEOACTIVIN* (Agilent whole mouse genome microarray), expressed as the fold change relative to the parental 4T1 population. **B.** Northern blot analysis of *in vivo* selected, aggressively bone metastatic 4T1 sub-populations probed with *OSTEOACTIVIN* and *GAPDH* as a loading control. Quantitative phosphorimager analysis was performed and the values for *OSTEOACTIVIN* were first normalized to *GAPDH* and then expressed relative to the 4T1 parental population. **C.** Immunoblot analysis of Osteoactivin expression in weakly and aggressively bone metastatic breast cancer populations in culture. **D.** Immunoblot analysis of Osteoactivin expression in primary tumors arising from mammary fat pad injection of parental 4T1 cells. Lysates from cultured 4T1 (4T1p) and bone metastatic (592 BM2) cells are included as negative and positive controls, respectively. The membranes in panel C and D were probed for α-Tubulin as a loading control.

Figure 6. Osteoactivin expression is required for the invasive phenotype of *in vivo* selected bone metastatic 4T1 breast cancer cells. **A.** 72 hours post-transfection, immunoblot analyses were performed on control and *OSTEOACTIVIN* siRNA-transfected 592 and 593 BM2 cells, using antibodies against Osteoactivin (*upper panel*), MMP-3 (*middle panel*) and α-Tubulin (*lower panel*) (data shown for 592 BM2 cells). **B.** Motility *(upper panel*) and invasion (*lower panel*) assays were performed as described in Fig. 3. Significant differences in invasion were observed between 592 and 593 BM2 cells treated with *OSTEOACTIVIN* siRNAs compared to 592 and 593 BM2 cells treated with control

siRNAs (*, $P < 0.01$; ** $P < 0.03$). Results are derived from at least three independent experiments. **C.** Representative images are shown for both motility (*upper panels*) and invasion (*lower panels*). **D.** Quantitative real-time PCR analysis was performed to examine *MMP*-3 expression in the *in vivo* selected bone metastatic populations compared to parental 4T1 cells. *MMP*-3 expression was first normalized to *GAPDH* levels and expressed as the fold change over 4T1 parental cells.

Figure 7. Osteoactivin overexpression is sufficient to enhance the motility of parental 4T1 cells and induce MMP-3 expression. **A.** Immunoblot analyses demonstrating Osteoactivin expression within pooled cultures and individual clones derived from 4T1p cells (*upper panel*). The membrane was reprobed with antibodies against MMP-3 (*middle panel*) and α-Tubulin (*lower panel*). **B.** Motility *(upper panel*) and invasion (*lower panel*) assays were performed as described in Fig. 3. Motility assays: pooled versus VC (*, $P <$ 0.018), clone 3 versus VC (**, $P < 0.023$), clone 20 versus VC (***, $P < 0.041$) and clone 24 versus VC (****, $P <$ 5.0E-05). Differences in invasion between the indicated cell lines were not significant when compared to the 4T1p VC. Results were obtained from three independent experiments. **C.** Representative images are shown for both motility (*left panels*) and invasion (*right panels*) assays.

Figure 8. Osteoactivin enhances the bone metastatic ability of 66cl4 breast cancer cells. **A.** Immunoblot analyses demonstrating Osteoactivin expression within pooled cultures compared to 66cl4 vector control (VC) and 592 BM2 cells (*upper panel*). The lower portion of the membrane was probed with antibodies against α-Tubulin as a loading control (*lower panel*). **B.** Motility *(upper panel*) and invasion (*lower panel*) assays were

performed as described in Fig. 3. Motility assays: Osteoactivin pooled versus VC (*, $P <$ 0.0003). Invasion assays: Osteoactivin pooled versus VC (**, P<0.0084). Results were obtained from three independent experiments. Representative images are shown for both motility (**B**, *upper right panels*) and invasion (**B**, *lower right panels*) assays. **C.** Cardiac injection of Osteoactivin-expressing 66cl4 cells and vector controls. The percentage of mice developing osteolytic bone metastases (*upper left panel*) and the average number of lesion per mouse (*lower left panel*) are shown. Representative hindlimb x-rays are shown from mice injected with either Osteoactivin-expressing or vector control 66cl4 cells (*right panels*). **D.** Quantitative real-time PCR analysis for *OSTEOACTIVIN* and *MMP-3* levels in 66cl4-OA pool cells that have been flushed from osteolytic lesions. *OA* and *MMP-3* expression was normalized to *GAPDH* and expressed as the fold change relative to 66cl4 VC cells.

Figure 9. Osteoactivin is highly expressed in human breast cancer and correlates with an aggressive tumor phenotype. **A.** *OSTEOACTIVIN* expression in a series of human-derived breast cancer cell lines from a recently published microarray dataset [221]. The data is expressed as the fold change in *OSTEOACTIVIN* relative to MCF10A cells, an immortalized but non-transformed human breast epithelial line. Only those breast cancer cells lines with a five-fold or greater increase in *OSTEOACTIVIN* expression are shown. **B.** Quantitative real-time PCR analysis performed on selected human breast cancer cell lines confirmed *OSTEOACTIVIN* expression. Analysis of publicly available gene expression datasets reveal that high levels of *OSTEOACTIVIN* are found in primary breast tumors versus normal breast epithelium (**C.** P=1.7x10^{-5}; [245]) and is significantly correlated with ERα negative tumors (**D.** P=7.5 x10^{-5}; [74]) of increasing grade (**E.**

P=0.001; [67]). In each box plot (**D-E**), the upper and lower limits of the box indicate the 75th and 25th percentile respectively whereas the lines (whiskers) emerging from above and below the box indicate the 90th and 10th percentiles. In each plot, the black dots represent the maximum and minimum values within the dataset. The sample sizes in each category are indicated in brackets and statistical significance was calculated using a Student;s *t*-test.

Figures

Figure 1

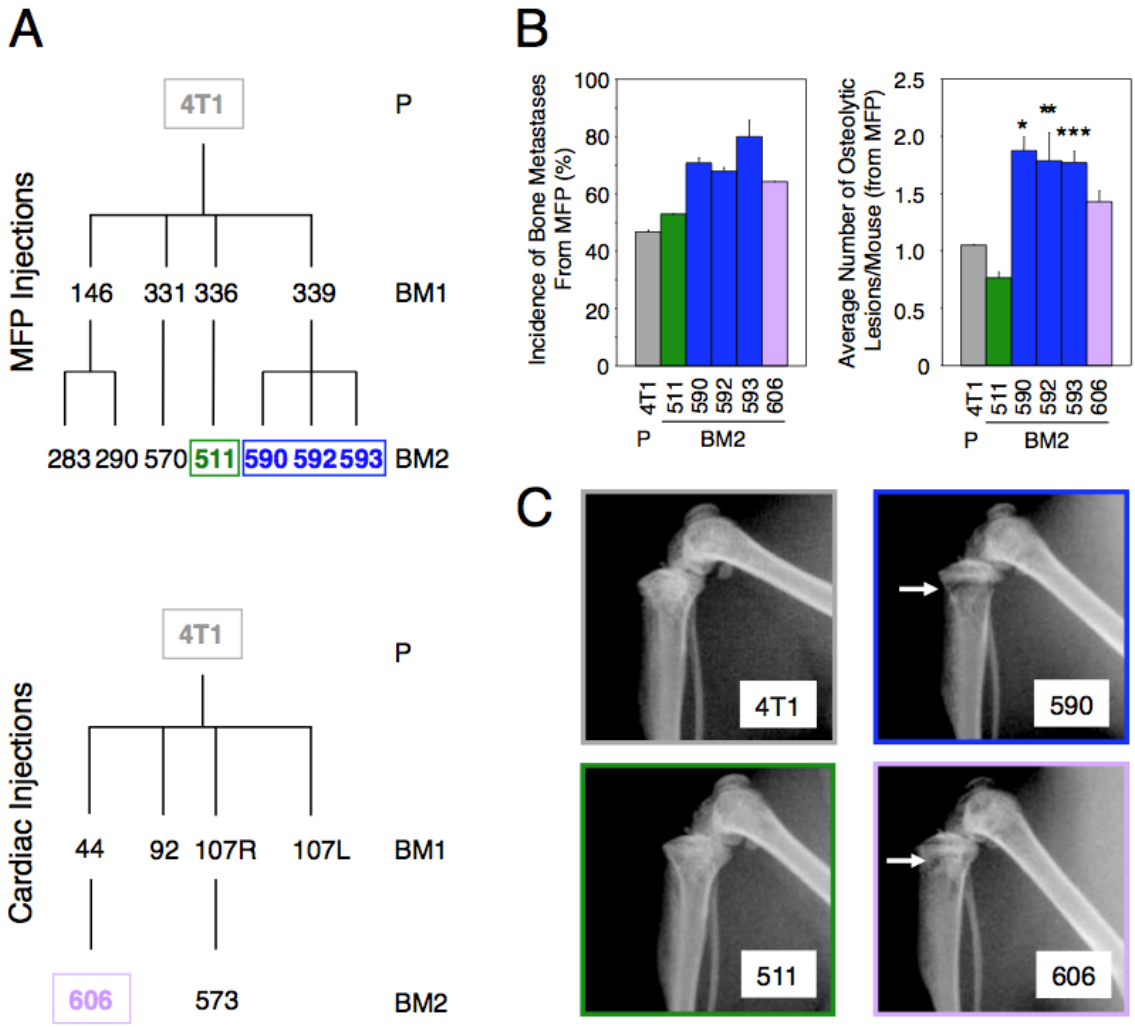


Figure 2

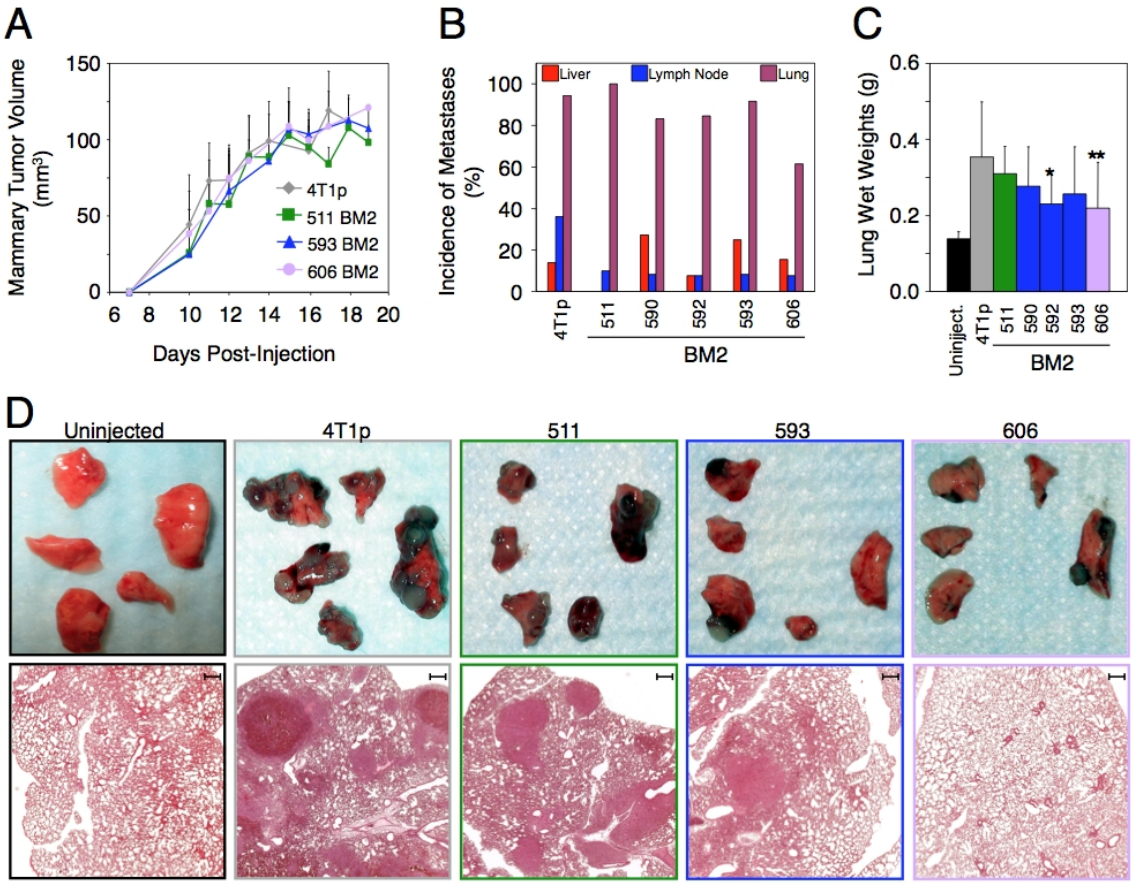

A
Mammary Tumor Volume (mm³)
150
100
50
0
6 8 10 12 14 16 18 20
Days Post-Injection
4T1p
511 BM2
593 BM2
606 BM2
B
Incidence of Metastases (%)
100
80
60
40
20
0
Liver
Lymph Node
Lung
4T1p
511
590
592
593
606
BM2
C
Lung Wet Weights (g)
0.6
0.4
0.2
0.0
*
**
Uninject.
4T1p
511
590
592
593
606
BM2
D
Uninjected
4T1p
511
593
606

Figure 3

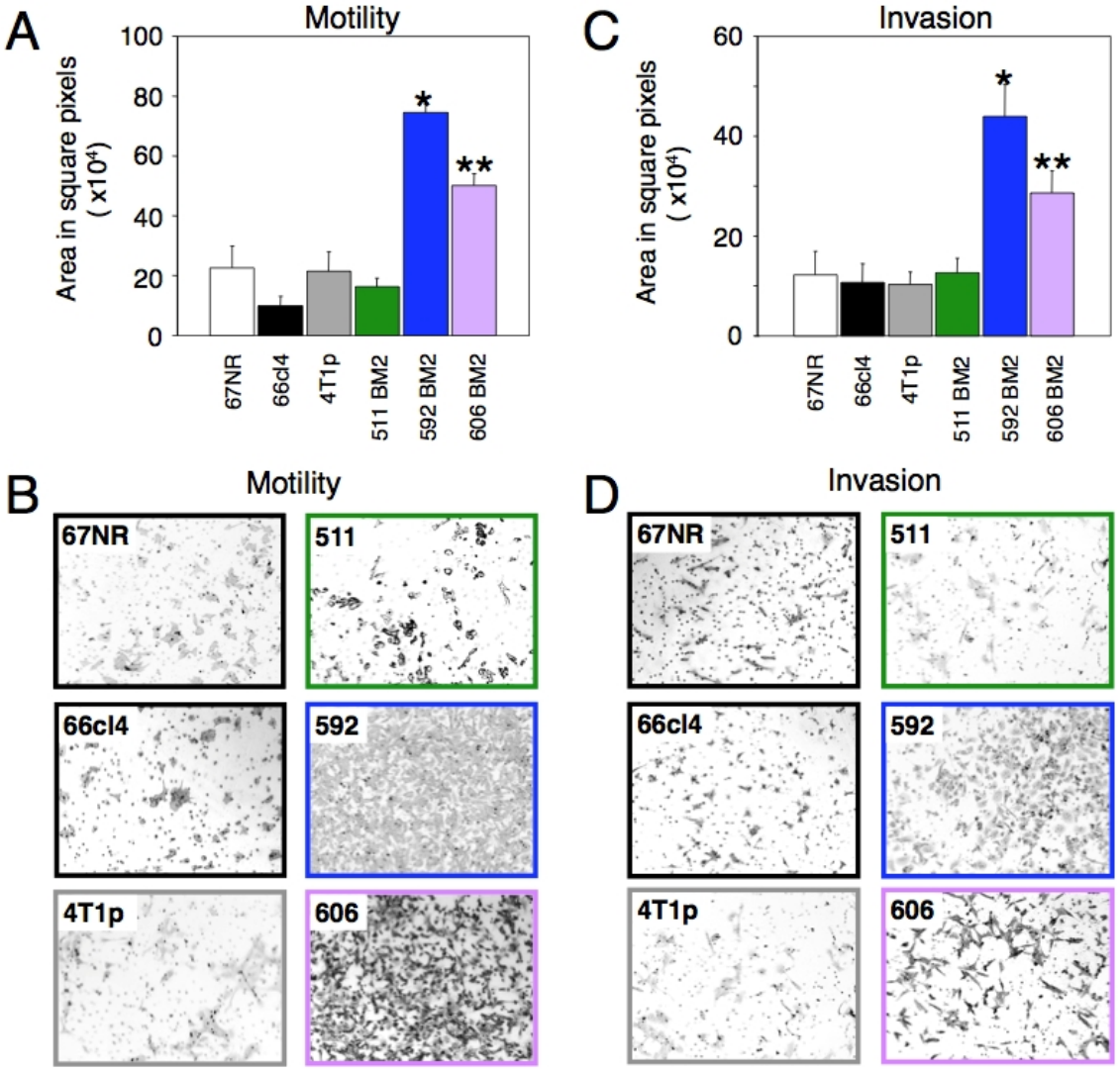

A
Motility
Area in square pixels (x10⁴)
100
80
60
40
20
0
*
**
67NR
66cl4
4T1p
511 BM2
592 BM2
606 BM2
C
Invasion
Area in square pixels (x10⁴)
60
40
20
0
*
**
67NR
66cl4
4T1p
511 BM2
592 BM2
606 BM2
B
Motility
67NR
511
66cl4
592
4T1p
606
D
Invasion
67NR
511
66cl4
592
4T1p
606

Figure 4

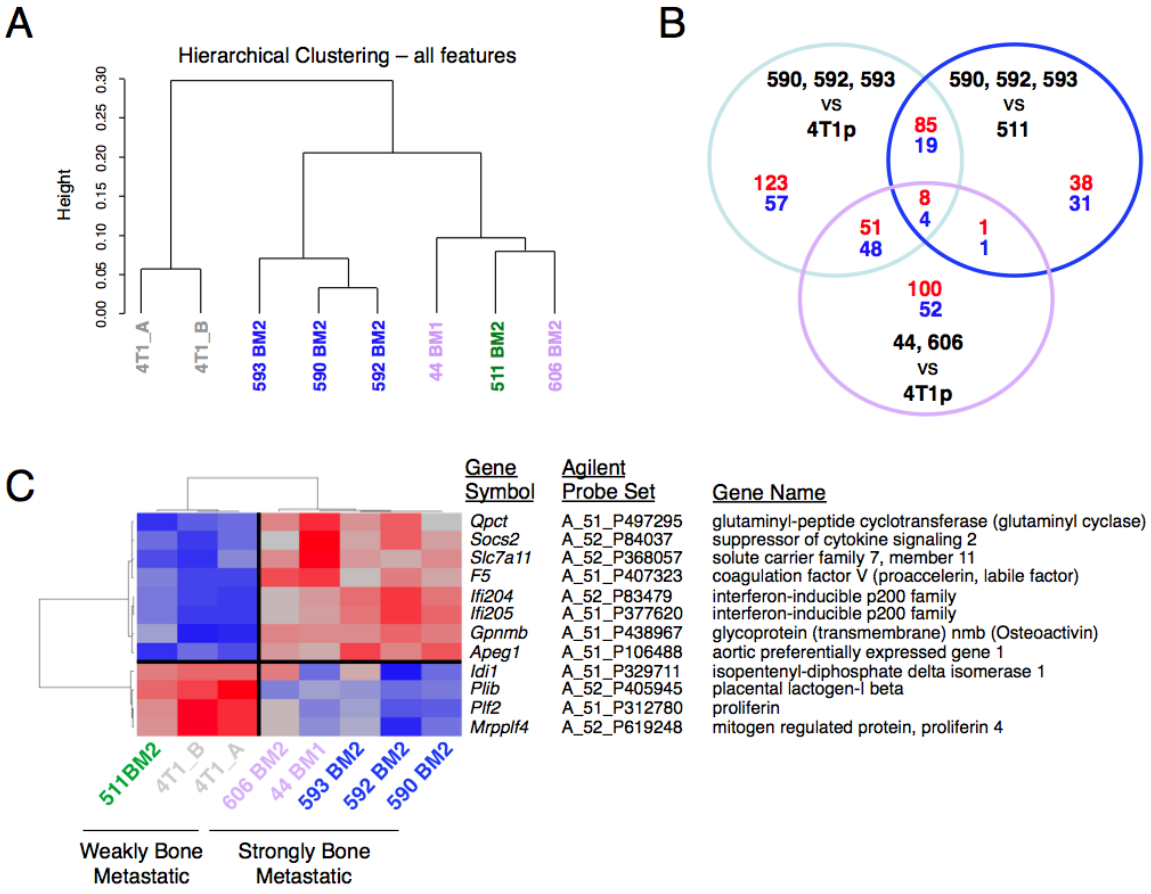

A
Hierarchical Clustering – all features
Height
0.00
0.05
0.10
0.15
0.20
0.25
0.30
4T1_A
4T1_B
593 BM2
590 BM2
592 BM2
44 BM1
511 BM2
606 BM2
B
590, 592, 593
vs
4T1p
590, 592, 593
vs
511
44, 606
vs
4T1p
85
19
123
57
8
4
38
31
51
48
1
1
100
52
C
Gene Symbol
Agilent Probe Set
Gene Name
Qpct A_51_P497295 glutaminyl-peptide cyclotransferase (glutaminyl cyclase)
Socs2 A_52_P84037 suppressor of cytokine signaling 2
Slc7a11 A_52_P368057 solute carrier family 7, member 11
F5 A_51_P407323 coagulation factor V (proaccelerin, labile factor)
Ifi204 A_52_P83479 interferon-inducible p200 family
Ifi205 A_51_P377620 interferon-inducible p200 family
Gpnmb A_51_P438967 glycoprotein (transmembrane) nmb (Osteoactivin)
Apeg1 A_51_P106488 aortic preferentially expressed gene 1
Idi1 A_51_P329711 isopentenyl-diphosphate delta isomerase 1
Plib A_52_P405945 placental lactogen-I beta
Plf2 A_51_P312780 proliferin
Mrpplf4 A_52_P619248 mitogen regulated protein, proliferin 4
511BM2
4T1_B
4T1_A
606 BM2
44 BM1
593 BM2
592 BM2
590 BM2
Weakly Bone Metastatic
Strongly Bone Metastatic

Figure 5

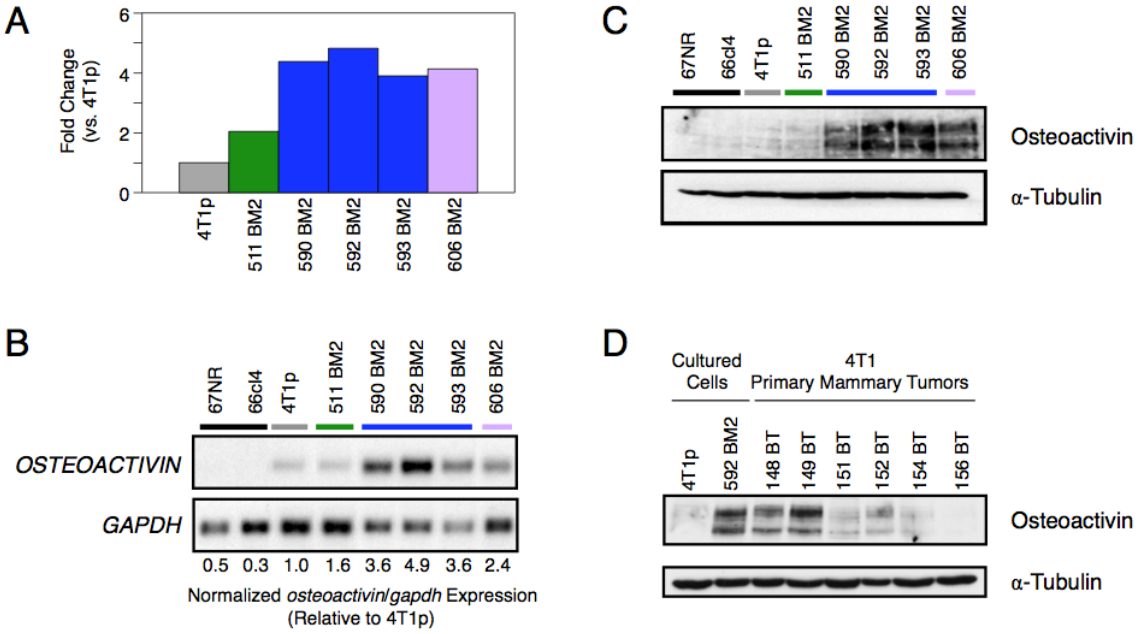

A
Fold Change (vs. 4T1p)
6
4
2
0
4T1p
511 BM2
590 BM2
592 BM2
593 BM2
606 BM2
B
67NR
66cl4
4T1p
511 BM2
590 BM2
592 BM2
593 BM2
606 BM2
OSTEOACTIVIN
GAPDH
0.5 0.3 1.0 1.6 3.6 4.9 3.6 2.4
Normalized osteoactivin/gapdh Expression
(Relative to 4T1p)
C
67NR
66cl4
4T1p
511 BM2
590 BM2
592 BM2
593 BM2
606 BM2
Osteoactivin
α-Tubulin
D
Cultured Cells
4T1 Primary Mammary Tumors
4T1p
592 BM2
148 BT
149 BT
151 BT
152 BT
154 BT
156 BT
Osteoactivin
α-Tubulin

Figure 6

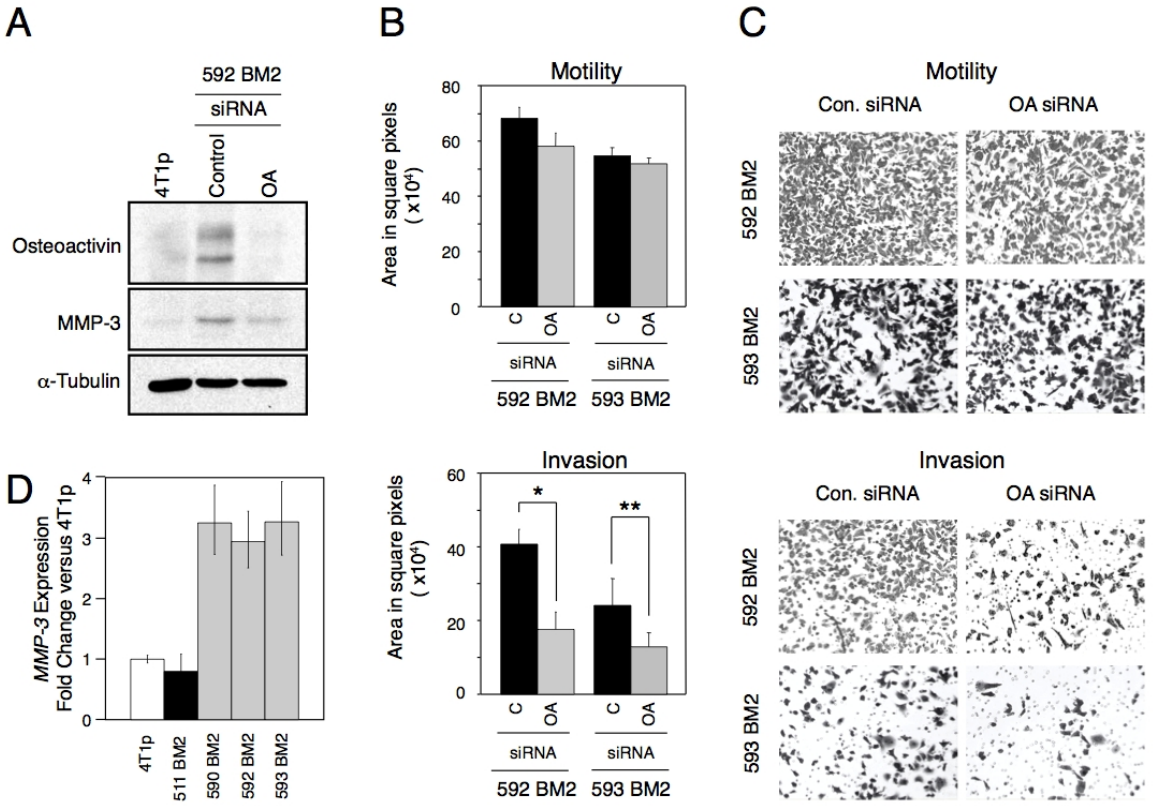

A
592 BM2
siRNA
4T1p
Control
OA
Osteoactivin
MMP-3
α-Tubulin
B
Motility
Area in square pixels (x10⁴)
80
60
40
20
0
C
OA
siRNA
592 BM2
593 BM2
Invasion
*
**
C
Motility
Con. siRNA
OA siRNA
592 BM2
593 BM2
Invasion
D
MMP-3 Expression Fold Change versus 4T1p
4
3
2
1
0
4T1p
511 BM2
590 BM2
592 BM2
593 BM2

Figure 7

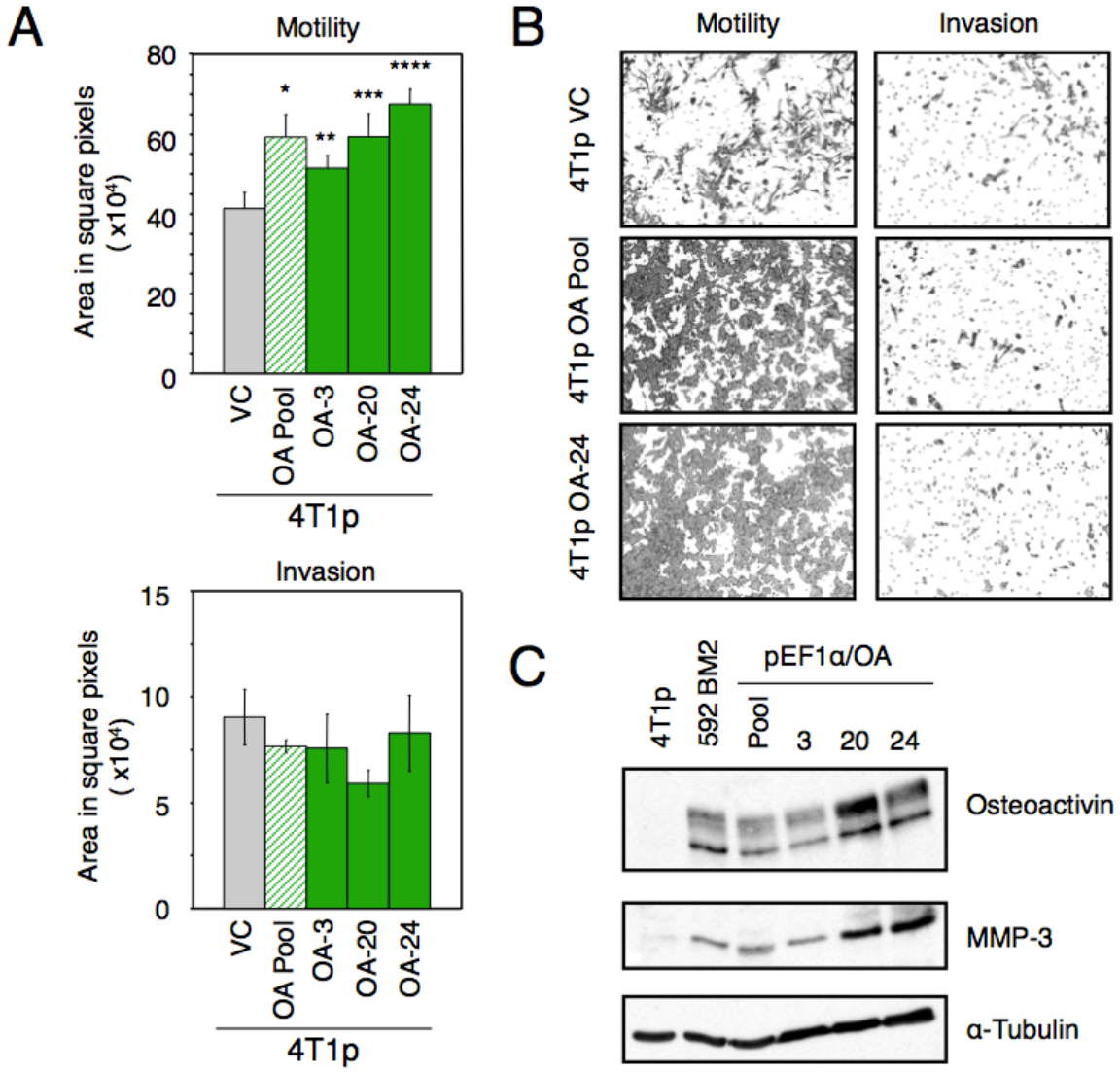

A
Motility
Area in square pixels (x10⁴)
80
60
40
20
0
*
**
***
****
VC
OA Pool
OA-3
OA-20
OA-24
4T1p
Invasion
15
10
5
0
B
Motility
Invasion
4T1p VC
4T1p OA Pool
4T1p OA-24
C
4T1p
592 BM2
pEF1α/OA
Pool
3
20
24
Osteoactivin
MMP-3
α-Tubulin

Figure 8

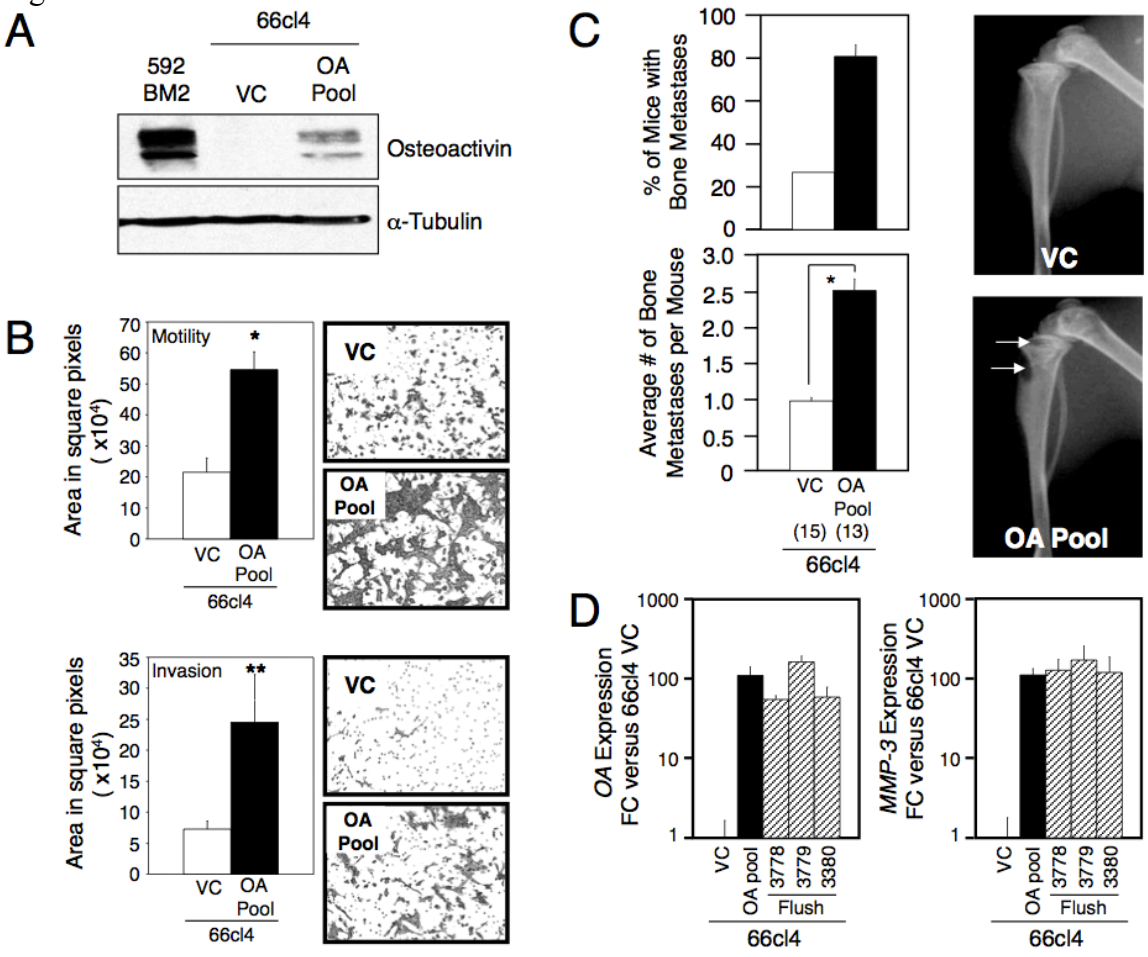
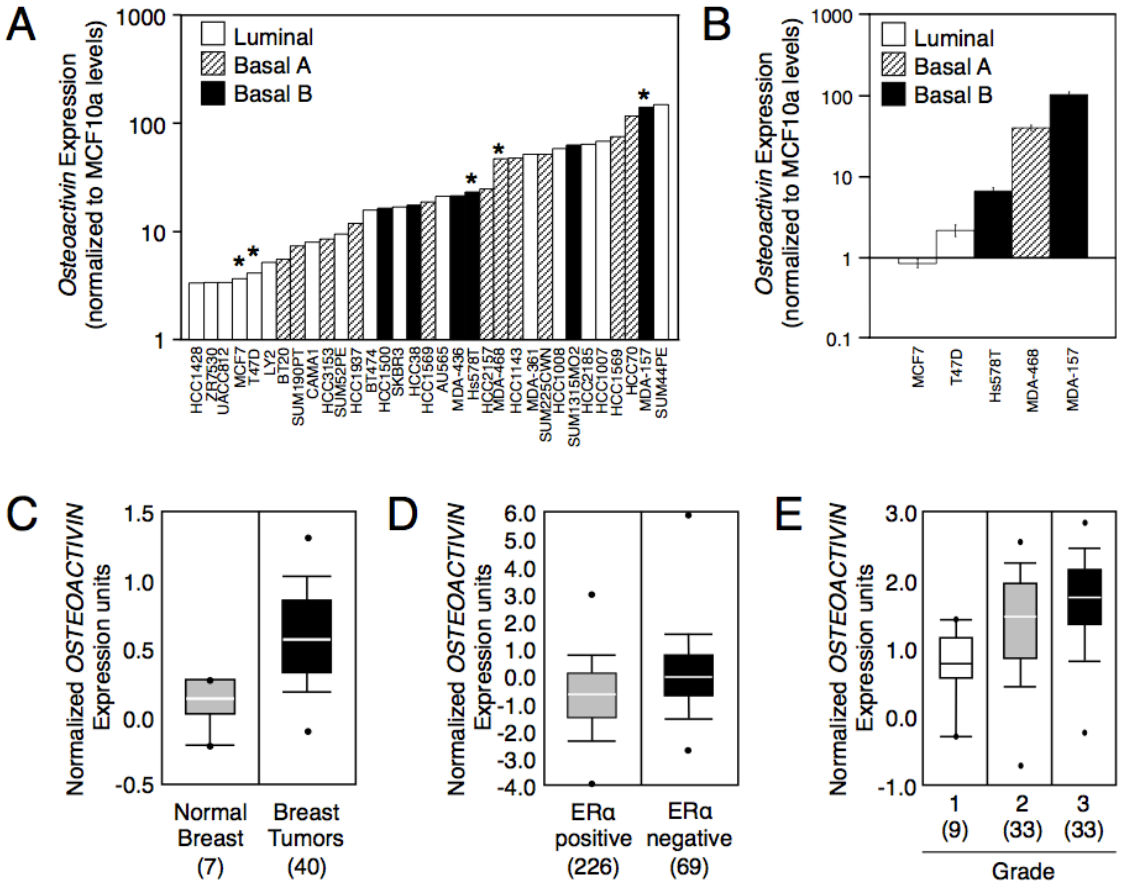

A
66cl4
592 BM2
VC
OA Pool
Osteoactivin
α-Tubulin
B
Motility
Area in square pixels (x10⁴)
VC
OA Pool
66cl4
Invasion
C
% of Mice with Bone Metastases
Average # of Bone Metastases per Mouse
(15) (13)
D
OA Expression FC versus 66cl4 VC
MMP-3 Expression FC versus 66cl4 VC
OA pool
3778
3779
3380
Flush

Figure 9


A
Osteoactivin Expression
(normalized to MCF10a levels)
1000
100
10
1
Luminal
Basal A
Basal B
HCC1428
ZR330
UACC812
MCF7
T47D
LY2
BT20
SUM190PT
CAMA1
HCC3153
SUM52PE
HCC1937
BT474
HCC1500
SKBR3
HCC38
HCC1569
AU565
MDA-436
Hs578T
HCC2157
MDA-468
HCC1143
MDA-361
SUM225CWN
HCC1008
SUM1315MO2
HCC2185
HCC1007
HCC1569
HCC70
MDA-157
SUM44PE
B
Osteoactivin Expression
(normalized to MCF10a levels)
1000
100
10
1
0.1
Luminal
Basal A
Basal B
MCF7
T47D
Hs578T
MDA-468
MDA-157
C
Normalized OSTEOACTIVIN
Expression units
1.5
1.0
0.5
0.0
-0.5
Normal
Breast
(7)
Breast
Tumors
(40)
D
Normalized OSTEOACTIVIN
Expression units
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
ERα
positive
(226)
ERα
negative
(69)
E
Normalized OSTEOACTIVIN
Expression units
3.0
2.0
1.0
0.0
-1.0
1
(9)
2
(33)
3
(33)
Grade

Chapter 3 – Glycoprotein nonmetastatic B is an independent prognostic indicator of recurrence and a novel therapeutic target in breast cancer

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Preface

In our previous work, we identified GPNMB as a gene that was up-regulated in bone metastatic breast cancer cells, which encoded a protein capable of promoting the formation of bone metastases *in vivo*. In recognition that CDX-011, a GPNMB-targeted therapy, was being investigated in clinical trials for the treatment of melanoma, we wanted to establish whether our findings using a mouse model of breast cancer metastasis were relevant to human breast cancer. To accomplish this, we investigated correlations between GPNMB expression (mRNA and protein) and patient outcomes in several gene expression and tissue microarray datasets. In an effort to provide further rationale for investigating CDX-011 in the treatment of metastatic breast cancer, we also set out to confirm that CDX-011 could effectively target and kill GPNMB-expressing breast cancer cells *in vitro* and using pre-clinical animal models.

Abstract

Purpose: While the murine orthologue of GPNMB (Glycoprotein non-metastatic B), Osteoactivin, promotes breast cancer metastasis in an *in vivo* mouse model, its importance in human breast cancer is unknown. We have examined the significance of GPNMB expression as a prognostic indicator of recurrence and assessed its potential as a novel therapeutic target in breast cancer.

Experimental Design: The clinical significance of GPNMB expression in breast cancer was addressed by analyzing GPNMB levels in several published gene expression datasets and two independent tissue microarrays derived from human breast tumors. GPNMB-expressing human breast cancer cell lines were further used to validate a toxin-conjugated anti-GPNMB antibody as a novel therapeutic agent.

Results: GPNMB expression correlates with shorter recurrence times and reduced overall survival of breast cancer patients. Epithelial-specific GPNMB staining is an independent prognostic indicator for breast cancer recurrence. GPNMB is highly expressed in basal and triple-negative (TN) breast cancers and is associated with increased risk of recurrence within this subtype. GPNMB expression confers a more migratory and invasive phenotype on breast cancer cells and sensitizes them to killing by CDX-011 (*glembatumumab vedotin*), a GPNMB-targeted antibody-drug conjugate.

Conclusions: GPNMB expression is associated with the basal/TN subtype and is a prognostic marker of poor outcome in patients with breast cancer. CDX-011

(*glembatumumab vedotin*) is a promising new targeted therapy for patients with metastatic TN breast cancers, a patient population that currently lacks targeted-therapy options.

Translational Relevance

Triple negative tumors constitute an aggressive subtype of breast cancer that is associated with poor disease outcome. Currently, no targeted therapies are available that effectively treat triple-negative tumors. However, there is substantial molecular heterogeneity within this subtype and some patients with triple negative tumors do not experience recurrence. Thus, there is great interest in identifying molecular markers that can identify the most aggressive of these tumors - particularly those representing targets for therapeutic intervention. Here we present the first evidence that GPNMB enhances the metastatic phenotype in triple negative breast cancer cells. Moreover, its expression predicts breast cancer recurrence across subtypes and specifically among patients with triple negative disease. Finally, GPNMB-expressing breast cancer cells are effectively killed by a novel toxin-conjugated anti-GPNMB antibody, termed CDX-011, which is currently being investigated in Phase II clinical trials as a promising therapy for patients with triple negative breast cancer.

Introduction

Breast cancer is a heterogeneous disease with respect to its histopathology and response to treatment. Gene expression analyses have classified primary human breast tumors into distinct molecular subtypes, which include normal-like, luminal, HER2-positive (HER2+) and basal-like breast cancers [65,67], which has implications for disease management [68]. Recent work indicates that tumors within a particular subtype display distinct organ-specific patterns of recurrence [71,273]. Basal-like breast cancers are more aggressive in nature, preferentially metastasize to brain and lung and are responsible for a disproportionate number of deaths [58]. Luminal breast tumors are generally responsive to hormonal therapies [274] whereas HER2+ tumors are treated primarily with HER2-targeted therapies such as trastuzumab or lapatinib. In contrast, no targeted therapeutic is currently available for patients with TN breast cancers. This deficiency in targeted treatment options, coupled with the frequency and pattern of metastasis associated with this subtype, accounts for the poor outcomes of patients with basal-like breast cancer.

GPNMB, also known as Osteoactivin, Dendritic Cell–Heparin Integrin Ligand (DC-HIL) or Hematopoietic Growth Factor Inducible Neurokinin-1 type (HGFIN), is a type I transmembrane protein [187,203,223]. The human and murine orthologues of this protein will be referred to as GPNMB and Osteoactivin, respectively. GPNMB is expressed at higher levels in several malignant human tissues relative to corresponding normal tissue [210,212,223]. Moreover, ectopic overexpression of GPNMB/Osteoactivin promotes invasion and metastasis of hepatocellular carcinoma, glioma and breast cancer cells [114,210,212]. Given its role as a mediator of metastasis and its cell surface expression, GPNMB is an attractive candidate for cancer therapy. In this regard, a GPNMB-specific

antibody conjugated to a cytotoxic drug, monomethylauristatin E, induces complete regression of GPNMB-expressing tumors derived from melanoma cell lines [226]. This agent (formerly CR011-vcMMAE, CuraGen) has recently been assigned the generic name of *glembatumumab vedotin*, also known as CDX-011, by Celldex Therapeutics.

In this study, we investigated GPNMB as a potential therapeutic target in human breast cancer. We analyzed GPNMB expression in several published breast cancer gene expression datasets and in primary human breast tumors. Our results indicate that GPNMB may serve as an important target for therapeutic intervention in breast cancer, particularly for patients with TN disease who do not benefit from currently available targeted therapies.

Results

GPNMB mRNA expression in human breast tumors is associated with reduced metastasis-free and overall survival. Our previous studies demonstrated that Osteoactivin enhances breast cancer cell motility, invasion and metastasis [114]. To determine the clinical relevance of this observation, we compared *GPNMB* mRNA levels with clinical outcome in three published datasets. *GPNMB* expression varied widely amongst the 295 breast tumors comprising the first dataset [74], with a 74-fold difference between tumors with highest and lowest *GPNMB* expression (Fig. 1*A*). Fewer tumors with high *GPNMB* expression belonged to the luminal A subtype (12.1%) compared to tumors with low and intermediate *GPNMB* expression (43.9% and 34.7%, respectively). Conversely, high *GPNMB*-expressing tumors were preferentially classified as basal-like (25.3%) relative to low and intermediate *GPNMB*-expressing tumors (11.2% and 10.5%,

respectively) (Fig 1*B*). Moreover, high *GPNMB* expression was associated with shorter metastasis-free (Fig. 1*C*) and overall survival times (Fig. 1*D*). We examined *GPNMB* transcript levels in two additional datasets, which contained 118 (Fig. S1*A*) [70] and 99 breast tumors (Fig. S2*A*) [104]. High *GPNMB*-expression was again enriched in the basal-like subtype (Fig. S1*B, S2B*) and correlated with poor outcome in breast cancer patients (Fig. S1C, S1D, S2*C*).

GPNMB protein expression in tumor epithelium is associated with poor outcome in human breast cancer. We next performed immunohistochemical staining for GPNMB using a breast tissue microarray (TMA1; Table S1), which contains normal breast, ductal carcinoma *in situ* (DCIS), tumor and lymph node metastasis tissue samples. All tissues were first classified as either GPNMB negative (< 5% positively stained tissue) or GPNMB positive (≥ 5% positively stained tissue). Using these criteria, only 3.5% of the normal breast tissue samples were considered GPNMB positive (Fig. S3*A*). In contrast, significantly higher percentages of DCIS lesions (26.8%) and malignant tissues (41.3 % of tumors; 15% of lymph node metastases) expressed GPNMB (Fig. S3*A*).

Given that GPNMB is expressed in both the tumor epithelium and stroma (Fig. S3*B*), we assessed the association between tumor epithelial or stromal GPNMB positivity and poor clinical outcome. GPNMB was localized in both epithelial and stromal tissue within the majority of GPNMB-positive cores (Fig. S3*B*). However, the degree to which GPNMB staining segregated between these tissue compartments was highly variable. We therefore classified tumors into three categories: negative (Fig. 2*A*), stromal positive (Fig. 2*B*) or epithelial-positive (Fig. 2*C*) based on where the predominant GPNMB staining occurred. Following these criteria, the majority of tumors were found to be GPNMB-

stromal (64.1%), followed by GPNMB-negative (25.2%) and finally GPNMB-epithelial (10.7%) (Fig. S4*A*). We demonstrate that high GPNMB levels within the tumor epithelium is significantly associated with reduced recurrence-free survival relative to patients that either lack, or display predominantly stromal patterns of GPNMB expression (Fig. 2*D*, S4*B*). Moreover, no significant difference was observed in recurrence-free survival in patients with GPNMB-negative versus GPNMB-stromal breast cancers (P = 0.3822) (Fig. S4*B*). When analyzed concurrently with established prognostic factors in a multivariate Cox model for recurrence-free survival, epithelial GPNMB staining stood out as an independent prognostic indicator of recurrence (P = 0.0199) (Table 1).

To validate the observation that GPNMB-epithelial staining is specifically associated with decreased overall survival, we interrogated a gene expression dataset derived from laser captured-dissected epithelial and stromal tissues from breast cancer patients [275,276]. High *GPNMB* mRNA expression within the epithelial, but not stromal compartment, was associated with reduced overall survival in breast cancer patients (Table S2). Taken together, these results demonstrate that high GPNMB expression within the tumor epithelium functions as an independent prognostic indicator of breast cancer recurrence.

GPNMB expression correlates with recurrence within the TN breast cancer subtype. The correlation between epithelial GPNMB expression and disease recurrence may be explained by the observation that GPNMB is most often expressed in aggressive basal/TN breast cancers (Fig. 1*B*). To facilitate and extend these analyses, we interrogated a second independent cohort of breast cancers (TMA2), which are enriched in TN breast cancer samples (Table S3). Using both TMAs (TMA1 and TMA2), we defined three subtypes

based on immunohistochemical staining for ER, PR and HER2. Thus, ER and/or PR positive tumors that were HER2 negative were classified as luminal (n=194), HER2 expressing tumors were classified as belonging to the HER2 subtype (n=69) and finally those tumors lacking ER/PR and HER2 expression were defined as TN (n=103). We investigated whether GPNMB expression, specifically in the tumor epithelium, correlated with histological subtype among 366 breast tumors. We observe that 29.1% of TN tumors are GPNMB-epithelial positive compared to only 3.6% of luminal and 11.6% of HER2 tumors (Fig. 3*A*). Given that epithelial GPNMB staining is an independent prognostic indicator of recurrence (Table 1), we determined whether epithelial-specific GPNMB expression is associated with breast cancer recurrence specifically within the TN subtype. Within the TN subtype, Kaplan-Meier survival analysis demonstrates that patients with GPNMB-epithelial positive tumors (n=30) display significantly shorter recurrence-free survival times relative to patients with GPNMB-negative or GPNMB-stromal positive tumors (combined, n=70) (Fig. 3*B*). Moreover, multivariate Cox regression survival analysis revealed that GPNMB still functioned as an independent prognostic indicator of distant metastasis in TN tumors (Table S4). This association between high GPNMB expression and increased incidence of distant metastasis was corroborated in an independent gene expression dataset [104] consisting of 30 TN breast cancer patients (Table S5). Thus, not only is GPNMB-epithelial expression more common in TN breast cancers, but even within this subtype its expression correlates with an increased risk of recurrence.

GPNMB is a therapeutic target for CDX-011 in breast cancer cells. We have reported that GPNMB levels vary widely across established human breast cancer cell lines [114].

To determine whether GPNMB is sufficient to promote a motile and invasive phenotype in human breast cancer cells, we selected BT549 cells that represent a basal cell line [221] lacking endogenous GPNMB expression (Fig. 4*A*). Ectopic GPNMB expression significantly increased the invasiveness of BT549 breast cancer cells (Fig. 4*A*). Importantly, overexpression of GPNMB did not induce cell growth in BT549 cells (Fig. S5), indicating that GPNMB-mediated effects on breast cancer cell invasion cannot be attributed to the enhancement of cell growth. To examine whether GPNMB expression is necessary for an invasive phenotype, we transiently-expressed a GPNMB siRNA in SUM1315 cells, a human basal breast cancer cell line that expresses high endogenous GPNMB levels. We confirmed that GPNMB protein levels were reduced in SUM1315 cells transfected with GPNMB-specific siRNAs relative to cells treated with a scrambled control. We observed a statistically significant reduction in breast cancer cell invasion in GPNMB-siRNA expressing cells relative to control cells (Fig. 4*B*). These results confirm our earlier results and support a role for GPNMB in promoting the motility and invasion of basal breast cancer cells.

Our data establishing an association between GPNMB expression and poor outcome in TN breast cancer, coupled with its cell surface expression and ability to promote breast cancer motility and invasion, make it an attractive candidate for targeted therapies. Indeed, cell surface expression of GPNMB was readily detectable in cells that endogenously (MDA-MB-361, MDA-MB-468) or exogenously (BT549, MDA-MB-453) expressed GPNMB (Fig. 5*A*, Fig. S6*A*). To determine if GPNMB represents a feasible target for breast cancer therapy, we tested the effects of CDX-011, an antibody-drug conjugate that specifically targets GPNMB, on tumor cell growth and survival. This antibody conjugate can kill GPNMB-expressing melanoma cells [223,226]. Breast cancer

cells expressing low (MDA-MB-453-VC, BT549-VC), moderate (MDA-MB-361, MDA-MB-468) or high (MDA-MB-453-GPNMB, BT549 GPNMB) levels of cell surface GPNMB were incubated with increasing concentrations of CDX-011 (Fig. 5*B*). The growth of both moderate and high GPNMB-expressing cells was inhibited by CDX-011 in a dose-dependent manner, whereas an IC_{50} was not achieved with concentrations up to 10ug/mL CDX-011 in low GPNMB-expressing cells. Unconjugated CDX-011 or isotype control antibodies were unable to induce this effect (Fig. S6*B*). Treatment of breast cancer cells with the CDX-011 drug conjugate lead to elevated apoptosis in GPNMB-expressing breast cancer cells, as indicated by increased cleaved caspase-3 levels (data not shown).

We next examined whether administration of CDX-011 could impair breast cancer growth *in vivo*. To accomplish this, MDA-MB-468 cells were injected into the mammary fat pads of nude mice and allowed to grow to a tumor volume of 125 mm^3. Tumor bearing mice were then divided into two groups, one which received a single injection of 20mg/kg of CDX-011 while the other cohort received control PBS injection. We observed a significant diminishment in tumor growth in mice receiving the CDX-011 conjugate compared to PBS controls (Fig. 5*C*). These data show that CDX-011 effectively targets and kills breast cancer cells that express GPNMB at the cell surface. Given that tumor epithelial-GPNMB expression is associated with poor outcome within the triple negative subtype, this GPNMB-targeted conjugate represents a novel therapeutic option for treating basal-type breast cancer patients.

Discussion

The molecular classification of breast cancer underscores the heterogeneity of this disease [65,67,68]. The poor prognosis associated with TN breast cancer, coupled with the lack of therapeutic targets, has created intense clinical interest in these tumors [57,59,62,277]. Recent studies have reported that TN tumors with basal-like features (those expressing some or all of the following proteins: CK5/6, CK14, CK17, EGFR) are associated with worse clinical outcomes than TN tumors lacking these markers [57,278]. Although it is unknown whether GPNMB contributes to the basal–like phenotype, our observations identify GPNMB as a prognostic marker in TN breast cancers and support the clinical development of GPNMB-targeted therapies. Interestingly, recent evidence suggests that signaling through the estrogen receptor can suppress GPNMB expression [279,280], which is consistent with our observation that GPNMB is more commonly expressed in TN breast cancers.

An unexpected finding of this study was the heterogeneous GPNMB staining observed among the various tumor compartments, with high levels of GPNMB evident in tumor stroma relative to normal tissue. This is supported by independent gene-expression profiling studies that reveal higher GPNMB levels in tumor-associated stroma compared to that derived from normal breast [275,281]. Within the stromal compartment, an independent study identified increased GPNMB expression in tumor-derived endothelium relative to normal endothelial cells [215]. GPNMB is highly expressed in dendritic cells [165] and macrophages [203], raising the possibility that some of the stromal staining within primary breast tumors may represent immune cell infiltrates. Moreover, Osteoactivin expression has been linked to fibroblast activation [205], and thus might be expressed in cancer-associated fibroblasts. While our studies indicate that GPNMB expressed within the cancer epithelium is associated with disease recurrence, the role of

stromal GPNMB in supporting the tumor microenvironment is intriguing, and warrants further investigation.

We provide the first evidence of a relationship between GPNMB expression in primary breast tumors and metastatic occurrence. We are the first to demonstrate that GPNMB-expressing breast cancer cells can be selectively killed by a toxin-conjugated antibody directed against GPNMB (CDX-011). Cancer therapy employing toxin/drug-conjugated antibodies is becoming increasingly popular [225] and includes a cytotoxin-conjugated version of Herceptin, Trastuzumab-DM1, which is currently being investigated in clinical trials for metastatic breast cancer [225,282]. In a Phase I/II clinical trial for the treatment of melanoma, CDX-011 was shown to have clinical activity and was well tolerated [225]. Moreover, initial results from an ongoing Phase I/II trial show that tumor shrinkage was observed in CDX-011-treated patients with metastatic breast cancer. Our observations that GPNMB is highly expressed in recurrent breast cancers but rarely in normal breast tissue, coupled with our observations that CDX-011 effectively inhibits the growth of GPNMB-expressing breast cancer cells *in vitro*, suggest that GPNMB represents a promising therapeutic target in breast cancer.

We demonstrate that epithelial-specific GPNMB expression is an independent prognostic indicator of recurrence. Therefore, IHC staining of biopsy material for epithelial GPNMB expression could be used to predict responders to CDX-011 in future clinical trials. The molecular processes that modulate cell surface expression of GPNMB, such as trafficking, internalization, and shedding of its extracellular domain [190,283] must be characterized in order to optimize GPNMB-targeted therapies. Such research will provide important insights into the molecular mechanisms through which GPNMB exerts its effects on breast cancer progression.

Materials and Methods

Analysis of published gene expression datasets. GPNMB expression levels were studied in published human breast cancer datasets [70,74,104] using the following probes: probe ID 1855, NM_002510 [74] or probe ID: 201141_at [70,104]. Fold-change expression values were generated by first normalizing the expression value for an individual tumor to the average expression value across all tumors. Normalized expression values were then $\log_{10}$ transformed, tumors segregated into three equivalent groups and subsequently defined as possessing "high", "intermediate", and "low" GPNMB expression. The Kruskal-Wallis non-parametric test, with normalized GPNMB expression used as the measurement variable, was employed to measure the statistical significance of its variance according to subtype. Associated clinical data for each tumor were used to generate Kaplan-Meier survival curves. Statistical analyses were performed with MedCalc (v9) software (MedCalc Software, Mariakerke, Belgium).

Patient information and tissue microarray (TMA). Two independent TMAs were employed to study GPNMB expression in breast cancer. The first study cohort (TMA1) consisted of 234 patients who underwent breast surgery between 1999 and 2003 at the McGill University Health Centre (MUHC). Paraffin blocks and corresponding slides were retrieved from the clinical pathology archive and assessed by an attending clinical pathologist for inclusion in a TMA. 169 areas containing invasive carcinoma, 31 areas containing ductal carcinoma *in situ* (DCIS), 50 areas of lymph nodes with evidence of metastatic disease, and 50 areas of normal/benign breast tissue were identified under

microscopic investigation, and 2 x 0.6 mm cores were extracted from each of the corresponding areas of the paraffin blocks and used to construct TMA blocks.

The second study cohort (TMA2) consisted of 209 patients diagnosed with primary breast cancer between 2003 and 2008 at the Centre Hospitalier de l'Université de Montréal (CHUM). Histological grade was diagnosed according to Nottingham's classification, as modified by Elston and Ellis [20]. The cohort consisted of both low- (n=36) and high-grade (n=140) ductal carcinomas and typical (n=13) and atypical (n=20) medullary carcinomas.

Data for pathological variables reported as per the clinical criteria in use at time of examination (pathological stage, histological grade, tumor size, ER, PR and HER2 receptor status) was collected from the original pathology reports. In cases where HER2 status was equivocal (TMA1), fluorescence in situ hybridization (FISH) was performed to derive a definitive assignment. For TMA2, only tumors that stained 3+ were considered HER2 positive. Clinical data was collected from initial interviews with patients as well as examination of medical records housed at the MUHC and the CHUM. Tables S1 and S3 contain further information on the clinico-pathologic characteristics of patients whose tumors were included on TMA1 and TMA2, respectively. Clinical follow-up for patients on both TMAs was conducted through annual review of medical records between the surgery date and November 2009. In this period, we documented death from breast cancer or from other causes unrelated to cancer (TMA1), as well as distant metastasis and/or local recurrence of disease (TMA1 and TMA2). These studies were approved by the Research Ethics Board of the MUHC (TMA1; studies SDR-99-780, SDR-00-966 and SDR-04-022) or the Research Centre Ethics Committee at the CHUM (TMA2; study SL05.019).

TMA immunohistochemical staining and analysis. Immunohistochemical staining was performed according to standard procedures using a polyclonal goat anti-GPNMB antibody (1:500 dilution; R&D Systems, Cat. # AF2550) and a biotin-conjugated donkey anti-goat secondary antibody (1:500 dilution; Jackson ImmunoResearch Laboratories; Cat. #705- 065-147). Sections were developed with 3-3-diaminobenzidine-tetrahydrochloride and counterstained with hematoxylin.

The initial analysis of GPNMB staining on TMA1 (normal, DCIS, tumor and lymph nodes) is described in detail in the supplementary methods. Subsequent analysis of both TMA1 and TMA2 was performed as follows: each individual core was evaluated for GPNMB positivity using a two-tiered system. Staining intensity (0: negative; 1+: mild; 2+ moderate; 3+: strong) and percentage of positive cells belonging to either the epithelial or stromal compartments were reported by a pathologist (L.G.) and an independent observer (A.R.).

Statistical analysis of TMA data. Survival curves were calculated according to the Kaplan-Meier method with a logrank test for probability of survival. Recurrence-free survival was computed from the date the primary tumor was surgically removed to the date of disease recurrence or last follow-up. Dichotomization for survival analysis using multivariate Cox proportional hazards model (TMA1) were performed as follows: GPNMB status as epithelial or non-epithelial (stromal or negative), age as ≥ 45 y or < 45 y, HER2 status as positive or negative, estrogen receptor status as positive or negative, histological grade as grade 3 versus grades 1 or 2 and tumor size as > 20 mm or ≤ 20 mm. The median follow-up period for survival analysis was 6.11 years (range: 0.03-9.18

years). All statistical analyses were performed with MedCalc (v9).

Cell culture, transfections and FACS analysis. Cell lines used in this study were obtained from cultured according to ATCC guidelines. SUM1315 cells were obtained from Asterand Inc. (Detroit, MI., USA). The pEF1-GPNMB vector was constructed by ligating the full-length human GPNMB cDNA (Open Biosystems; Accession: BC032783) into a pEF1/V5-His expression vector (Invitrogen) using 5' *Eco* RI and 3' *Not* I restriction enzyme sites. BT549 and MDA-MB-453 cell lines were engineered to express GPNMB by LipofectAMINE 2000 (Invitrogen)–mediated transfection. GPNMB expressing cells are pools of 3 independent clones. Stable cell lines were maintained under 1 mg/mL G418 antibiotic selection. For flow cytometric analysis, cells were stained for cell surface GPNMB expression as previously described (11). Data analysis was performed with FlowJo software (v7.5) (Tree Star, Inc., Ashland, Oregon).

Immunoblotting. The following antibodies were used: GPNMB (1:2500 dilution; R&D Systems, Cat. # AF2550) and α-Tubulin (1:10,000 dilution; Sigma-Aldrich, Cat. #T6199). Appropriate HRP–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, USA) were used at a dilution of 1:10,000 and proteins were visualized by chemiluminescence (Millipore, Cat. #: WBKLS0500).

Motility and invasion assays. Motility and invasion assays were performed as previously described with minor modifications [114]. Briefly, $2x10^4$ BT549 cells were used for

migration assays. For invasion assays, $5x10^4$ BT549 cells were used and transwell inserts were pre-coated with a 6% Matrigel solution.

Transient knockdown of GPNMB in SUM1315 cells was accomplished by transfection (LipofectAMINE 2000, Invitrogen) using 75 nmol/L of the ON-TARGET*plus* SMARTpool (pool of 4 GPNMB-targeted siRNAs, Dharmacon) at $t = 0$ h. An ON-TARGET*plus* pool of four non-targeting (scrambled) siRNAs was used as a control. Cells were plated in transwell inserts for invasion assays at t=24h. Protein lysates were prepared at the end of the invasion assays (t=48h) to confirm efficient GPNMB knockdown over the duration of the experiment. For the invasion assays, $4x10^5$ SUM1315 cells were plated in triplicate wells, and results are cumulative from two experiments.

In vitro and in vivo growth inhibition/cytotoxicity assays. Breast cancer cells ($5x10^4$ cells for MDA-MB-453 and MDA-MB-361; $2.5x10^4$ cells for MDA-MB-468; $1x10^4$ cells for BT549) were seeded in 24-well tissue culture plates and allowed to adhere overnight. The following day, the medium was changed and cells were grown for 4 days in the absence or presence of the indicated concentrations of CDX-011, as previously described [223]. Viable cells were counted by Trypan blue exclusion using an automated cell counter (Cellometer Auto-T4, Nexcelcom Bioscience). CD1 nude mice (Charles River, USA) were injected with $5x10^6$ MDA-MB-468 breast cancer cells and monitored until tumors reached 125 mm^3. Tumor-bearing animals were divided into two groups and one cohort was injected I.V. with a single dose of CDX-011 (20mg/kg) suspended in PBS while the other was injected with PBS as a control. Tumor growth was monitored weekly by caliper measurement for 6 weeks post-treatment. Results from two independent experiments are shown.

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Tables

Table 1. Cox regression analysis for recurrence-free survival in 145 breast cancer patients (TMA1)

Prognostic Factors	Univariate			Multivariate		
	RR	95% CI	P Value	RR	95% CI	P Value
Epithelial GPNMB expression	2.63	1.21 – 5.74	0.0155	2.73	1.18 - 6.32	0.0199
Histological Grade (III vs. I and II)	7.06	2.74 – 18.17	< 0.0001	7.08	2.54 - 19.77	0.0002
ER (positive vs. negative)	0.34	0.15 – 0.55	0.0002	0.49	0.24 - 1.00	0.0505
HER2 (positive vs. negative)	2.74	1.37 – 5.50	0.0047	2.03	0.95 – 4.29	0.0666
Age (≥ 45 yrs vs. < 45 yrs)	0.22	0.26 – 1.35	0.2167	0.24	0.09 – 0.66	0.0057
Tumor Size (> 20mm vs. ≤ 20 mm)	3.73	1.89 – 7.32	0.0001	3.88	1.83 – 8.25	0.0004

Figures Legends

Figure 1. High *GPNMB* mRNA levels are associated with poor prognosis in human breast cancer. *A,* Relative *GPNMB* mRNA levels in a human breast tumor gene expression dataset [74]. *B,* Distribution of high, intermediate and low *GPNMB*-expressing breast tumors with respect to molecular subtype. Statistically significant differences between the variance in *GPNMB* expression across subtypes were determined using the Kruskal-Wallis test ($P < 0.0001$). Specifically, the distribution of low, intermediate and high GPNMB-expressing tumors in the Her2 and basal subtypes were distinct ($P<0.05$) from both the luminal A and normal subtypes, and luminal B was distinct ($P<0.05$) from the luminal A subtype. Kaplan-Meier survival analysis reveals that patients with high

GPNMB expressing tumors had significantly shorter *(C)* metastasis-free survival (*; P = 0.032) and *(D)* overall survival (*; P = 0.007).

Figure 2. GPNMB expression in breast tumor epithelium is a novel predictor of breast cancer recurrence. Representative images of breast tumor cores from TMA1 illustrating negative (*A*) stromal- (*B*) or epithelial-specific (*C*) GPNMB staining. *D,* Kaplan-Meier analysis of recurrence-free survival for patients with GPNMB-epithelial positive tumors and those with negative or GPNMB-stromal staining (*; P = 0.0024 for patients with GPNMB-epithelial positive tumors versus all other patients). Panels *A*-*C*, scale bar represents 100 μm.

Figure 3. GPNMB expression is associated with recurrence in TN breast tumors. *A,* Tumors on the tissue microarrays (TMA1 and TMA2) were classified as: luminal (n=194), HER2+ (n=63) or TN (n=101). The percentage of tumors within each subtype that are GPNMB-epithelial positive is shown. *; P < 0.0001 for patients with TN tumors relative to luminal tumors; **, P = 0.0082 for patients with TN tumors versus HER2 tumors. *B,* Kaplan-Meier analysis of recurrence-free survival for patients with TN breast cancer is shown. *; P = 0.0401 for GPNMB-epithelial TN tumors (n=30) versus GPNMB-negative/GPNMB-stromal positive TN tumors (n=70).

Figure 4. GPNMB expression is necessary and sufficient to promote breast cancer cell invasion. *A*, Immunoblot analyses in left panel showing ectopic expression of GPNMB in BT549 cells relative to empty vector controls (VC). Two glycosylated forms of GPNMB, which migrate at 115kDa and 80 kDa, are indicated by the arrows. α-Tubulin (55kDa)

was used as a loading control. Modified Boyden chamber assays were used to assess the migration and invasion of VC and GPNMB expressing BT549 cells. Quantification of migration and invasion assays is shown in the center panel and representative images are shown in the right panels. *; $P < 0.001$, **; $P < 0.0001$, T-test for independent samples (2-tailed). *B*, Immunoblot analysis in left panel showing transient siRNA-mediated reduction in GPNMB expression in SUM1315 cells versus cells treated with a scrambled siRNA control (as described in panel A). Quantification of invasion assays is shown in the center panel and representative images are shown in the right panels. *; $P < 0.0008$. T-test for independent samples (2 tails).

Figure 5. GPNMB is expressed at the cell surface of breast cancer cells and is a target of the novel therapeutic, CDX-011. *A,* FACS analysis on breast cancer cells that exogenously (BT549 and MDA-MB-453) or endogenously (MDA-MB-468 and MDA-MB-361) overexpress GPNMB. *B,* Cells were incubated with CDX-011 for the times indicated and the percentage of remaining adherent cells was quantified. The data is represented as a percentage of the adherent cells remaining in mock-treated cultures. Experiments were done in triplicate wells for a minimum of three independent experiments. *; $P < 0.05$, **; $P < 0.0001$, T-test for independent samples. *C*, Mice were injected with MDA-MB-468 cells into the mammary fat pad, allowed to reach a tumor volume of 125 mm^3 and then injected with a single dose of CDX-011 (20mg/kg) (n=7) or PBS (n=9) as a control. Values represent the percent tumor volume relative to the tumor volume measured one day prior to injection of the toxin-conjugated antibody or PBS control. Statistical differences in tumor growth between antibody-treated and PBS

injected controls were determined using the non-parametric Mann-Whitney test for serial measurements ($P = 0.0002$).

Figures

Figure 1

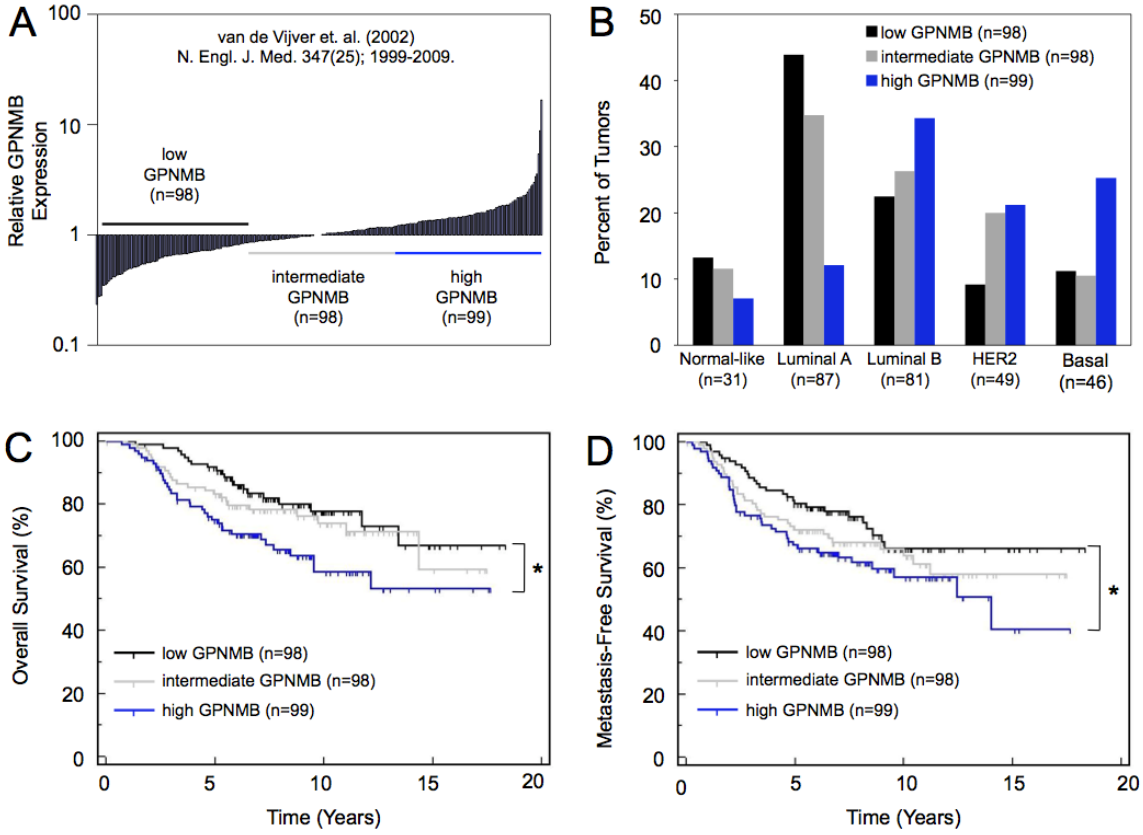
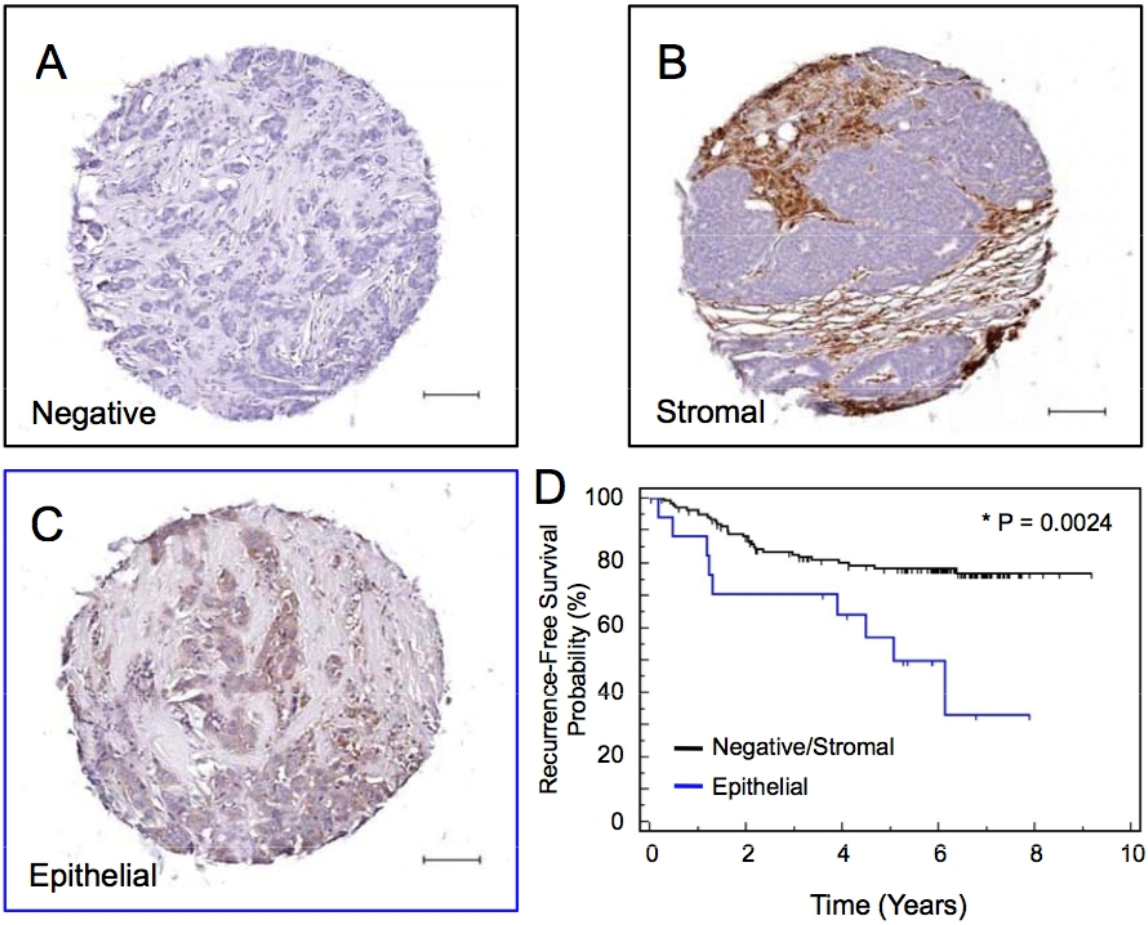


Figure 2


A
Negative
B
Stromal
C
Epithelial
D
Recurrence-Free Survival Probability (%)
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40
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0
* P = 0.0024
Negative/Stromal
Epithelial
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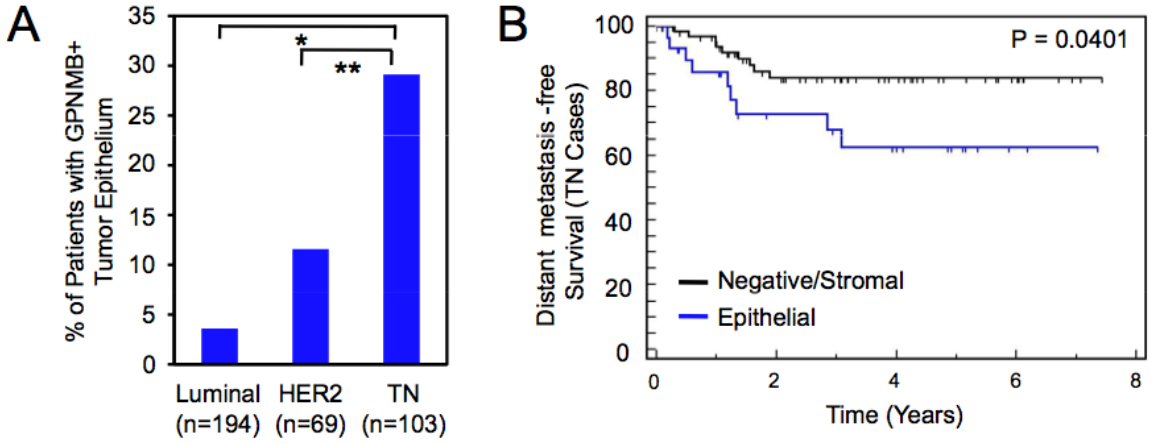
2
4
6
8
10
Time (Years)

Figure 3


A
% of Patients with GPNMB+ Tumor Epithelium
35
30
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Luminal (n=194)
HER2 (n=69)
TN (n=103)
B
Distant metastasis -free Survival (TN Cases)
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80
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P = 0.0401
Negative/Stromal
Epithelial
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4
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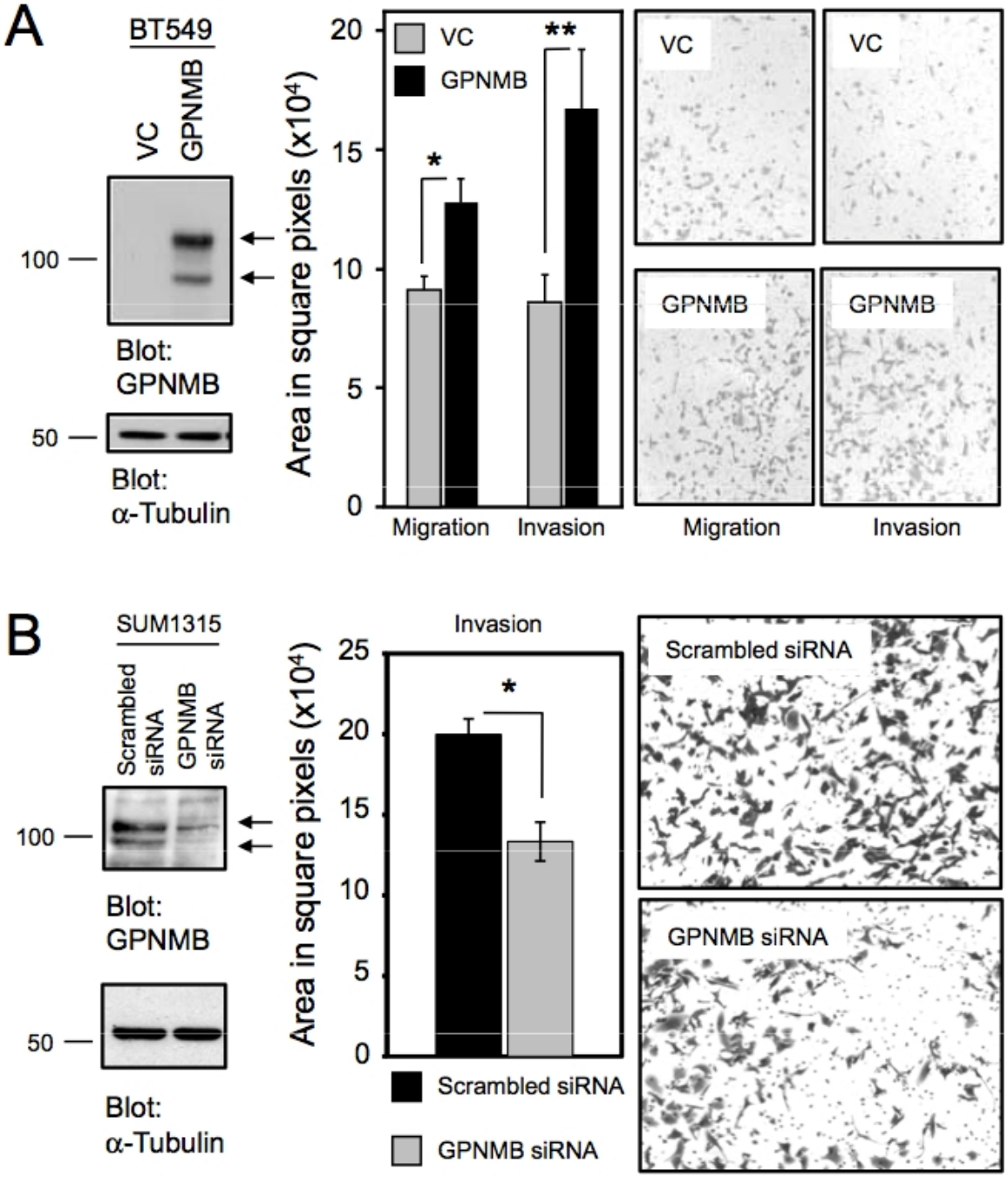
8
Time (Years)

Figure 4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BT549
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SUM1315
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α-Tubulin
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Area in square pixels (x10⁴)
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Scrambled siRNA
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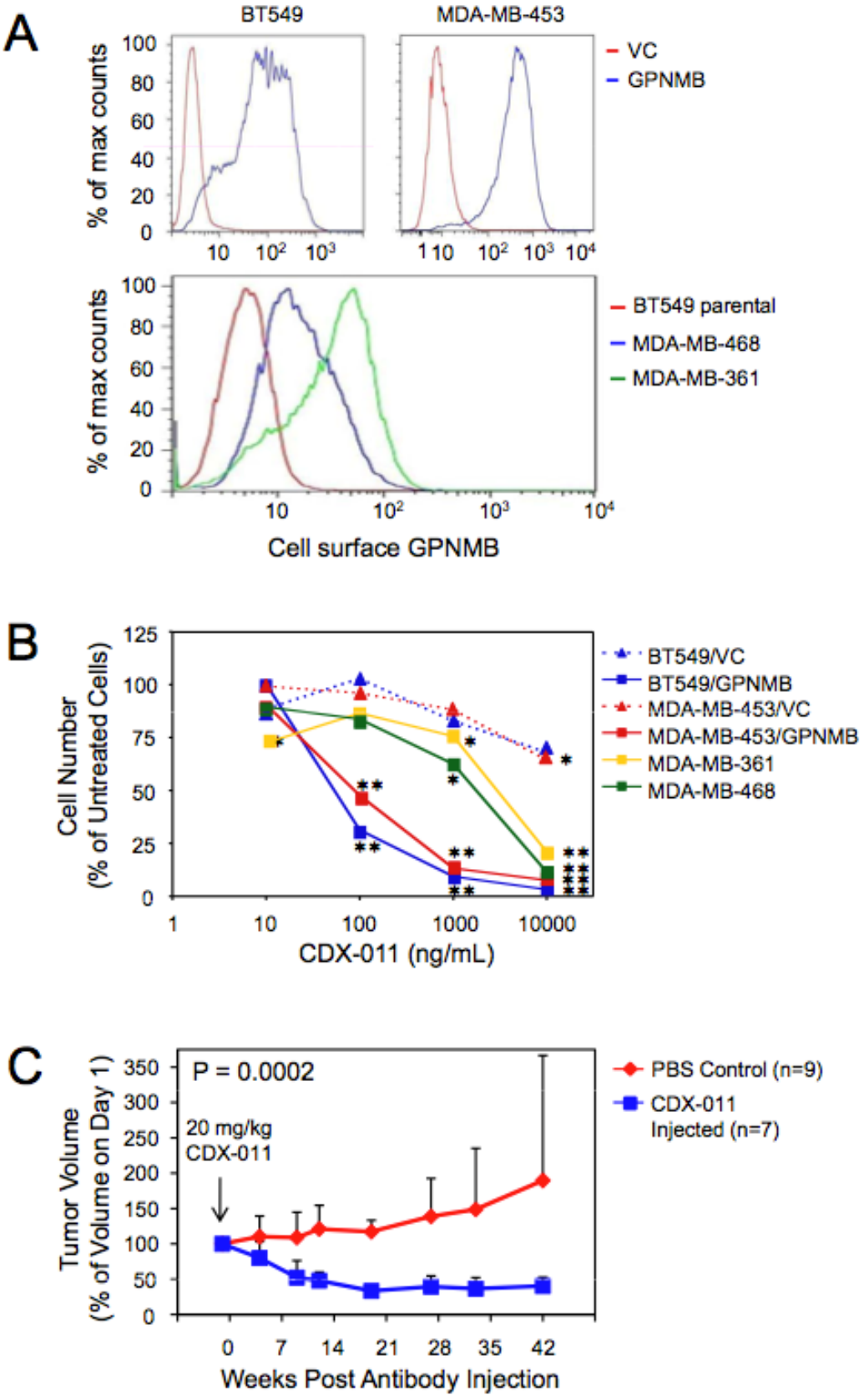
Figure 5


A
BT549
MDA-MB-453
% of max counts
VC
GPNMB
BT549 parental
MDA-MB-468
MDA-MB-361
Cell surface GPNMB
B
Cell Number
(% of Untreated Cells)
BT549/VC
BT549/GPNMB
MDA-MB-453/VC
MDA-MB-453/GPNMB
MDA-MB-361
MDA-MB-468
CDX-011 (ng/mL)
C
Tumor Volume
(% of Volume on Day 1)
P = 0.0002
20 mg/kg
CDX-011
PBS Control (n=9)
CDX-011
Injected (n=7)
Weeks Post Antibody Injection

Supplemental Materials and Methods

Analysis of published gene expression data. To interrogate clinical associations between *GPNMB* expressed in the epithelium or stroma of human breast cancers, *GPNMB* mRNA expression values derived from of laser-capture microdissected breast tumor epithelium and stroma were obtained from a published gene expression dataset [275,276]. Normalized continuous *GPNMB* expression values were used in a Cox proportional hazards regression model to analyze the statistical significance of correlations between epithelial or stromal *GPNMB* expression levels and survival among breast cancer patients (Table S2).

For analysis of the association between *GPNMB* expression and metastasis in triple negative tumors, all tumors (regardless of subtype) in the Minn *et. al.* dataset [104], were ordered based on levels of *GPNMB* expression as described in the Methods section. Tumors were then split into two equivalently sized groups and classified as "high" or "low" *GPNMB* expressers. For the majority of tumors, ER, PR and HER2 status, which was inferred from IHC staining, was available. Triple negative (TN) tumors were classified as those tumors lacking ER, PR and with a Her2 score < 3+. The number of involved metastatic sites was then compared for *GPNMB*-high and *GPNMB*-low patients with TN breast cancer (Table S5).

Quantification of immunohistochemical staining. 57 normal breast, 56 DCIS, 312 tumor and 93 lymph node metastasis cores with suitable integrity were present on TMA1. To quantify GPNMB staining (Supplemental Figure 3), the TMAs were first scanned at 20X magnification using an Aperio Scanscope XT slidescanner. Automated image

analysis was performed using Aperio Image scope (v9.1.19.1567) software and quantified with the Positive Pixel Count algorithm (v2). GPNMB positive pixels were sub-divided as being weakly (+1), moderately (+2) or strongly positive (+3) and the percentage of GPNMB positive pixels was calculated as: +2 and +3 positive pixels/total pixels. Tissue cores were considered positive if ≥ 5% of the core stained positively for GPNMB. In most cases two or more cores on the array represented a single tissue sample. The percentage of positive pixels was calculated for each core individually and the value associated with a given tissue represents the average positivity of all cores derived from that tissue. Fisher's exact test was used to assess statistically significant differences in the percentage of GPNMB-positive samples in DCIS, tumor or lymph node metastases relative to normal breast samples.

Cell Culture. For cell counting experiments (BT549, $5x10^3$ cells; MDA-MB-453; $1x10^4$ cells), cells were seeded on Day 0 and allowed to adhere overnight. The following day the media was changed and cells were counted 5 days after plating.

Flow Cytometry. Data analysis was performed with FloJo software (v7.5) (Tree Star, Inc., Ashland, Oregon) and the mean fluorescence intensity (MFI) was determined for each sample. Geometric mean ratios (GMR) of MFI for each cell line were calculated by dividing the MFI of cells stained with both primary and secondary antibodies by the MFI of cells stained with secondary antibody alone.

Supplementary Tables

Table S1. Clinicopathologic Characteristics of Patients with Invasive Cancer (TMA1).

Prognostic Factors	Total (%)
All Cases	159 (100)
Mean Age (range)	59.1 (27.1-89.1)
≥45	63 (39.6)
<45	96 (60.4)
Grade	
I	76 (51.0)
II	58 (36.5)
III	18 (11.3)
Estrogen Receptor (ER)	
Positive	123 (77.4)
Negative	35 (22.0)
Progesterone Receptor (PR)	
Positive	84 (52.8)
Negative	73 (45.9)
HER2	
Positive	28 (17.6)
Negative	127 (79.8)
Triple Negative	
Yes	19 (11.9)
No	139 (87.4)
Lymph Node	
Positive	70 (44.0)
Negative	62 (38.9)

Note: The number of cases in each category may not reach 159 due to lack of clinical data.

Table S2. Association of GPNMB expression in laser capture dissected epithelial or stromal tissue with overall survival in patients with breast cancer.

GPNMB mRNA expression[a]	P value[b]	Relative Risk (RR)[c]	Confidence Interval (95%)[d]
Epithelial Tissue[e]	0.035	2.84	1.08-7.47
Stromal Tissue[f]	0.254	2.28	0.56-9.41

[a] normalized GPNMB values expressed as a continuous variable
[b] derived from univariate Cox proportional hazards model
[c] The instantaneous relative risk of recurrence for an individual with an increase of 1 in the value of GPNMB, compared with another individual.
[d] 95% confident that the relative risk falls within the range indicated
[e] Data from 63 patients
[f] Data from 53 patients

Note: In 50 breast cancer samples, the Laser Capture microdissected material for epithelium and stoma from the same patient.

Table S3. Clinicopathologic Characteristics of all Patients (TMA2).

Prognostic Factors	Total (%)
All Cases	208 (100)
Mean Age (range)	58.0 (29-89)
≥45	175 (84.1)
<45	32 (15.3)
Grade	
I	35 (16.8)
II	15 (7.2)
III	157 (75.4)
Estrogen Receptor (ER)	
Positive	83 (39.9)
Negative	124 (59.6)
Progesterone Receptor (PR)	
Positive	74 (35.5)
Negative	133 (63.9)
HER2	
Positive	41 (19.7)
Negative	166 (79.8)
Triple Negative	
Yes	84 (40.3)
No	123 (59.1)
Lymph Node	
Positive	73 (35.1)
Negative	104 (50.0)

Note: The number of cases in each category may not reach 208 due to lack of clinical data.

Table S4. Cox proportional hazards regression model for distant metastasis-free survival in 85 TN breast cancer patients

Prognostic Factors	Univariate			Multivariate		
	RR	95% CI	P Value	RR	95% CI	P Value
Epithelial GPNMB expression	2.55	1.01-6.39	0.0477	4.12	1.30-13.06	0.0165
Histological Grade (III vs I and II)[a]	>100	0- >100	0.9644	>100	0- >100	0.9637
LN metastases (positive vs negative)	3.29	1.14-9.46	0.0280	3.40	1.10-10.49	0.0342
Age (≥45 versus <45)	0.38	0.14-0.94	0.0382	0.54	0.18-1.66	0.2871
Tumor Size (>20 versus </= 20)	1.32	0.51-3.39	0.5652	1.29	0.32-5.21	0.7205

[a] Of the 85 patients included in the multivariate analysis, only two were Grade I or II, the rest were Grade III

Table S5. High GPNMB mRNA expression correlates with metastasis in patients with triple negative breast cancer[a]

	Low GPNMB Expression (n=12)	High GPNMB Expression (n=18)	P value
Number of Patients with Lung Metastases	2	9	0.121[b]
Number of Patients with Bone Metastases	0	5	0.128[b]
Average Number of Metastases per patient	0.167	0.778	0.010[c]

[a] Data taken from Minn et al., (2005) Nature 436 (7050) 518-524.
[b] Fishers exact test (one tail)
[c] Student's T-test (two tail)

Supplemental Figure Legends

Figure S1. High levels of *GPNMB* mRNA expression are associated with poor prognosis in human breast cancer. *A,* Relative levels of *GPNMB* mRNA expression in human breast tumors from a published gene expression dataset [70]. *(B)* Distribution of high, intermediate and low *GPNMB*-expressing breast tumors with respect to molecular subtype. Statistically significant differences between the variance in *GPNMB* expression across subtypes were determined using the Kruskal-Wallis test (P =0.0495). Specifically, the distribution of low, intermediate and high *GPNMB*-expressing tumors in the HER2 and basal subtypes were distinct (P<0.05) from the luminal A subtype. Kaplan-Meier survival analysis reveals that patients with high *GPNMB*-expressing tumors had significantly shorter *(C)* recurrence-free survival (*; P = 0.029) and *(D)* overall survival (*; P = 0.013).

Figure S2. High levels of *GPNMB* mRNA expression are associated with poor prognosis in human breast cancer. *A,* Relative levels of *GPNMB* mRNA expression in human breast tumors from a published gene expression dataset [104]. *B,* High, intermediate and low *GPNMB* expressing breast tumors partition differently with respect to molecular subtype: Luminal (ER and/or PR positive, Her2<3+), Her2 (Her2 3+), Triple Negative (ER and PR negative, Her2< 3+). Differences between the proportion of *GPNMB* high versus low and intermediate expressing breast tumors belonging to each subtype were analyzed using the Kruskal-Wallis test (P = 0.059). Kaplan-Meier survival analysis reveals that patients with high *GPNMB* expressing tumors had significantly shorter *(C)* metastasis-free survival (*, P = 0.038).

Figure S3. GPNMB is expressed in malignant human breast tissue. Immunohistochemical staining with an anti-GPNMB antibody was performed on TMA1. These arrays consisted of 517 undamaged cores representing 34 normal, 35 DCIS, 161 breast tumor and 47 lymph node metastasis samples. All patient samples were represented by multiple (2-4) cores on the array. *A,* Cores with ≥ 5% of the tissue expressing GPNMB were considered positive. The indicated P values for each sample type relative to normal tissue (*;P = 0.001, **; P = 9.460×10^{-8}, ***; P = 0.030) were obtained using Fisher's Exact Test. *B*, Representative images of normal (*i*), DCIS (*ii*), breast tumor (*iii*) and lymph node metastasis (*iv*) samples are shown. In panel *Biii*, the red arrow indicates epithelial staining and the black arrow denotes stromal staining. Scale bar represents 100 μm.

Figure S4. Epithelial-specific GPNMB staining is associated with shorter time to recurrence in patients with breast cancer. *A*, The percentage of invasive cancers (TMA1) that were negative, GPNMB-stromal positive or GPNMB-epithelial positive are shown. *B*, Kaplan-Meier analysis of recurrence-free survival for patients with GPNMB-negative, GPNMB-stromal positive and GPNMB-epithelial positive tumors. Overall; P = 0.0073; GPNMB-epithelial positive versus GPNMB-negative, P = 0.0406; GPNMB-epithelial positive versus GPNMB-stromal positive, P = 0.0020; GPNMB-stromal positive versus GPNMB-negative, P = 0.3822.

Figure S5. GPNMB expression correlates with diminished growth of breast cancer cells *in vitro*. Cell counting experiments in BT549 and MDA-MB-453 breast cancer cells

engineered to overexpress wild type (WT) GPNMB compared to those harboring an empty vector control. *, P = 0.025; **, P < 0.001).

Figure S6. The growth of GPNMB-expressing cells are specifically impaired by CDX-011. *A*, Quantification of GPNMB cell surface expression by FACS analysis. For each cell line, the Geometric Mean Ratio (GMR) of cell surface GPNMB expression is given. *B*, BT549 VC and GPNMB expressing cells were incubated for four days in the absence of antibody (ctrl) or with 100ng/mL of the following antibodies: CDX-011, a cytotoxin conjugated irrelevant control antibody, PK16.3-VCMMAE, or an unconjugated GPNMB targeted antibody, CDX-011. At the end of the assay, adherent treated cells were counted and represented as a percentage of the adherent, untreated cells for each cell line. Experiments were done in triplicate wells for a minimum of three independent experiments. *; P < 0.0001, T-test for independent samples.

Supplemental Figures

Figure S1

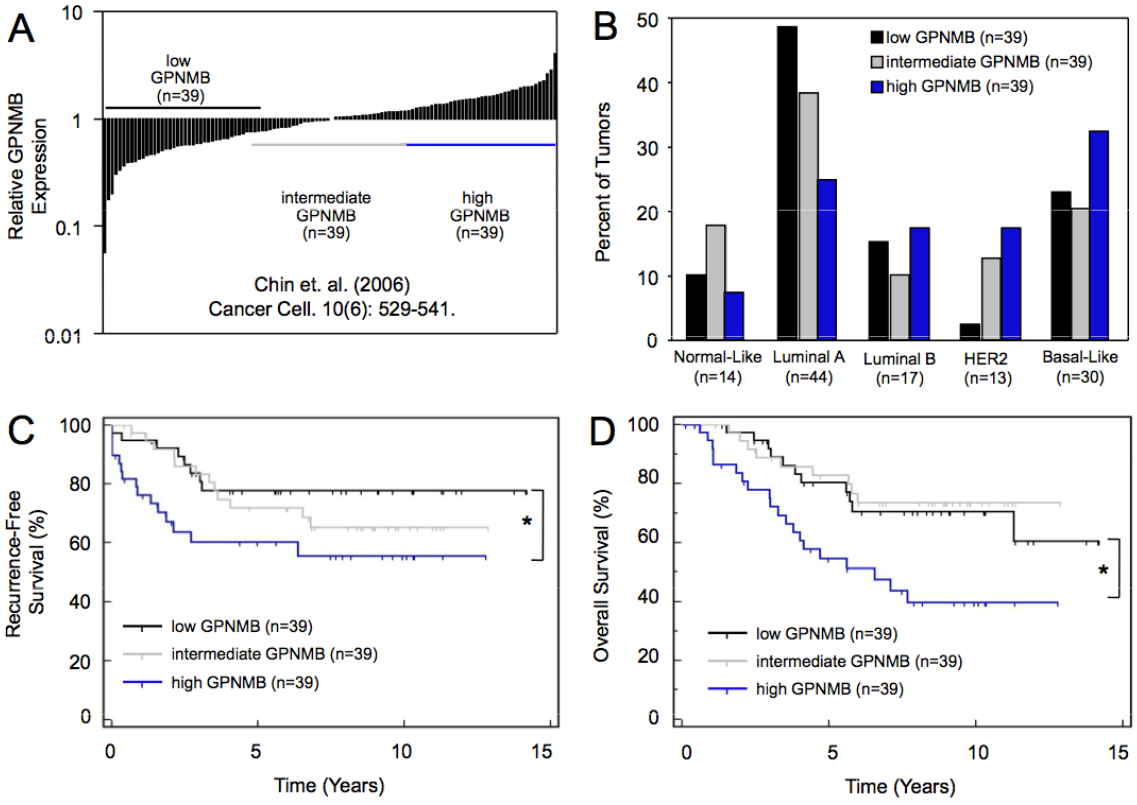


Figure S2

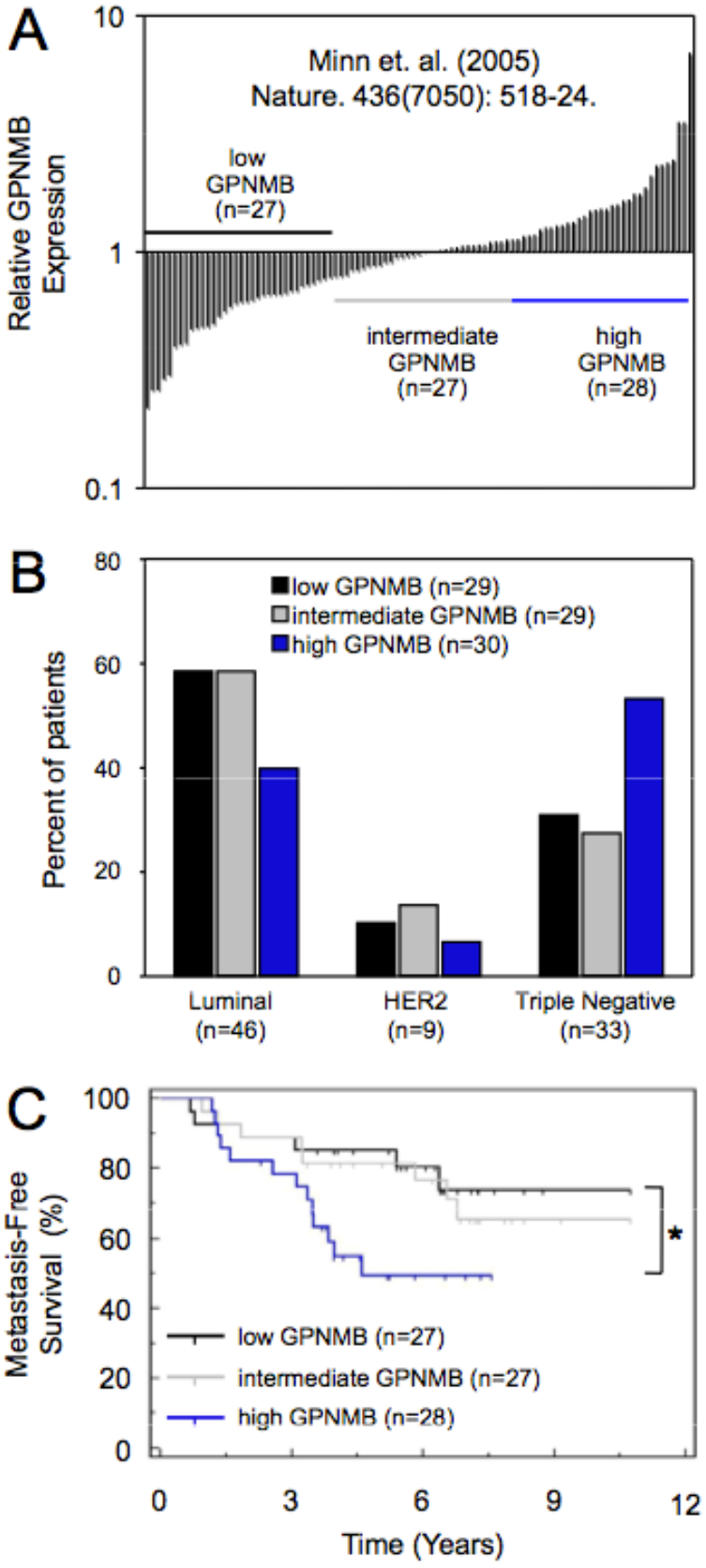

A
Minn et. al. (2005)
Nature. 436(7050): 518-24.
Relative GPNMB Expression
10
1
0.1
low GPNMB (n=27)
intermediate GPNMB (n=27)
high GPNMB (n=28)
B
Percent of patients
80
60
40
20
0
low GPNMB (n=29)
intermediate GPNMB (n=29)
high GPNMB (n=30)
Luminal (n=46)
HER2 (n=9)
Triple Negative (n=33)
C
Metastasis-Free Survival (%)
100
80
60
40
20
0
*
low GPNMB (n=27)
intermediate GPNMB (n=27)
high GPNMB (n=28)
0
3
6
9
12
Time (Years)

Figure S3

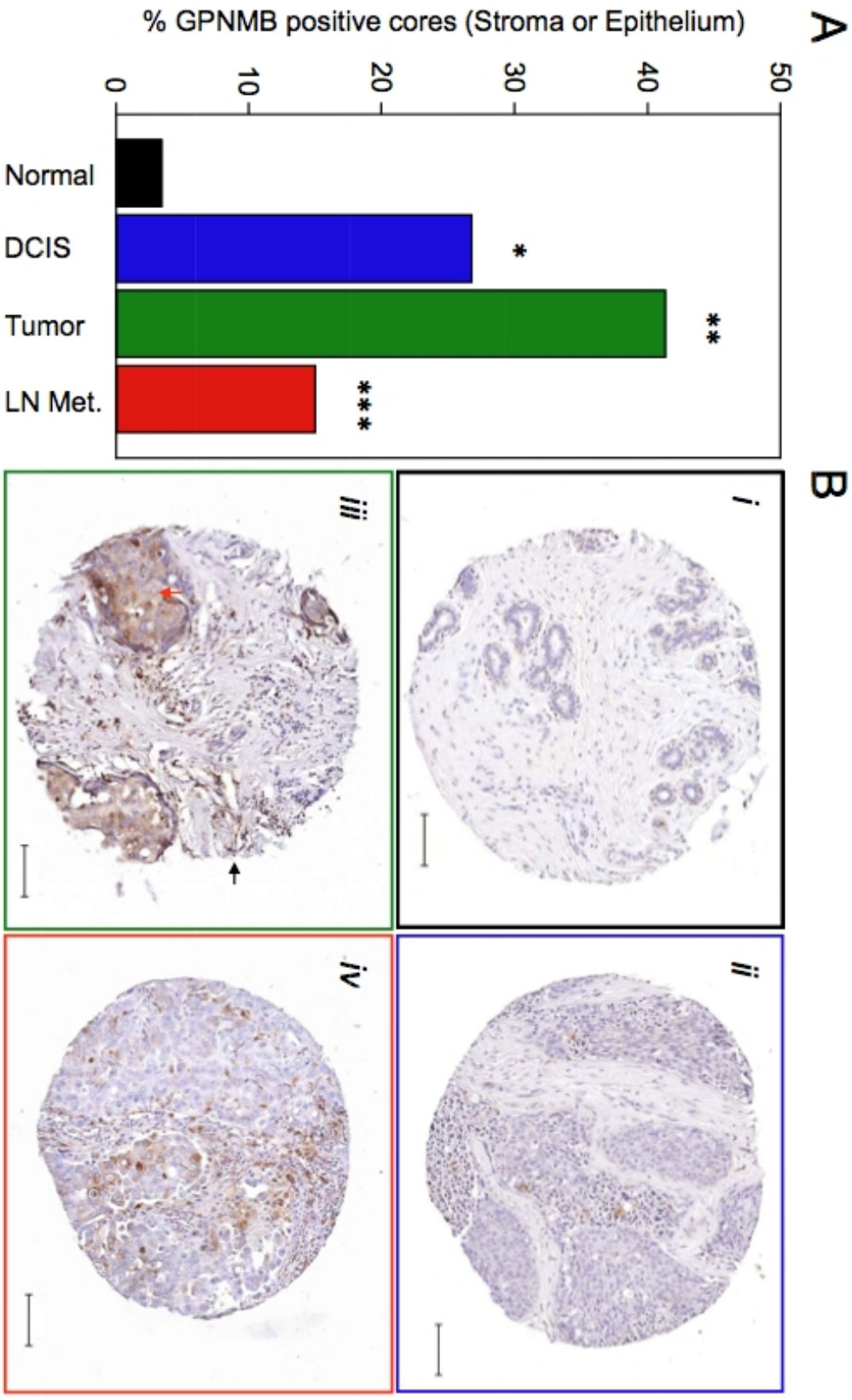

A
% GPNMB positive cores (Stroma or Epithelium)
0
10
20
30
40
50
Normal
DCIS
Tumor
LN Met.
*
**
***
B
i
ii
iii
iv

Figure S4

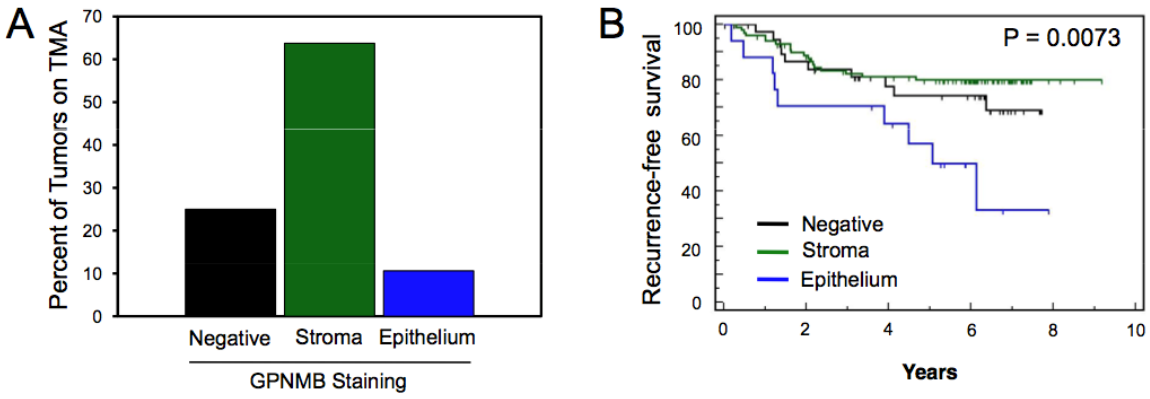

A
Percent of Tumors on TMA
70
60
50
40
30
20
10
0
Negative
Stroma
Epithelium
GPNMB Staining
B
Recurrence-free survival
100
80
60
40
20
0
P = 0.0073
Negative
Stroma
Epithelium
0
2
4
6
8
10
Years

Figure S5

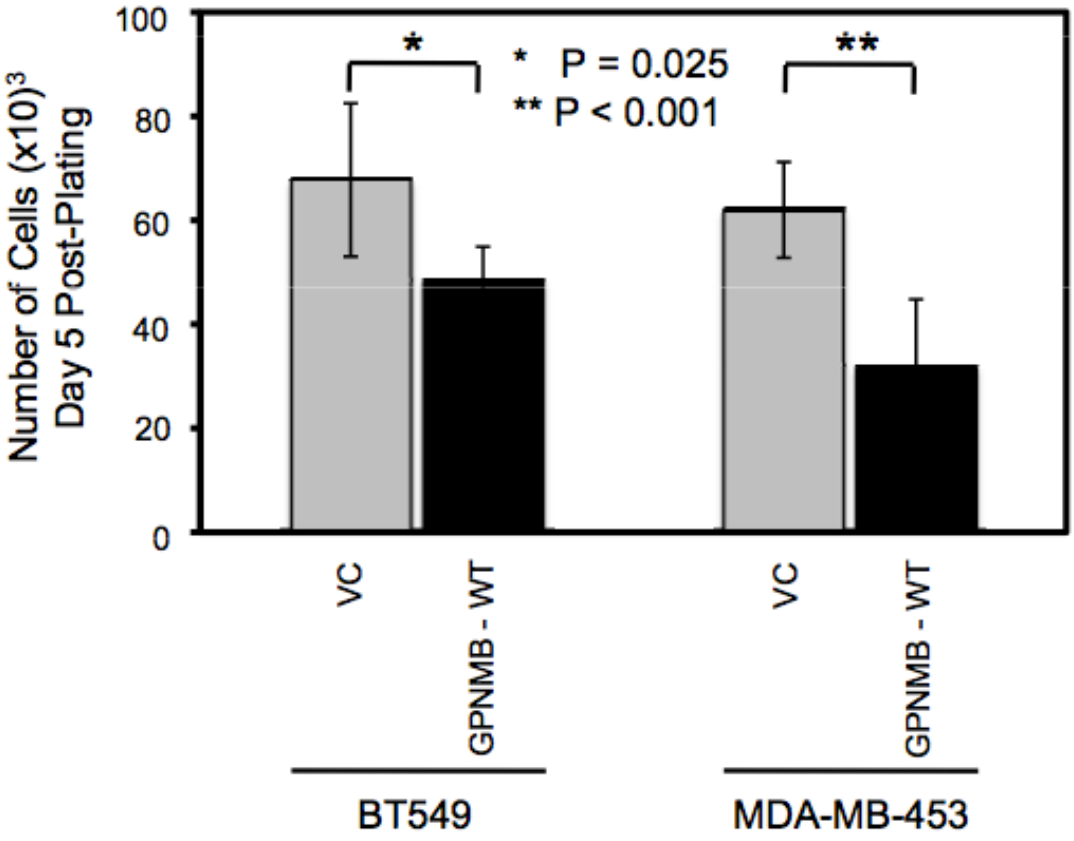
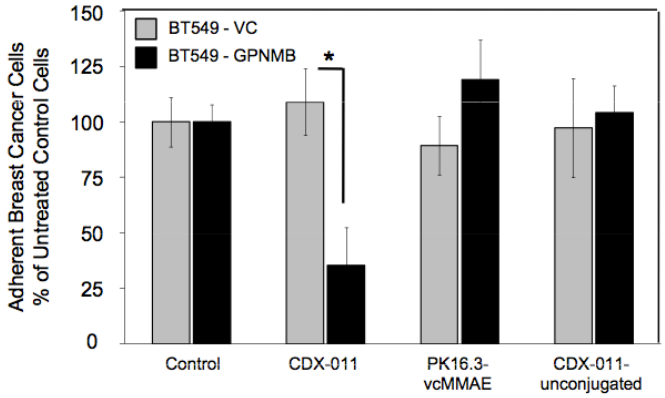

Number of Cells (x10)³
Day 5 Post-Plating
100
80
60
40
20
0
*
**
* P = 0.025
** P < 0.001
VC
GPNMB - WT
VC
GPNMB - WT
BT549
MDA-MB-453

Figure S6

A

Cell Line	GMR
BT549/VC	1.15
BT549/GPNMB	25.1
MDA-MB-453/VC	1.61
MDA-MB-453/ GPNMB	44.8
MDA-MB-361	7.72
MDA-MB-468	3.35

B



Supplemental References

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CHAPTER 4 - ADAM10 releases a soluble form of the GPNMB/Osteoactivin extracellular domain with angiogenic properties

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Preface

In our previous studies, we identified GPNMB as a novel mediator of breast cancer metastasis, a prognostic marker for recurrence and a viable therapeutic target. However, very little was known about mechanisms of GPNMB function in breast cancer progression. In this study, we set out to elucidate GPNMB function(s) in promoting tumor growth and to begin characterizing the functional domains that are important for GPNMB function.

Abstract

BACKGROUND: Glycoprotein non-metastatic melanoma protein B (GPNMB)/Osteoactivin (OA) is a transmembrane protein expressed in 40-75% of breast cancers. GPNMB/OA promotes the migration, invasion and metastasis of breast cancer cells; it is commonly expressed in basal/triple-negative breast tumors and is associated with shorter recurrence-free and overall survival times in patients with breast cancer. Thus, GPNMB/OA represents an attractive target for therapeutic intervention in breast cancer; however, little is known about the functions of GPNMB/OA within the primary tumor microenvironment.

METHODOLOGY/PRINCIPLE FINDINGS: We have employed mouse and human breast cancer cells to investigate the effects of GPNMB/OA on tumor growth and angiogenesis. GPNMB/OA-expressing tumors display elevated endothelial recruitment and reduced apoptosis when compared to vector control cells. Primary human breast cancers characterized by high vascular density also display elevated levels of GPNMB/OA when compared to those with low vascular density. Using immunoblot and ELISA assays, we demonstrate the GPNMB/OA ectodomain is shed from the surface of breast cancer cells. Transient siRNA-mediated knockdown studies of known sheddases identified ADAM10 as the protease responsible for GPNMB/OA processing. Finally, we demonstrate that the shed extracellular domain (ECD) of GPNMB/OA can promote endothelial migration *in vitro*.

CONCLUSIONS/SIGNIFICANCE: GPNMB/OA expression promotes tumor growth, which is associated with enhanced endothelial recruitment. We identify ADAM10 as a sheddase capable of releasing the GPNMB/OA ectodomain from the surface of breast

cancer cells, which induces endothelial cell migration. Thus, ectodomain shedding may serve as a novel mechanism by which GPNMB/OA promotes angiogenesis in breast cancer.

Introduction

Glycoprotein non-metastatic melanoma protein B (GPNMB) is a type I transmembrane protein that is also known as Osteoactivin (OA), Dendritic Cell–Heparin Integrin Ligand (DC-HIL) or Hematopoietic Growth Factor Inducible Neurokinin-1 type (HGFIN). GPNMB/OA is expressed in a wide array of normal tissue types including: the bone, hematopoietic system and the skin. Within the bone, GPNMB/OA has been shown to promote the differentiation of both osteoclasts [195,198] and osteoblasts [187,197]. GPNMB/OA is also readily detectable in immune cells, such as macrophages and dendritic cells [202,203], and has been shown to functionally impair T-cell activation [192,193]. Within the skin, GPNMB/OA has been proposed to be expressed specifically in melanocytes [168], while others suggest a broader pattern of expression that includes keratinocytes, melanocytes and Langerhans cells [193].

In addition to its diverse roles in normal cells, aberrant GPNMB/OA expression has been linked to various pathological disorders such as glaucoma [284], kidney disease [204], osteoarthritis [285] and several types of cancer, including: uveal melanoma [213], glioma [170,210], hepatocellular carcinoma [212] and cutaneous melanoma [223]. Recently, we demonstrated that GPNMB/OA is highly expressed in several aggressively bone-metastatic sub-populations of the 4T1 mouse mammary carcinoma cell line. Moreover, we showed that ectopic expression of GPNMB/OA in poorly metastatic 66cl4 mouse mammary carcinoma cells is sufficient to induce MMP-3 expression and increases their invasion *in vitro* and promotes bone metastasis *in vivo* [114]. Subsequently, we employed IHC-based analysis of tissue microarrays to investigate the relevance of GPNMB/OA expression in human breast cancer, and found that GPNMB/OA is

expressed in the tumor epithelium of approximately 10% of human breast cancers and the stromal compartment of nearly 70% of breast tumors. Moreover, epithelial, but not stromal, GPNMB/OA expression is a prognostic indicator of cancer recurrence across all breast cancer subtypes, and specifically within "triple negative" breast cancers [171].

GPNMB/OA is localized to diverse subcellular locations within the cell, including the plasma membrane of cancer cells [171,223], within melanosomes of melanoma cells [193] and within endocytic/lysosomal vesicles in osteoclasts [198]. Two *GPNMB/OA* mRNA isoforms encoding 560 and 572 amino acid proteins have been identified; the longer isoform corresponds to a splice variant that contains an in-frame 12 amino acid insertion within the extracellular domain [170]. Both isoforms contain a large extracellular domain (ECD), a single pass transmembrane domain and a short cytoplasmic tail. The GPNMB/OA ECD contains an integrin-binding RGD domain that is required for the GPNMB/OA-dependent adhesive interaction between melanocytes and keratinocytes [193] and a polycystic kidney disease (PKD) domain whose function in GPNMB/OA remains unknown. Moreover, several groups have reported that GPNMB/OA is proteolytically cleaved in an MMP-dependent manner [168,190,191]. Interestingly, NIH-3T3 fibroblasts stimulated with a recombinant GPNMB/OA ECD displayed enhanced Erk and p38 phosphorylation along with the upregulation of *Mmp-3* mRNA [190].

Given the utility of GPNMB/OA expression as a prognostic indicator of recurrence and its potential as a therapeutic target in human breast tumors [147,286], we aimed to investigate the functional role of GPNMB/OA in the primary breast tumor microenvironment. We demonstrate that GPNMB/OA expression enhances primary tumor growth, which is associated with diminished apoptosis and elevated recruitment of endothelial cells. GPNMB/OA is constitutively shed from breast cancer cells in an

ADAM10-dependent manner and the shed GPNMB/OA ECD is capable of inducing endothelial cell migration *in vitro*. Thus, we are the first to implicate ADAM10 as a sheddase that liberates GPNMB/OA ECD and to describe a functional role for the GPNMB/OA ECD in promoting endothelial cell migration.

Results

Ectopic GPNMB/OA expression enhances primary tumor growth

Previously we have reported that GPNMB/OA expression is increased in *in vivo* selected aggressively bone metastatic subpopulations of 4T1 mammary carcinoma cells [114]. In addition to bone metastatic sub-populations (592, 593), GPNMB/OA is also overexpressed in 4T1 sub-populations that are either aggressively metastatic to lung (526), liver (2776, 2792) or that have been explanted from primary tumors (066) (**Figure 1A**). This is consistent with our previous observations that GPNMB/OA is also overexpressed in human breast tumors [114,171], and suggests that GPNMB/OA may be functionally implicated in regulating tumor growth in addition to promoting invasion and metastasis. To investigate this hypothesis, we employed[114,171] an independent, less aggressive mammary tumor cell line in which we generated one pooled vector control (VC), and two clonal populations (GPNMB/OA4, GPNMB/OA5) of 66cl4 mouse mammary carcinoma cells. Variable levels of GPNMB/OA could be detected in the cell lysates of 66cl4-OA4 and 66cl4-OA5 cells (**Figure 1B**). To assess the consequences of GPNMB/OA expression on primary mammary tumor growth, 66cl4 cells were injected into the mammary fat pads of Balb/c mice. GPNMB/OA increased the incidence of mammary tumor formation (**Figure 1C**) and also accelerated tumor outgrowth relative to

VC tumors (**Figure 1D**). Moreover, the kinetics of tumor outgrowth correlated with the level of GPNMB/OA expressed in these cells (**Figure 1B, D**). To rule out the possibility that these findings reflect phenotypes associated with clonal breast cancer populations, we generated a population of pooled GPNMB/OA expressing cells (**Supplemental Figure 1A**) and found that these too enhanced tumor growth relative to vector control cells (**Supplemental Figure 1B**).

GPNMB/OA expressed on antigen presenting cells can suppress T-cell activation [179,192,193]. Recently, it has been shown that GPNMB/OA expressed in melanoma cells promotes their growth by impairing the activation of melanoma-reactive T-cells [287]. To assess whether a similar mechanism could account for GPNMB/OA-induced mammary tumor growth observed in Balb/c mice, we performed a second set of mammary fat pad injections into athymic mice that lack functional T-cells. Importantly, the GPNMB/OA-associated increase in tumor outgrowth observed in Balb/c mice was maintained even when cells were injected into immunodeficient mice, although to a lesser degree when compared to injections performed in Balb/c mice (**Supplemental Figure 1B**). Thus, the tumor growth promoting effects of GPNMB/OA cannot be explained through a mechanism involving suppression of T-cell activation.

GPNMB/OA expression in breast cancer cells is associated with decreased apoptosis and increased vascular density in vivo

To better characterize the functional role of GPNMB/OA in promoting tumor growth, we removed the primary tumors and subjected them to IHC analysis to assess differences in proliferation, apoptosis and angiogenesis. Using antibodies against Ki67 as a proliferation marker [37], we observed no significant differences in the mean percentage

of proliferation control (28.7%) versus GPNMB/OA-expressing mammary tumors (25.1%) (**Figure 2A**). We next quantified the number of apoptotic cells in non-necrotic regions of these mammary tumors and found that, on average, fewer cells in GPNMB/OA-expressing tumors (1.1%) were undergoing apoptosis when compared to control mammary tumors (2.6%) (**Figure 2B**). Finally, we assessed the vascular density of these tumors by quantifying the degree of CD31 positivity, a routinely used endothelial cell marker. These analyses revealed that the vascular density in GPNMB/OA-expressing mammary tumors (3.5%) was significantly higher when compared to control tumors (0.9%) (**Figure 2C**). We next investigated whether this increase in angiogenesis could be attributed to VEGF induction by GPNMB/OA. Similar quantities of VEGF were detected in cell lysates and conditioned media from vector control and GPNMB/OA expressing 66cl4 cells (**Supplemental Figure 2 A, B**); however, tumors derived from GPNMB/OA expressing cells (mean 652ng/ml) produced nearly twice as much VEGF as vector control tumors (mean = 328 ng/mL), suggesting that GPNMB/OA may indirectly upregulate VEGF *in vivo* via interactions with stromal cells in the tumor microenvironment (**Supplemental Figure 2C**).

To address whether the GPNMB/OA-associated angiogenic phenotype was specific to the 66cl4 mouse mammary tumor model, we next interrogated the association between GPNMB/OA expression and vascular density in human breast cancer cells and primary tumors. We ectopically expressed GPNMB/OA in BT549 cells, a basal breast cancer model. Although vector control and GPNMB/OA-expressing BT549 cells were incapable of forming tumors when injected into athymic mice (data not shown), we analyzed whether GPNMB/OA is capable of enhancing the angiogenic phenotype in these cells by performing matrigel plug assays. Matrigel plugs containing either vector control or

GPNMB/OA-expressing BT549 cells were harvested 10 days post-injection and subjected to immunohistocytochemical analysis for CD31 expression. These analyses, in agreement with our results from GPNMB/OA-expressing 66cl4 mouse mammary tumors, revealed that matrigel plugs containing GPNMB/OA-expressing BT549 cells displayed greater endothelial recruitment (11.8%) when compared to matrigel plugs composed of empty vector control cells (8.5%) (**Supplemental Figure 3A, B**).

We next interrogated gene expression data from laser capture microdissected tumor epithelium isolated from breast tumors that were categorized into those with high versus low MVD, based on quantification of CD31 staining [288] (**Figure 3A**). Interestingly, we observed a 2-fold increase in *GPNMB/OA* mRNA levels in the epithelium of breast tumors characterized as high MVD (average expression value = 1.452) versus those with low MVD (average expression value = 0.734) (**Figure 3B**). These data, together with our observations from our mouse and human breast cancer models, suggest a role for GPNMB/OA in promoting endothelial recruitment during mammary tumorigenesis.

GPNMB/OA extracellular domain is shed by ADAM10

It has been previously reported that GPNMB/OA can be cleaved and shed from the cell surface, producing an ECD fragment that has signaling capacity in stromal cells [190,191]. The GPNMB/OA ECD, when fused to the immunoglobulin Fc region, is also capable of binding to the surface of endothelial cells [165]. Given these observations, we hypothesized that the shed, soluble fragment of GPNMB/OA might facilitate the GPNMB/OA-dependent pro-angiogenic phenotype. To investigate this hypothesis, we determined whether the GPNMB/OA ECD was consistently shed into the conditioned media of breast cancer cells. Indeed, we can detect a soluble form of GPNMB/OA in

conditioned media from GPNMB/OA-expressing 66cl4 cells (**Figure 4A**). To extend these observations to a human breast cancer model, we engineered two cell lines overexpressing GPNMB/OA. Full length GPNMB/OA, containing a C-terminal V5-epitope tag, was readily detectable in the basal-like BT549 breast cancer cells and luminal-like MDA-MB-453 cells engineered to overexpress this protein (**Figure 4B**). In addition to full length GPNMB/OA, we also identified two small C-terminal fragments with molecular weights of approximately ~25kDa and ~13kDa, which we labeled CTF1 and CTF2, respectively (**Figure 4B**). These fragments have previously been described as the result of post-translational proteolytic processing in C2C12 myoblast cells engineered to overexpress GPNMB/OA, as well as in melanocytes and melanoma cells endogenously expressing GPNMB/OA [168,190]. Notably, processing of GPNMB/OA was less efficient in MDA-MB-453 cells relative to that observed BT549 cells, despite comparable expression levels of the full length protein in both cell lines (**Figure 4B**). In addition, we observed that less GPNMB/OA ECD was shed into conditioned media of MDA-MB-453 cells when compared to conditioned media harvested from BT549 cells (**Figure 4C**).

The mechanism governing GPNMB/OA shedding has been the subject of growing interest, yet the specific proteases involved in this process have yet to be elucidated. The ADAM (A Disintegrin And Metalloproteinase) subfamily of matrix metallopreoteinases (MMPs), known for their sheddase abilities, have been recently postulated to be candidate proteases that could mediate GPNMB/OA ectodomain shedding [168]. To test this possibility, we first investigated whether ADAM10, 12 or 17 were differentially expressed between BT549 and MDA-MB-453 cells, which differ in their degree of GPNMB/OA shedding. We found that both ADAM10 and ADAM17 were expressed at higher levels in BT549 cells compared to MDA-MB-453 cells, whereas ADAM12

expression was higher in MDA-MB-453 cells (**Figure 5A**). We next asked whether ADAM10 or ADAM17 - which are the primary sheddases for most ectodomains [289] - were functionally required for GPNMB/OA shedding. To accomplish this, we performed transient siRNA mediated knockdown of ADAM10 and ADAM17, independently or in combination, in GPNMB/OA-expressing BT549 cells and found that the amount of the GPNMB/OA ECD detectable in the conditioned media was diminished only when ADAM10 expression was reduced (**Figure 5B, *upper panels***). Moreover, co-suppression of ADAM17 and ADAM10 did not further diminish release of the soluble GPNMB/OA ECD. Immunoblots for GPI were performed to control for the total amount of protein in the condition media (**Figure 5B, *upper panels*)**. Immunoblots for ADAM10 and ADAM17 revealed that the siRNA-mediated knock-down of these proteins was effective (**Figure 5B, *lower panels***). To confirm whether ADAM10 was required for GPNMB/OA shedding in an independent cell line, we chose the basal-like MDA-MB-468 cell line that endogenously expresses GPNMB/OA. Indeed, transient siRNA-mediated knockdown of ADAM10 in these cells also greatly diminished shedding of the GPNMB/OA ECD into the culture media (**Figure 5C**). Together, these data indicate that ADAM10 is able to release the GPNMB/OA ectodomain from the surface of breast cancer cells.

GPNMB/OA ECD promotes endothelial cell migration

Having determined that GPNMB/OA is constitutively shed in an ADAM10-dependent manner in our breast cancer model systems, we next investigated whether this shed GPNMB/OA ECD possessed angiogenic properties. Given that the migration of endothelial cells is a requisite step during tumor angiogenesis, we investigated whether the GPNMB/OA ECD was capable of promoting this process. First, we collected

conditioned media (CM) from empty vector control (VC) or GPNMB/OA-expressing BT549 cells and used this as chemoattractant for HPMEC endothelial cells *in vitro*. We found that CM from VC cells induced limited endothelial migration; however, this increase did not achieve statistical significance when compared to the effects of serum free media (DMEM) (**Figure 6A**). In contrast, CM from GPNMB/OA-expressing BT549 cells induced a >2-fold enhancement in endothelial migration when compared to serum free media (**Figure 6A**). To determine whether this effect on endothelial migration was specific to shed GPNMB/OA, we used a V5-tagged recombinant protein encoding only the ECD of GPNMB/OA (rhECD). In this assay, known inducers of endothelial migration such as FGF-2 and VEGF, promoted a >2 fold or 1.5 fold increase in endothelial migration, respectively, when compared to serum free media (DMEM) (**Figure 6B**). By comparison, we found that recombinant human GPNMB/OA ECD induced a 1.5-fold increase in endothelial migration compared to serum free media (**Figure 6B**). Together, these observations support the hypothesis that shed GPNMB/OA augments breast tumor angiogenesis by directly stimulating endothelial migration.

Discussion

We have previously demonstrated that GPNMB/OA expression is elevated during the formation of primary mammary tumors; its expression is further elevated in breast cancer bone metastases and plays a functional role in this process [114]. GPNMB/OA belongs to a group of osteomimetic proteins (ie. Osteopontin, Osteonectin and Osteocalcin) [290] that are normally expressed by osteoblasts/osteoclasts, which when expressed in cancer cells, promote the development of bone metastases. Indeed, GPNMB/OA is emerging as a

critical mediator of osteoblast and osteoclast differentiation, two cell types important for bone remodeling and turnover [187,195,197]. In addition, GPNMB/OA expression is up-regulated in bone pathologies such as osteoarthritis and during fracture repair [285,291]. However, in the current study, we demonstrate that GPNMB/OA expression is also elevated in 4T1 subpopulations that preferentially metastasize to lung and liver, in addition to those that spread to bone. This data suggests that the GPNMB/OA may play a more generalized role in promoting tumor progression, but does not preclude the possibility that certain GPNMB/OA-related functions specifically favor the development of bone metastases.

We have observed that in certain cell-based models, such as 66cl4 mouse mammary carcinoma cells, GPNMB/OA expression can enhance tumor growth *in vivo*. Our data suggests that GPNMB/OA-dependent augmentation of tumor growth is attributed to decreased apoptosis and increased angiogenesis in GPNMB/OA expressing tumors. It is not clear whether the predominant tumor growth stimulatory effect of GPNMB/OA stems from impaired apoptosis or enhanced vascular recruitment; however, it is likely that the two processes are interrelated. Indeed, breast cancer cells that overexpress GPNMB/OA, when grown in complete media, tend to display slower *in vitro* growth rates when compared to empty vector control cells [171], suggesting that the reduced apoptosis observed in GPNMB/OA-expressing mammary tumors may be secondary to tumor/stromal interactions that occur only *in vivo*. Recently, an alternate mechanism, involving GPNMB/OA-mediated suppression of T-cell activation, has been proposed to explain how GPNMB/OA can promote the growth of melanoma tumors [287]. In this study, shRNA mediated reduction in GPNMB/OA expression in B16 melanoma cells was shown to cause a reduction in sub-cutaneous tumor growth compared to control cells

when injected into syngeneic mice. Interestingly, this difference in melanoma growth between GPNMB/OA-expressing cells and those with the GPNMB/OA knockdown was not observed when these cells were injected into immunodeficient mice [287]. The mechanism by which GPNMB/OA promoted melanoma tumor outgrowth was through suppression of T-cell activation, which normally serves to limit tumor outgrowth [287]. Given that we employed a syngeneic mouse breast cancer model to initiate our studies, we examined this possibility and found that GPNMB/OA expression was able to promote the growth of 66cl4 cells in both an immunocompetent and immunocompromised background. These observations indicate that GPNMB/OA can contribute to tumor growth through mechanisms other than suppression of anti-tumor immunity.

The observation that primary human breast tumors with high MVD express elevated levels of GPNMB/OA in the tumor epithelium provides a clinical correlate that substantiates our *in vivo* studies with the 66cl4 mammary carcinoma model. Importantly, we restricted these analysis to examining GPNMB/OA expression in the tumor epithelium of high and low MVD primary breast tumors; therefore, it remains to be determined whether GPNMB/OA expression in the tumor stroma is also associated with enhanced angiogenesis. Of interest is the observation that tumor–derived endothelial cells express high levels of GPNMB/OA relative to endothelial cells derived from normal tissues [215]. However, it is unclear whether GPNMB/OA expressed within endothelial cells functions to promote angiogenesis. Our data suggests that GPNMB/OA, when expressed in breast cancer cells, can increase vascular recruitment and enhance tumor growth.

Interestingly, VEGF levels in GPNMB/OA expressing 66cl4 cells is similar to empty vector control cells when measured *in vitro*; however, VEGF expression is upregulated

~2-fold in GPNMB/OA-expressing compared to VC mammary tumors. Given that GPNMB/OA is only capable of inducing VEGF expression *in vivo*, it is likely that GPNMB/OA promotes interactions with and/or recruitment of stromal cells, which in turn produce increased amounts of VEGF. Potential stromal cell types that could be involved in this process are tumor-associated macrophages (TAMs). These cells are actively recruited into breast tumors and are known to produce VEGF, which contributes to angiogenesis and breast tumor growth [292]. Whether GPNMB/OA-expressing mammary tumors are characterized by increased numbers of infiltrating TAMs requires further investigation.

In addition to its ability to indirectly upregulate VEGF *in vivo*, we investigated whether GPNMB/OA may be able to promote angiogenesis via direct interactions with endothelial cells. Recent studies demonstrating that GPNMB/OA can undergo proteolytic processing led us to investigate the possibility that this protein was subject to ectodomain shedding in breast cancer cells. We are the first to identify ADAM10 as specific protease capable of cleaving and releasing the ECD of GPNMB/OA. This observation is consistent with published reports showing that GPNMB/OA shedding can be inhibited by GM6001, a broad spectrum MMP-inhibitor [168,190,191]. GPNMB/OA processing can also be induced through the use of a calmodulin inhibitor or via PMA stimulation [168]. It has been proposed that ADAM10 can promote the constitutive shedding of target proteins, such as CD44, whereas PMA-induced CD44 shedding is mediated through ADAM17 [293]. In our study, we specifically investigated whether ADAM10 and ADAM17 were responsible for constitutive shedding of GPNMB/OA in breast cancer cells, thus it is possible that ADAM17 is also capable of shedding GPNMB/OA in the context of PMA stimulation.

Our data indicate that the soluble extracellular domain of GPNMB/OA can function as a chemoattractant for endothelial cells, which is capable of inducing the migration of this cell type. While the receptor for the GPNMB/OA ECD in endothelial cells is not known, the literature implicates a number of interesting candidates. For example, GPNMB/OA can be immunoprecipitated with either integrin β1 or integrin β3 in differentiating osteoclasts [195]. Presumably this interaction occurs via the N-terminal RGD domain in GPNMB/OA, which is functionally required for its ability to adhere to endothelial cells [195]. An increasing body of evidence supports a role for the β1 class of integrins in regulating endothelial adhesion, migration and survival during tumor induced angiogenesis [294]. The β3 integrin, as part of the αVβ3 receptor, is expressed on the surface of endothelial cells during angiogenesis and has been reported to interact with and potentiate FGF-2 signaling in endothelial cells [295]. Thus, integrins may serve as receptors for the GPNMB/OA ECD and transduce signals that promote endothelial migration.

The effects of GPNMB/OA rhECD on endothelial migration are significant but more modest than the effects of CM containing shed GPNMB/OA ECD, suggesting that GPNMB/OA cooperates with other factors to promote endothelial migration and angiogenesis. It is conceivable that the shed form of GPNMB/OA can act directly to induce endothelial migration, which in concert with an indirect upregulation of VEGF, leads to a robust angiogenic response.

Given the growing interest in GPNMB/OA targeted agents in breast cancer [147,286], our observations that ADAM10 functions as a sheddase for GPNMB/OA have potentially important therapeutic implications. CDX-011 is an anti-GPNMB/OA antibody-drug conjugate whose efficacy is proportional to the levels of cell surface GPNMB/OA expressed

on cancer cells [171,191]. Thus, GPNMB/OA shedding from the cell surface may limit the efficacy of GPNMB/OA-targeted therapies. It is possible that agents such as CDX-011 might be improved when used in combination with ADAM10 inhibitors that would reduce ECD shedding of GPNMB/OA. One such inhibitor, INCB7839, has been shown to cooperate with receptor tyrosine kinase inhibitors that target EGFR and ErbB2 to impair breast tumor growth [296]. Similarly, epirubicin, a chemotherapeutic drug known to down-regulate ADAM10 expression in cancer cells [297], could be used in combination with CDX-011, to potentially enhance its efficacy.

Materials and Methods

Cell culture and transfections

The murine 4T1 and human BT549, MDA-MB-453 and MDA-MB-468 breast cancer cell lines used in this study were obtained from the ATCC and cultured according to their guidelines. The 66cl4 murine mammary carcinoma cells were a generous gift from Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, MI). All 4T1-derived subpopulations were generated by *in vivo* selection in our lab [114]. Human pulmonary microvascular endothelial cells (HPMEC-ST1-6R) have been described previously [298] and were a generous gift from Dr. Vera Krump-Konvalinkova (IPEK-LMU, Munich, Germany). The pEF1-GPNMB/OA vector was constructed by ligating the full-length human GPNMB/OA cDNA (Open Biosystems; Accession: BC032783) into a pEF1/V5-His expression vector (Invitrogen) using 5' *Eco* RI and 3' *Not* I restriction enzyme sites. BT549 and MDA-MB-453 cell lines were engineered to express GPNMB/OA by LipofectAMINE 2000 (Invitrogen)–mediated transfection. GPNMB/OA-expressing cells

are pools of 3 independent clones. Osteoactivin-expressing 66cl4 cells have been described previously [114]. Transient knockdown of ADAM10 and ADAM17 was accomplished by transfection (Lipofectamine 2000, Invitrogen) using 15 nM of the ON-TARGETplus SMARTpool [pool of four ADAM10 or ADAM17-targeted small interfering RNAs (siRNA), Dharmacon]. An ON-TARGETplus pool of four non-targeting (scrambled) siRNAs was used as a control. Twenty-four hours later the transfection media was removed, the cells were washed once with PBS and media was changed to serum free media (SFM). Conditioned media, used for immnoblot or ELISA analysis, was collected after 48 hours.

Immunoblotting

Sub-confluent cells were lysed for 20 min. on ice in TNE lysis buffer. Protein concentrations were determined by Bradford assay (Bio-Rad) and 30-45 μg of total protein were used in gel electrophoresis. For immunoblotting of conditioned media (CM), 1mL of CM was concentrated using microcentricon tubes (30kDa MWCO, Millipore) and 10uL of protein the concentrate was loaded on a gel. The antibodies used were as follows: GPNMB/OA (1:2,500 dilution; R&D Systems), Osteoactivin (1:2,500 dilution; R&D Systems), ADAM10 (1:1,000 dilution; Millipore), ADAM17 (1:1,000 dilution; Millipore), ADAM12 (1:200 dilution; rb122) [299], GPI (1:1,000 dilution; Santa Cruz), V5 (1:5,000 dilution, Sigma) and α-Tubulin (1:10,000 dilution; Sigma-Aldrich). Appropriate horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at a dilution of 1:10,000 and proteins were visualized by chemiluminescence (Millipore).

In vivo tumor growth assays

Female Balb/c mice (4-6 weeks) were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed in facilities managed by the McGill University Animal Resources Centre and all animal experiments were conducted under a McGill University approved Animal Use Protocol in accordance with guidelines established by the Canadian Council on Animal Care. For the tumor growth assays, 66cl4 mammary carcinoma cells were harvested from sub-confluent plates, washed once with PBS and resuspended (10^4 cells) in 50µl of a 50:50 solution of matrigel (BD Biosciences) and PBS. This cell suspension was injected into the right abdominal mammary fat pad of Balb/c mice and measurements were taken beginning on day 10 post injection for the time periods indicated. Tumor volumes were calculated using the following formula: $\pi LW^2/6$, where L is the length and W is the width of the tumor. Tumors were surgically removed, using a cautery unit, once they reached a volume between 200-300 mm^3.

Matrigel plug assays

Subconfluent BT549 cells were trypsinized, washed once in PBS and resuspended at a final concentration $1x10^7$ cells/mL in matrigel. A 100µL cell suspension was injected subcutaneously into athymic mice. Mice were sacrificed and matrigel plugs were removed 10 days post-injection. The matrigel plugs were then fixed overnight in 4% paraformaldehyde and prepared for immunohistocytochemical staining.

Immunohistocytochemical staining and analysis of 66cl4 mammary tumors

Primary mammary tumors were fixed overnight in 4% paraformaldehyde. Immunohisto-cytochemistry was performed with the following antibodies: Ki67 (1:100 dilution; BD Pharmingen; Mississauga, ON) and CD31 (1:200 dilution; BD Pharmingen). Appropriate Biotin-SP-conjugated anti-IgG secondary antibodies were purchased from Jackson Laboratories (Bar Harbor, ME). Apoptotic cells were detected using an ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon® International; Temecula, CA) in accordance with the manufacturer's instructions. Sections were developed with 3-3-diaminobenzidine-tetrahydrochloride and counterstained with hematoxylin. Slides were first scanned using a Scanscope XT digital slide scanner (Aperio, Vista, CA, USA) and further analyzed using Imagescope software (Aperio) using either positive pixel count or immunohistocytochemistry nuclear algorithms. For Ki67 and TUNEL staining, data was represented as a percentage of positive nuclei among total nuclei in each field. For CD31 analyses only moderate (+2) and strong (+3) staining were considered positive. The number of positive pixels is represented as a percentage of total pixels per field (66cl4 tumors) or as CD31-positive pixels per nuclei (BT549 plugs).

Assessment of GPNMB/OA expression and MVD in human breast tumors

Immunohistocytochemistry directed against PECAM1 (Cat. No. BBA7, R&D Systems, Minneapolis, MN) was performed for MVD quantitation according to antibody manufacturer's instructions. Quantitation of PECAM1 staining density was performed by averaging the stained pixel intensity of 3 fields captured at 10x magnification using imageJ (http://rsb.info.nih.gov/ij/). Tissue samples from 21 patients undergoing surgery for primary invasive ductal carcinoma (IDC) with no prior neoadjuvant therapy were subjected to Laser Capture Microdissection (LCM); these were selected from more than

100 tumors based on their having the highest and lowest MVD. From this cohort we obtained 17 samples of tumor-associated vasculature. LCM, RNA isolation and sample preparation, as well as microarray hybridization, were carried out as previously described [275,288]. This study was approved by the McGill University Health Centre Research Ethics Board (Protocols SUR-99-780 and SUR-00-966). All patients provided written, informed consent. Microarray data were extracted and analyzed as previously described [288]. Normalized GPNMB/OA expression values were determined from the following Agilent probe: A_23_P134426. To minimize the effect of outliers in this small subset of tumors, we removed the tumors with highest and lowest GPNMB/OA expression from both groups (high MVD and low MVD). This resulted in the following sample sizes low MVD (n=7) and high MVD (n=8). Student's T-test (2 tails) was used to assess statistical significance.

Quantification of shed GPNMB/OA and soluble VEGF

The GPNMB/OA ELISA assay was designed by coating 96-well plates with capture antibody (human anti-GPNMB/OA, 2.10.2), which was generously provided by Celldex Therapeutics, at 4°C overnight. After blocking with BSA and several wash steps, conditioned media from BT549 cells was allowed to adhere to the antibody-coated plate for one hour at 37°C. A goat anti-GPNMB/OA antibody (R&D systems, Minneapolis, MN) was used as a detection antibody and an HRP-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories) in concert with TMB (3,3´,5,5´-tetramethylbenzidine) chromogenic substrate (Pierce Thermoscientific, Rockford, IL) was used to visualize the reaction. Plates were quantified with 540/450 nm wavelength readings on a Bio-Plex Reader (Bio-Rad Laboratories, Hercules, CA) and data was

analyzed with Bio-Plex Manager 2.0 software (Bio-Rad Laboratories, Hercules, CA). Soluble VEGF was quantified using manufacturer's protocol with a commercially available ELISA kit (R&D systems, Minneapolis, MN).

Endothelial migration assays

For assessing endothelial migration, a GPNMB-specific ELISA was used to quantify the amount of GPNMB/OA sECD present in the CM of GPNMB/OA-expressing BT549 cells that had been cultured for two days in serum free media. A volume of media containing 50ng of the sECD was added to a final volume of 1 mL of serum-free media and placed in the bottom chamber of a modified Boyden chamber assay. The same volume of concentrated conditioned media was added from the vector control cells. Untreated refers to serum free media (SFM) that was not mixed with conditioned media harvested from GPNMB/OA-expressing or vector control cells. Briefly, $7.5x10^4$ HPMECs were seeded in the upper well and allowed to migrate through an 8μm porous membrane towards the conditioned media in the bottom chamber for a period of 18 hours. For EC migration experiments employing recombinant factors, recombinant FGF2 (50 ng/ml; BPS Bioscience, San Diego, CA), VEGF (50 ng/ml; BPS Bioscience, San Diego, CA) and GPNMB/OA (rhECD, 100ng/ml; Celldex, Needham, MA) was added to 1mL SFM in the bottom chamber, and $1x10^5$ HPMECs were plated in the upper chamber and allowed to migrate through the filter for a period of 18 hours. At the termination of each experiment, cells were fixed in formalin, stained with crystal violet (Sigma) and those cells remaining on the upper side of the membrane were removed by scraping. Five images were taken for each insert and the cells were quantified using Scion Image software (Scion Corporation).

Data for each insert are represented as the average pixel count from the five images. The data was obtained from at least three independent experiments, performed in triplicate wells.

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Figure Legends

Figure 1. GPNMB/OA enhances primary tumor growth. (**A**) Immunoblot analysis of GPNMB/OA expression in parental cells (4T1) and explants taken from primary tumors (066, 067) and the following metastatic sites: bone (592, 593), lung (526, 533) and liver (2776, 2792). (**B**) Expression of GPNMB/OA was confirmed by immunoblot analysis of total cell lysates from vector control (VC) and two clonal cell lines expressing GPNMB/OA (GPNMB/OA4 and GPNMB/OA5). As a loading control, total cell lysates were blotted for α-Tubulin (**A**, **B**). (**C**) Percentage of Balb/c mice that developed mammary tumors reaching $200mm^3$ by 6 weeks post-injection of VC (n = 13/30), GPNMB/OA4 (n = 20/28) or GPNMB/OA5 (n = 6/10) expressing 66cl4 cells. (**D**) Tumor growth curves in mice injected with VC (n = 13), GPNMB/OA4 (n = 20) and GPNMB/OA5 (n = 6) expressing 66cl4 cells.

Figure 2. Osteoactivin inhibits apoptosis and enhances angiogenesis in 66cl4-derived mammary tumors. Tumors derived from vector control or Osteoactivin-expressing 66cl4 cells were characterized using immunohistochemical analysis for (**A**) proliferation (Ki67), (**B**) apoptosis (TUNEL) and (**C**) vascular density (CD31). Representative images are shown for control tumors (VC) or GPNMB/OA5-expressing tumors (left panels). Proliferation and apoptosis are expressed as the percentage of Ki67 or TUNEL-positive nuclei/field, respectively. Vascular density is expressed as the percentage of total CD31-positive pixels/field.

Figure 3. GPNMB/OA-expressing human mammary tumors display enhanced vascular density. (**A**) Human breast tumors were stained with CD31 and classified into two groups, those with low or high microvascular density (MVD). (**B**) Laser capture microdissection was used to extract RNA specifically from the tumor epithelium of low or high MVD breast tumors. Analysis of *GPNMB/OA* mRNA expression values for each tumor revealed a significant correlation between high levels of GPNMB/OA expression in the tumor epithelium and high microvascular density. *, P = 0.008, Student's t-test.

Figure 4. The GPNMB/OA ectodomain is shed from breast cancer cells. (**A**) The shed form of GPNMB/OA was detected in the conditioned media from 66cl4 cells engineered to overexpress this protein. AMF/GPI is a secreted cytokine that served as a loading control for the conditioned media collected from VC, GPNMB/OA4 and GPNMB/OA5-expressing cells (**B**) Anti-V5 immunoblot identified full length GPNMB/OA as well two C-terminal fragments (CTF1 and CTF2) in human breast cancer cells engineered to overexpress GPNMB/OA (BT549-WT and MDA-MB-453-WT). Breast cancer cells harboring an empty vector (VC) served as negative controls. Immunoblots for α-Tubulin were performed to control for protein loading in whole cell lysates. (**C**) Immunoblot analysis with an antibody directed to the extracellular domain of GPNMB/OA identified shed GPNMB/OA in the conditioned media (CM) harvested from GPNMB/OA-expressing BT549 cells (WCL: whole cell lysate).

Figure 5. ADAM10 induces shedding of the GPNMB/OA ectodomain. (**A**) Immunoblot analysis of ADAM10, ADAM12 and ADAM17 expression in BT549 and MDA-MB-453 cells. Arrow indicates band corresponding to Adam17 and asterisk

denotes a doublet of non-specific bands. (**B**) siRNA-mediated knockdown of ADAM10, but not ADAM17, reduced shedding of GPNMB/OA in BT549 cells. *Upper panels*, immunoblot analysis for GPNMB/OA in the CM harvested from BT549-GPNMB/OA cells treated with the indicated control and ADAM-specific siRNAs. *Lower panels*, immunoblot analysis was performed to determine the degree of ADAM10 and ADAM17 knockdown. Arrow indicates band corresponding to Adam17 and asterisk denotes a doublet of non-specific bands. (**C**) A role for ADAM10 in GPNMB/OA ectodomain shedding is confirmed in MDA-MB-468 human breast cancer cells that endogenously express GPNMB/OA. An immunoblot for GPNMB/OA was performed on CM harvested from MDA-MB-468 breast cancer cells treated with control or ADAM10-specific siRNAs. Immunoblot analysis with antibodies specific for ADAM10 was performed to confirm knockdown of ADAM10 expression. Immunoblots for α-Tubulin were performed to control for protein loading in whole cell lysates (**A**, **B**, and **C**). Immunoblots for AMF/GPI were performed to control for protein loading in the CM samples (**B**, **C**). CM refers to conditioned media, Lysate indicates whole cell lysates prepared from these cells.

Figure 6. GPNMB/OA ECD promotes endothelial migration. (**A**) Human pulmonary microvascular endothelial cells (HPMECs) were plated onto the upper well and allowed to migrate towards serum free media (DMEM) or conditioned media (CM) harvested from vector control (VC) or GPNMB/OA-expressing cells (GPNMB/OA). The area in square pixels was quantified over fifteen images for each condition (*left panel*), one representative field for each condition is shown (*right panel*). The data is the average of three independent experiments performed in triplicate and the standard error is: *; P <

0.006; **; P < 0.0007. (**B**) HPMECs were plated in the upper chamber and allowed to migrate towards serum free media containing recombinant FGF2 (50 ng/ml), VEGF (50 ng/ml) or GPNMB/OA (rhECD, 100ng/ml). Quantification (*left panel*) was performed as described in (**A**) and one representative field for each condition is shown (*right panel*). The data is the average of three independent experiments performed in triplicate. The standard error, values relative to untreated control: *, P = 0.0014; **, P = 0.0138; #, P = 0.0005).

Figures

Figure 1

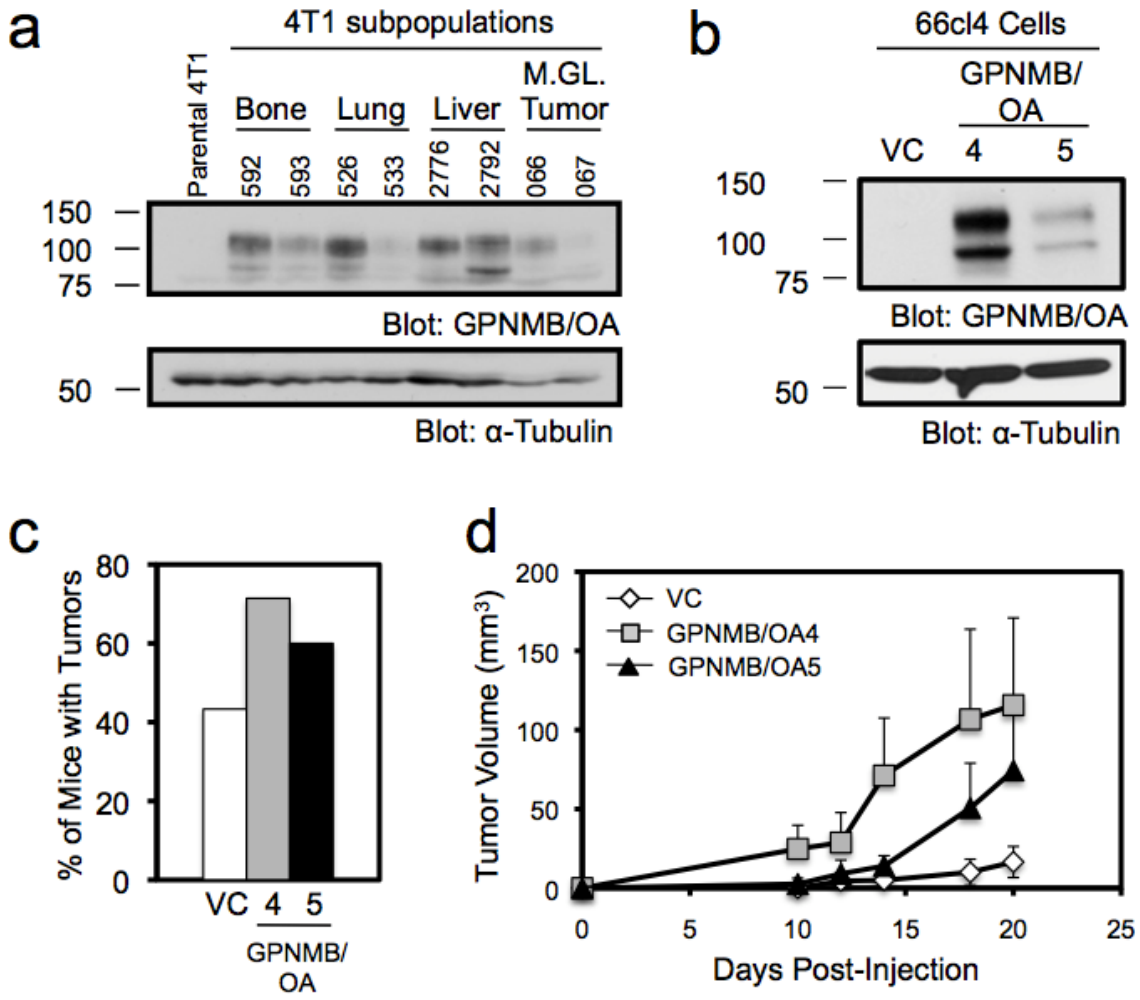


Figure 2

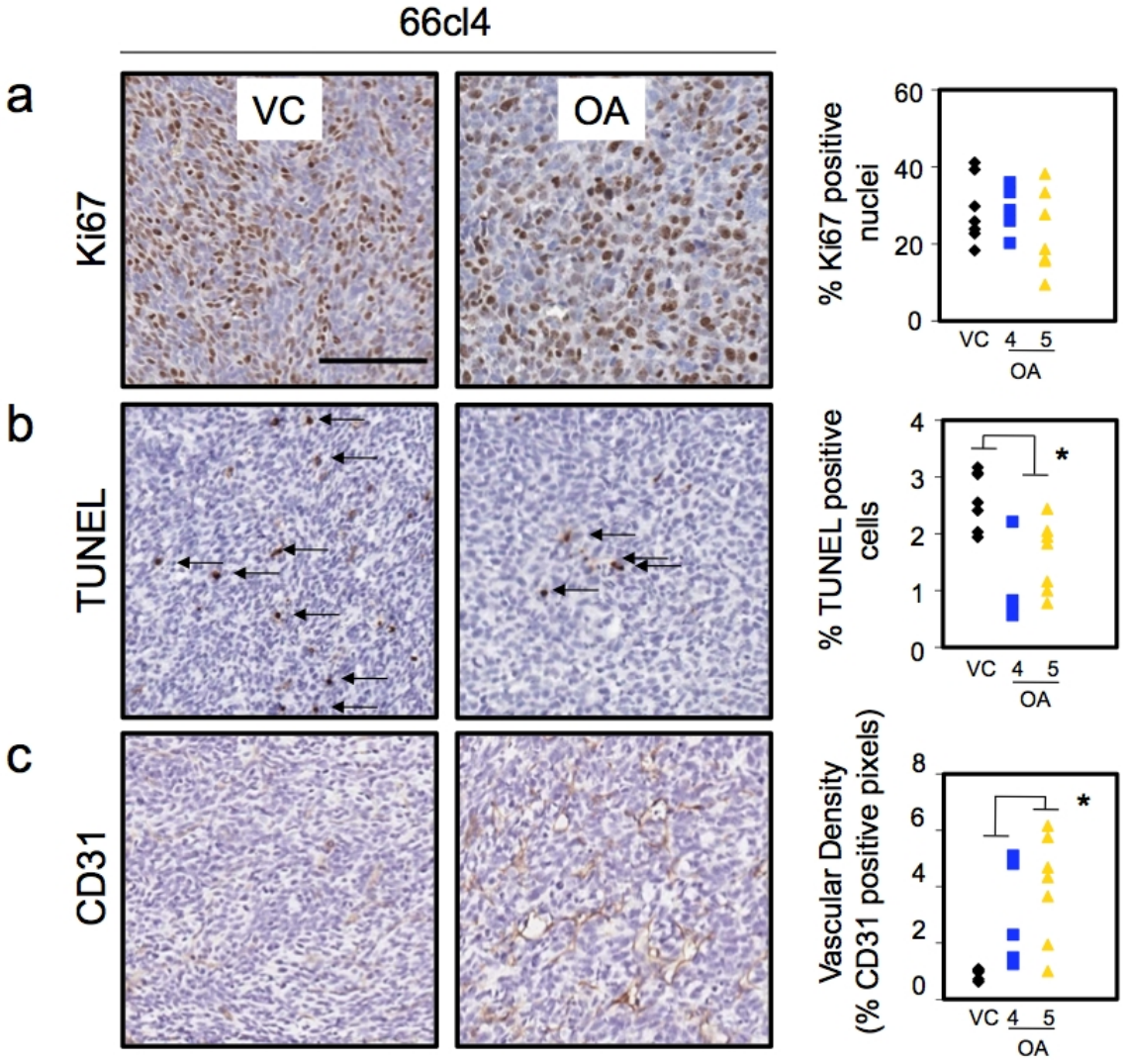

66cl4
a
VC
OA
Ki67
% Ki67 positive nuclei
60
40
20
0
VC 4 5
OA
b
TUNEL
% TUNEL positive cells
4
3
2
1
0
*
VC 4 5
OA
c
CD31
Vascular Density (% CD31 positive pixels)
8
6
4
2
0
*
VC 4 5
OA

Figure 3

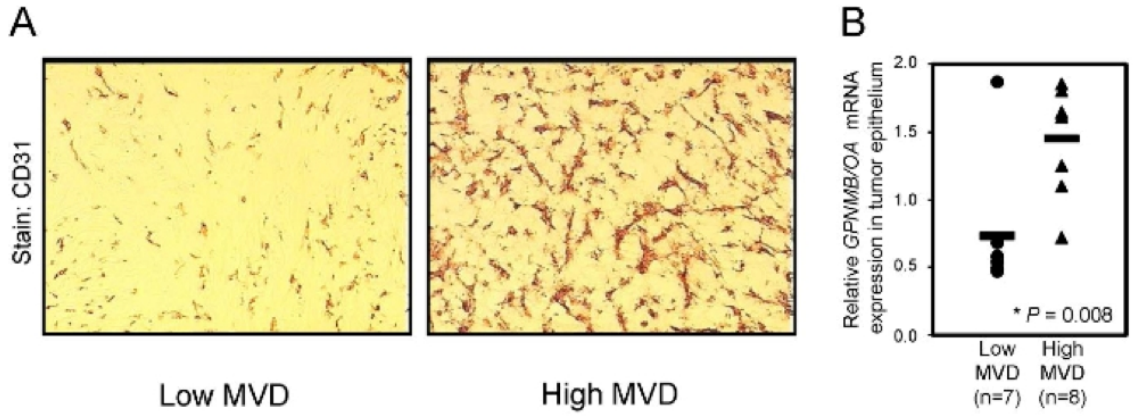

A
Stain: CD31
Low MVD
High MVD
B
Relative GPNMB/OA mRNA expression in tumor epithelium
2.0
1.5
1.0
0.5
0.0
* P = 0.008
Low MVD (n=7)
High MVD (n=8)

Figure 4

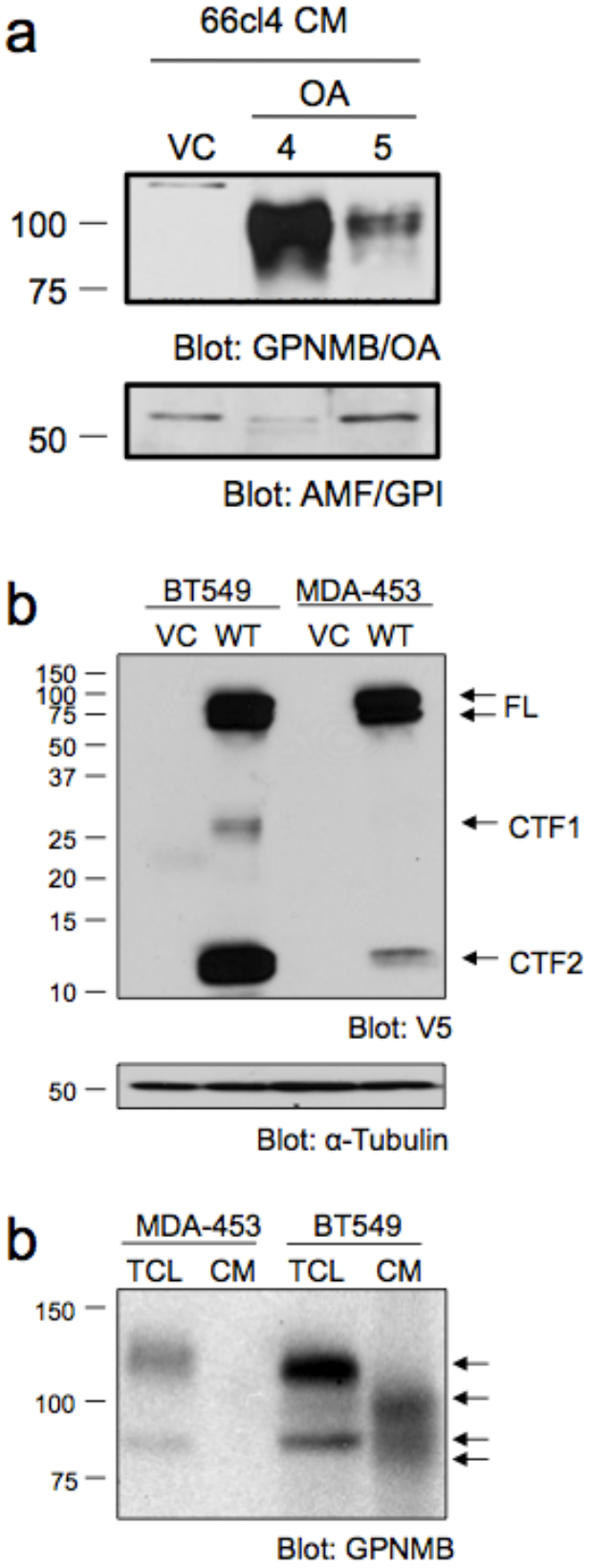

a
66cl4 CM
OA
VC
4
5
100
75
Blot: GPNMB/OA
50
Blot: AMF/GPI
b
BT549
MDA-453
VC
WT
VC
WT
150
100
75
50
37
25
20
15
10
FL
CTF1
CTF2
Blot: V5
50
Blot: α-Tubulin
b
MDA-453
BT549
TCL
CM
TCL
CM
150
100
75
Blot: GPNMB

Figure 5

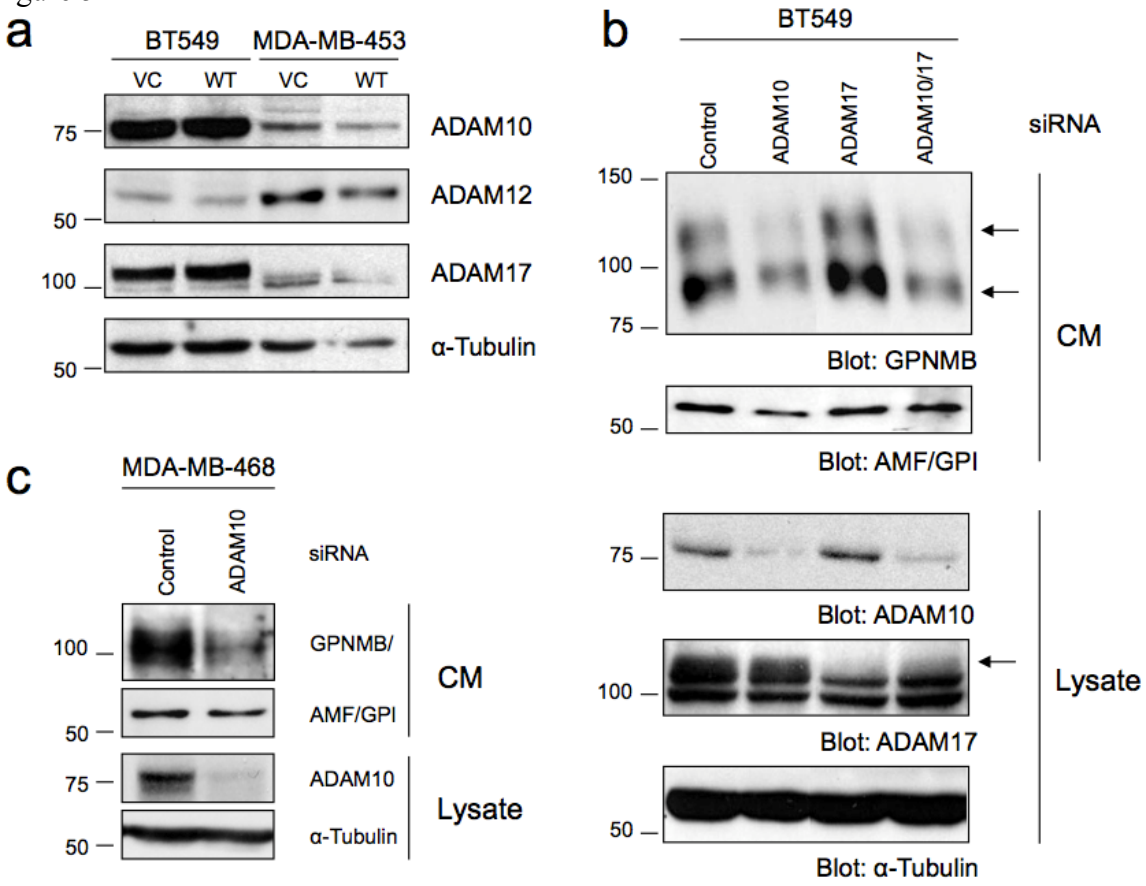
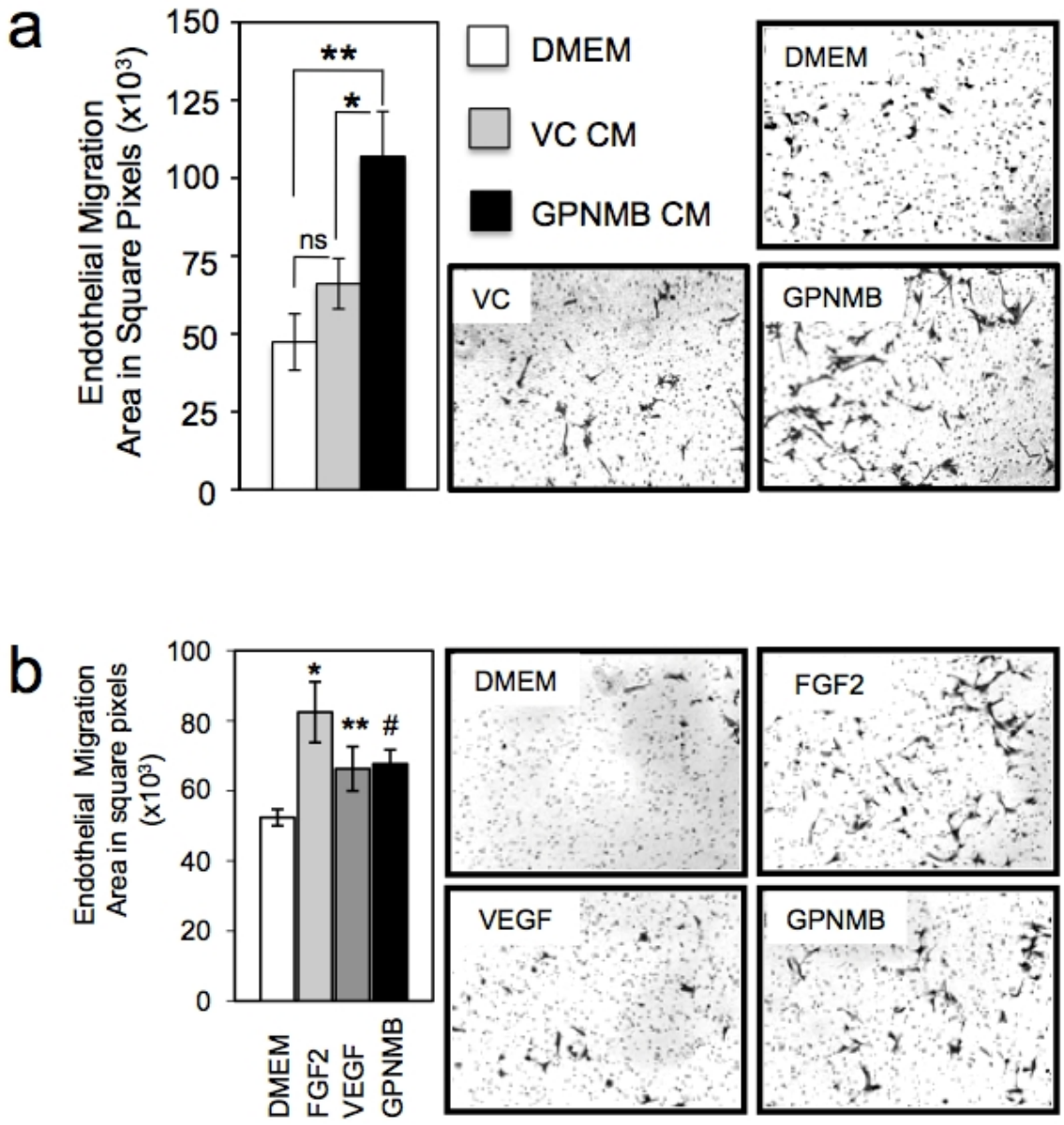

a
BT549
MDA-MB-453
VC
WT
VC
WT
75
ADAM10
ADAM12
50
ADAM17
100
α-Tubulin
50
b
BT549
Control
ADAM10
ADAM17
ADAM10/17
siRNA
150
100
75
Blot: GPNMB
CM
50
Blot: AMF/GPI
c
MDA-MB-468
Control
ADAM10
siRNA
100
GPNMB/
AMF/GPI
CM
50
75
ADAM10
α-Tubulin
Lysate
50
75
Blot: ADAM10
100
Blot: ADAM17
Lysate
50
Blot: α-Tubulin

Figure 6


a
Endothelial Migration
Area in Square Pixels (x10³)
150
125
100
75
50
25
0
**
*
ns
DMEM
VC CM
GPNMB CM
DMEM
VC
GPNMB
b
Endothelial Migration
Area in square pixels
(x10³)
100
80
60
40
20
0
*
**
#
DMEM
FGF2
VEGF
GPNMB
DMEM
FGF2
VEGF
GPNMB

Supplemental Figure Legends

Figure S1. Tumors derived from a pool of GPNMB/OA expressing 66cl4 cells display enhanced tumor outgrowth in immunocompetent Balb/c and athymic mice. (**A**) GPNMB/OA expression was confirmed by immunoblot analysis of total cell lysates from pooled vector control (VC) and GPNMB/OA-expressing (GPNMB/OA pool) 66cl4 cells. As a loading control, total cell lysates were blotted for α-Tubulin. (**B**) Tumor growth curves from Balb/c (triangles) and athymic (circles) mice injected with $1x10^5$ VC (open symbols) or GPNMB/OA pool (filled symbols) expressing 66cl4 cells. *, $P = 0.0003$, GPNMB/OA pool (athymic) vs. VC (athymic); **, $P < 0.0001$, GPNMB/OA pool (Balb/c) vs. VC (Balb/c); #, $P = 0.0001$, GPNMB/OA (Balb/c) vs. GPNMB/OA (athymic). All P-values were determined using a non-parametric Mann-Whitney test for serial measurements.

Figure S2. Analysis of VEGF expression and endothelial recruitment in breast cancer cells expressing GPNMB/OA. (A) Total cell lysates and **(B)** cell supernatants were extracted from vector control (VC, black bars) and GPNMB/OA-expressing (GPNMB/OA4, blue bars) 66cl4 cells grown *in vitro* and from **(C)** tumors grown *in vivo*. Tumors were excised at a volume of 200-300mm^3 and flash frozen in liquid nitrogen. VEGF protein was quantified using ELISA and normalized to the total amount of protein in the corresponding cell lysate (**A**, **B**) or tumor lysates (**C**). *, $P = 0.003$, Student's t-test.

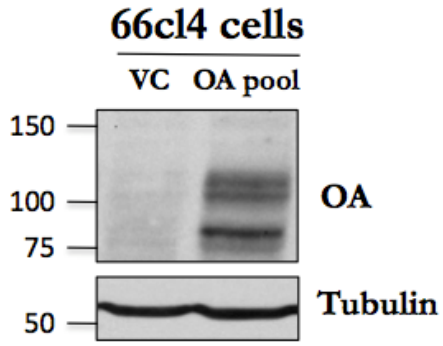
Figure S3. GPNMB/OA promotes angiogenesis in an in vivo human breast cancer model. VC or GPNMB/OA-expressing BT549 cells ($1x10^6$) were suspended in a 50:50

solution of PBS:matrigel and injected subcutaneously into athymic mice and the animals sacrificed 10 days later. **(A)** CD31 (endothelial marker)-stained pixels were quantified for each matrigel plug and normalized to the number of total nuclei in the section. *, $P = 0.021$, Student's t-test. (**B**) Vasculature recruited into the matrigel plugs was visualized on the inner surface of the skin (***upper panels***). Representative images of CD31 stains are shown (***lower panels***). Scale bars represent 100 μm.

Supplemental Figures

Figure S1

A



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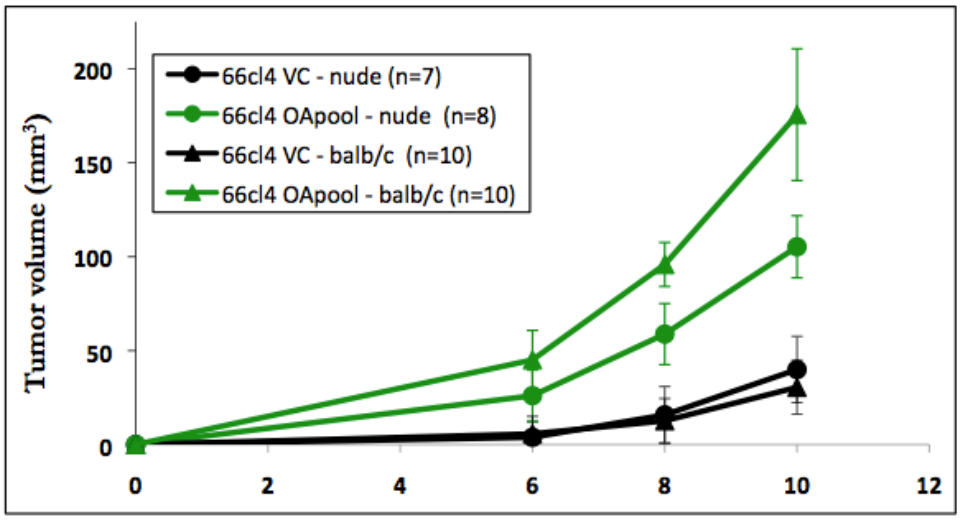


Figure S2

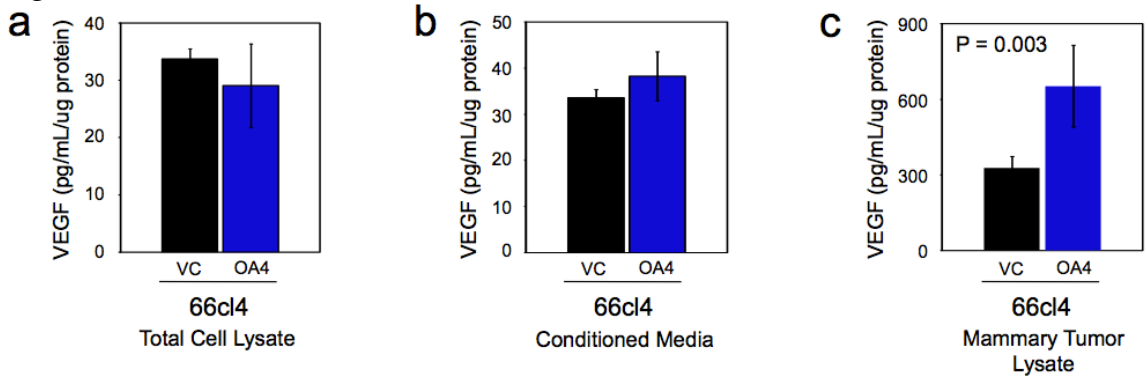
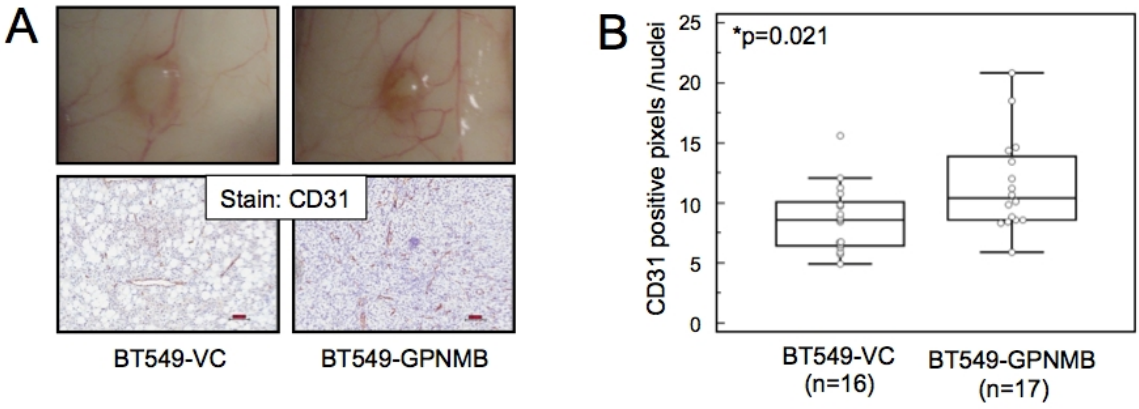

a
VEGF (pg/mL/ug protein)
40
30
20
10
0
VC
OA4
66cl4
Total Cell Lysate
b
VEGF (pg/mL/ug protein)
50
40
30
20
10
0
VC
OA4
66cl4
Conditioned Media
c
VEGF (pg/mL/ug protein)
900
600
300
0
P = 0.003
VC
OA4
66cl4
Mammary Tumor Lysate

Figure S3


A
Stain: CD31
BT549-VC
BT549-GPNMB
B
*p=0.021
CD31 positive pixels /nuclei
25
20
15
10
5
0
BT549-VC
(n=16)
BT549-GPNMB
(n=17)

CHAPTER 5 – DISCUSSION

5.1 Validation of GPNMB expression in bone-metastatic breast cancer cells

We first became interested in GPNMB when we found it to be highly expressed in sub-populations of 4T1 breast cancer cells that displayed an aggressively bone metastatic phenotype. However, a similar approach had previously been employed by Kang *et. al.*, who used MDA-MB-231 breast cancer cells injected into athymic nude mice to identify molecular mediators of bone metastasis [112]. In this study, GPNMB was not differentially expressed in MDA-MB-231 sub-populations with an aggressively bone metastatic phenotype. Nor was GPNMB differentially expressed in a separate study comparing gene expression profiles of primary human breast tumors that produced bone metastases and those that did not [137]. These inconsistencies raised questions about the relevance of GPNMB expression to human breast cancer bone metastases. However, in a study that was recently published in the form of a final grant report, Price *et. al.* performed *in vivo* selection for bone metastatic sub-populations of basal-like SUM149 breast cancer cells and identified GPNMB as a gene that was overexpressed in bone metastatic SUM149 cells [300]. It is clear from these findings that GPNMB is not

required for the formation of bone metastases by all breast cancer cells, but that its expression is associated with bone metastases in several breast cancer cell lines of mouse and human origin. Importantly, these findings by Price *et. al.* support the notion that human GPNMB - despite sharing only 69% identity with mouse GPNMB - is capable of supporting breast cancer metastasis in mice.

5.2 Functional roles of epithelial GPNMB in cancer progression

Our data indicate that epithelial GPNMB expression, but not stromal GPNMB expression, is prognostic for increased risk of metastasis in breast tumors [171]. We also found that GPNMB expressed by a variety of breast cancer cell lines is necessary and sufficient to promote cell migration and invasion *in vitro* [114,171] (Figure 1a), and that overexpression of GPNMB in 66cl4 cells enhances their ability to form metastases *in vivo* [114]. Moreover, others have observed the effects of tumoral GPNMB expression on migration, invasion and metastasis in glioma, hepatocellular carcinoma, and melanoma model systems [210,212,222]. Taken together, these observations support the notion that GPNMB expression by stromal cells in the tumor microenvironment may support primary tumor growth, but it is GPNMB expressed by the cancer cells themselves that confers a metastatic phenotype. The next question critical question is: how?

5.2.1 GPNMB-induced changes in gene expression

In dendritic cells, GPNMB is activated by ligand binding, which leads to tyrosine phosphorylation of its hemITAM and large-scale changes in gene expression. We postulated that ectopic GPNMB expression in breast cancer cells would also lead to gene expression changes that favor a pro-metastatic phenotype. To address this, we

investigated known downstream GPNMB-induced target genes. Indeed, we found that MMP-3, a known target gene of GPNMB [210] [190,205], was enhanced by ectopic GPNMB and reduced by GPNMB knockdown in breast cancer cells [114]. MMP-3 is capable of degrading abundant ECM molecules such as: type IV, V, IX, and X collagens, gelatin, fibronectin, and laminin [301] and Rich *et. al.* have shown that MMP-3 inhibition was sufficient to abrogate GPNMB-induced invasion of glioma cells [210] (Figure 1a). Transgenic overexpression of MMP-3 in mice induces mammary tumors with mesenchymal features [302], and treatment of breast cancer cells with MMP-3 induces an epithelial to mesenchymal transition (EMT) [302,303]. This is due, in part, to its ability to cleave E-cadherin, a cell-cell adhesion protein, and to up-regulate expression of the Snail transcription factor [301,303]. EMT is a widely studied phenomenon known for its ability to enhance the invasive and metastatic phenotype of cancer cells [304]. Given its well described role in tumor promotion, MMP-3 induction represents one mechanism by which GPNMB promotes cancer progression. However, GPNMB overexpression promotes invasion of BT549 breast cancer cells [171], despite the fact that it did not enhance MMP-3 expression in these cells (data not shown). As such, it appears that MMP-3 may contribute to GPNMB-dependent metastasis in some cases but is dispensable in other contexts.

To investigate if GPNMB was capable of inducing the expression of additional pro-metastatic genes, we performed microarray analysis on control and GPNMB-overexpressing 66cl4 and BT549 cells. In doing so we identified 13 genes that were up-regulated and 4 genes that were down regulated by GPNMB in both cell lines (Figure 2). Thus far, we have confirmed that proteins encoded by two of these genes, Neuropilin 1 (Nrp1) and clusterin (Clu), are up-regulated by GPNMB in breast cancer cells (Gordana

Maric and Peter Siegel, unpublished observations). Each of these genes has been associated with breast tumor progression. Clusterin is an enigmatic protein: it has two isoforms, a secreted isoform (sClu) and a nuclear isoform (nClu) with seemingly opposing functions in breast cancer [305]. Overexpression of sClu in MCF7 breast cancer cells protects them from TNF-α-induced apoptosis and promotes invasion *in vitro* while enhancing tumor growth and metastasis *in vivo* [306]. Nrp1 expressed by breast cancer cells and can activate latent TGF-β [307], which is known for its ability to promote EMT, invasion and metastasis of breast cancer cells [308]. It has also been reported to promote invasion and metastasis when expressed in lung cancer cells [309]. In addition to being a co-receptor for VEGF and promoting angiogenesis [310], Nrp1 can interact with and activate the c-Met tyrosine kinase in cancer cells to promote cell survival [311,312]. Thus we have identified downstream target genes of GPNMB that are linked to breast tumor progression, including some processes for which we have data that functionally implicates GPNMB, including: angiogenesis [172], protection from apoptosis [172], enhanced tumor growth [172], migration and invasion [171,220,309]. Interestingly, an intact RGD domain is necessary for GPNMB-induced upregulation of NRP1 (Gordana Maric and Peter Siegel, unpublished observations), but it remains to be seen whether this is the case for additional GPNMB-target genes. The requirement for Nrp1 and Clu in GPNMB-mediated breast tumor progression is currently being investigated in our laboratory.

5.2.2 Potential mechanisms of GPNMB-mediated signaling in breast cancer cells

An important question in elucidating the role of GPNMB in breast tumor progression will be: what functional domains of GPNMB are required for its pro-

metastatic effects? Given that treatment of GPNMB expressing cells with an antibody that recognizes its extracellular domain induces tyrosine phosphorylation of its hemITAM, along with large-scale changes in gene expression [186], it is likely that this motif is indeed a functional hemITAM. In support of this notion, data from our lab employing a panel of GPNMB mutants that were overexpressed in BT549 cells, has revealed that GPNMB mutants lacking the cytoplasmic domain, or those that harbour an RGD -> RAA mutation are unable to promote breast cancer cell invasion (Patricia MacDonald, Gordana Maric, Peter Siegel - unpublished observations). This suggests that GPNMB may interact with integrins on the cell surface, which could lead to activation of its hemITAM and subsequent induction of pro-invasive genes. Indeed, ITAM-containing transmembrane adapter proteins have recently been reported to form complexes with integrins and to participate in integrin-mediated signaling in osteoclasts and hematopoietic cells [181].

If we presume that the hemITAM tyrosine residue (Y525) is required for GPNMB-dependent gene-expression changes, it would be of interest to know through which downstream kinases and/or adaptor molecules GPNMB signals are transmitted. Other hemITAM harboring proteins have been shown to transmit signals via the tyrosine kinase, Syk [183], which in breast cancer is considered a tumor suppressor as it inhibits breast tumor growth and cell migration and promotes cell-cell contact [313,314]. However, recent data has also revealed anti-apoptotic [315] and transformation-promoting [316] functions of Syk in mammary epithelial cells. Thus, it will be of great interest to know whether Syk family kinases are required for hemITAM-mediated signals emanating from GPNMB in breast cancer or other cell types.

5.2.3 Effects of GPNMB shedding on intrinsic breast cancer cell properties

In the fourth chapter of this thesis, we reported that GPNMB is constitutively shed from breast cancer cells in an ADAM10 dependent manner. Importantly, we also showed that the shed ECD is functionally capable of inducing endothelial migration. But some important questions remain unanswered: 1) is there a stimulus, such as ligand binding, for GPNMB shedding? Studies employing overexpression or blocking of GPNMB ligands, such as SD4 or integrins would be useful in answering this question. 2) What is the functional relevance, if any, of the cytoplasmic domain that remains following ECD cleavage? Is the remaining C-terminal domain capable of signaling in a similar vain as ectodomain-shed Her2 or Notch [317,318,319]? Studies using a non-cleavable mutant of GPNMB, or ectopic expression of a recombinant protein encoding only the C-terminal domain would be useful for addressing these questions.

BT549 cells expressing a mutant GPNMB encoding only its ECD do not show enhanced invasion (Figure 3), suggesting that the functional domains required for invasion may lie in its cytoplasmic domain. This is somewhat surprising, given that shed GPNMB and recombinant human GPNMB ECD are both capable of inducing HPMEC endothelial migration [172], and that the ECD is sufficient to induce expression of GPNMB target genes, such as NRP1 (Gordana Maric and Peter Siegel, unpublished observations). It is possible that GPNMB ECD is capable of promoting migration but not invasion, whereas full length GPNMB is capable of promoting both migration and invasion. We have proposed that inhibition of GPNMB shedding represents a potential means to improve the efficacy of CDX-011 therapy in breast cancer [172]; however, we do not know what effect this will have on GPNMB function. It is possible that, by increasing the amount of cell surface GPNMB, inhibition of GPNMB shedding may in

fact promote breast cancer metastasis. Thus, approach will require further research in pre-clinical models before being examined in clinical trials.

5.3 Effects of GPNMB on tumor microenvironment

5.3.1 GPNMB induced MMP-3 – effects on ECM and stromal cells

MMP-3 is a stromelysin with a broad range of ECM substrates and has also been shown to increase the bioavailability or activate various pro-tumorigenic growth factors, including FGF-2, VEGF, IGF-1 and TGF-β [301]. It is commonly overexpressed in breast cancer; however, it is most commonly localized to tumor stroma rather than tumor epithelium [302]. In this thesis, we reported that GPNMB expression is associated with enhanced MMP-3 expression in breast cancer cells. However, it is also possible that GPNMB shed from breast cancer cells induces MMP-3 in the surrounding stromal cells; indeed, a recombinant protein encoding the ECD of GPNMB was sufficient to induce MMP-3 in fibroblasts [190]. Moreover, we have found that treatment of HPMEC endothelial cells with GPNMB ECD increases MMP-3 expression more than 3-fold in these cells (Figure 4). An additional role for MMP-3 in promoting metastasis was put forth recently when mice that were implanted subcutaneously with B16F10 melanoma cells (which are known to abundantly express GPNMB [175,222]) displayed upregulated MMP-3 expression in pre-metastatic lung mesenchyme. MMP-3 synergized with MMP-10 and angiopoietin-2 to disrupt vascular integrity and promote the formation of lung metastasis [320]. Thus MMP-3, a known downstream target of GPNMB in breast cancer cells – which may also be induced by shed GPNMB in stromal cells – appears to have a

number of functional roles which could contribute to the ability of GPNMB to enhance breast tumor progression.

5.3.2 GPNMB and Angiogenesis

In the fourth chapter of this thesis, we reported that GPNMB overexpression in breast cancer is associated with increased angiogenesis *in vivo*. We speculated that GPNMB may regulate this process indirectly, by up-regulating VEGF, as well as directly by being shed from the cell surface to induce endothelial cell migration (Figure 1b). At this point, the extent to which the direct effects and indirect effects of GPNMB are important for promoting angiogenesis, is unknown. Is GPNMB capable of promoting angiogenesis in the absence of increased VEGF? Also, it appears that GPNMB does not upregulate VEGF in breast cancer cells *in vitro* [172], which suggests that the source of tumoral VEGF may be stromal component. This observation raises the question – which cell types are the source of increased VEGF in GPNMB-expressing tumors, and by what mechanism are these cells interacting with GPNMB? As previously mentioned, GPNMB upregulates the VEGF co-receptor Nrp1, in breast cancer cells. Our preliminary data reveals that the GPNMB ectodomain is capable of inducing Nrp1 in endothelial cells (Figure 4), this would likely enhance their responsiveness to VEGF and promote an angiogenic phenotype.

It is a likely possibility that shed GPNMB mediates its pro-migratory functions directly, by interacting with integrins on the endothelial cell surface (Figure 1b). This notion is supported by the fact that GPNMB ECD interacts with endothelial cells in an RGD-dependent manner [165]. Our own unpublished observations illustrate that GPNMB-expressing breast cancer cells display an enhanced capacity to adhere to endothelial cells, which is abrogated in the presence of an RGDS competitive inhibitor. It

has been reported that murine GPNMB can physically interact with integrins β1 and β3 [195], and these observations have been recapitulated in our lab using human GPNMB (Gordana Maric and Peter Siegel, unpublished observations). The experiments required to definitively prove that an integrin-GPNMB interaction mediates endothelial cell migration are on-going in our laboratory.

5.3.3 GPNMB functions in anti-tumor immunity and inflammation

In melanoma cells, it was reported that GPNMB contributes to tumor growth and metastasis in immunocompetent but not immunocompromised mice. The authors provided evidence that this occurs through a mechanism involving inhibition of T-cell mediated anti-tumor immunity [222]. In support of this mechanism, we also found that GPNMB enhanced breast tumor growth more robustly in immunocompetent mice than it did in immunocompromised mice [172] (Figure 1c). However, unlike Tomihari *et. al.* we reported that GPNMB was still capable of promoting tumor growth in immunocompromised mice. This discrepancy in GPNMB-dependent tumor growth in immunocompromised mice may be due to additional functional interactions between GPNMB and cell types or molecules that are present in the mammary tumor microenvironment but not in the subcutaneous tumor microenvironment. There have been several reports indicating that GPNMB is capable of regulating immunity and inflammation in T-cells, dendritic cells and macrophages. It is upregulated by TNF-α, a pro-inflammatory cytokine that is abundantly expressed by breast tumors [321] and is associated with cell migration and invasion [321,322]. Interestingly, immunohistochemical staining for GPNMB in our MDA-MB-468 xenografts revealed that its expression was most robustly induced in cells that surrounding necrotic regions of

the tumor (data not shown), which represent areas most likely associated with increased inflammation [323]. Thus it appears that inflammatory processes affect GPNMB expression, and in turn, GPNMB is functionally capable of further regulating these processes. A high degree of inflammation within the tumor microenvironment is associated with tumor progression [323], and based on these preliminary observations, it is likely that GPNMB may play a contributing role in this process.

5.4 GPNMB as a prognostic and predictive marker in cancer

5.4.1 Tumoral GPNMB expression

Based on our analysis of gene expression profiles and tissue microarrays constructed from hundreds of breast tumors, we have characterized GPNMB as a gene (and protein) that is commonly expressed in breast cancer. Moreover, we demonstrate that epithelial GPNMB is associated with a significant increase in the risk of metastasis as well as shorter overall survival times [171]. We also found that epithelial GPNMB was much more commonly expressed in triple negative tumors than it was in luminal or Her2-positive tumors [171]. Validating these findings, in a recent proteomics analysis of 3 ER+ and 3ER- breast tumors, GPNMB was identified as one of 98 proteins that were selectively up-regulated in ER- tumors [324]. Although we confirmed that GPNMB expression was associated with poor outcome in several gene expression datasets, and in tumors from two distinct tissue microarrays, our findings will need to be independently validated in larger data sets. Tissue microarrays consisting of a large number of triple negative tumors will be particularly useful in confirming the utility of GPNMB expression as a prognostic marker specifically within this subtype.

5.4.2 GPNMB ECD as a serum marker

Analysis of tumoral protein expression is incredibly useful for research purposes – to characterize and validate prognostic and predictive markers - and it is also used routinely in the clinic to guide therapeutic strategies. However, for patients who have experienced recurrence years after their initial diagnosis, archived tumor samples are not always available. Moreover, protein expression in metastatic lesions often differs from the original primary tumor [156]. Another approach to facilitate prediction and prognosis in cancer is based on the use of serum markers. Our observations that the ectodomain of GPNMB is constituitively shed in breast cancer cells [172], along with our unpublished findings showing that GPNMB ECD is detectable in the serum of mice harboring MDA-MB-468 xenograft tumors, suggest that GPNMB may serve as a useful serum marker. However, it remains to be seen whether serum GPNMB levels vary between patients without cancer and those with GPNMB-expressing tumors. Given that GPNMB is expressed by a multitude of normal cells, there is a distinct possibility that such an approach may prove futile. Indeed, in two recent meta-analyses examining the utility of the Her2 ectodomain as a prognostic and predictive marker in breast cancer have concluded that assessment of serum Her2 ectodomain levels does not provide significant clinical benefit [325,326]. If the GPNMB ECD does not prove useful as a biomarker, perhaps there is hope for the usefulness of GPNMB found within exosomes containing GPNMB. Tomihari *et. al.* showed that GPNMB expression was abundant in exosomes released from melanoma cells, and postulated that this might represent a mechanism that facilitates the systemic immunosuppressive functions of GPNMB in tumor bearing mice [222].

5.5 GPNMB as a therapeutic target

To date, there have been two GPNMB-targeted therapies described in the literature: 1) DC-HIL-SAP, which consists of the extracellular domain of GPNMB fused to the Fc portion of human IgG_1 [327]. This construct is subsequently conjugated to saporin, a cytotoxin that elicits cell death by inhibiting ribosomes and shutting down protein synthesis. 2) CDX-011, which consists of a GPNMB-targeted antibody conjugated to the cytotoxin, monomethyl auristatin E (MMAE, aka vedotin) [223], which acts by blocking tubulin polymerization in dividing cells and inducing apoptosis.

5.5.1 DC-HIL-SAP

By nature of its ability to interact with SD4, DC-HIL-SAP can specifically target and kill a subset of T-cells that express this ligand, including those found in a specific kind of cutaneous T-cell lymphoma [327]. To date, this has been the only application investigated for this targeted therapeutic. However, given that GPNMB likely has other ligands, such as integrins, it would be of great interest to know what effect this conjugate would have in the context of the breast tumor microenvironment. We have shown that endothelial cells are one stromal cell type that is responsive to stimulation with the GPNMB ECD. If these cells were stimulated with DC-HIL SAP, would they internalize it and be killed by the cytotoxin? Would this be an effective means of inhibiting tumor angiogenesis, and if so, would it be sufficient to inhibit tumor growth? DC-HIL-SAP represents an interesting novel therapeutic that targets GPNMB-function and warrants further research into its anti-cancer capabilities.

5.5.2 CDX-011

In addition to being a prognostic marker for breast cancer recurrence, we have provided evidence that GPNMB represents a therapeutic target in breast cancer and may have value as a marker for predicting response to CDX-011 therapeutic intervention [171]. These findings were supported by preliminary data from clinical trials where there was an association between tumoral GPNMB expression and response to CDX-011. In the first IHC-based analysis of these tumors, using the same antibody and staining protocol we used in our own TMA analysis, 9 tumors were classified as GPNMB-positive, and it appeared that GPNMB was most commonly expressed in stromal cells [328], which was consistent with our observations [171]. However, it should be noted that, in a subsequent analysis using a different antibody, 30% of tumors had stromal GPNMB and 28% had stained positively for epithelial GPNMB expression [230]. It would be of great interest to further investigate this discrepancy and to determine whether the two antibodies are detecting unique GPNMB isoforms.

We found that epithelial GPNMB expression, but not stromal GPNMB expression, is associated with disease recurrence. However, clinical data suggests that targeting GPNMB-expressing stromal cells with CDX-011 is sufficient capable of inducing tumor regression [230]. This observation raises a number of important questions: 1) Are GPNMB expressing stromal cells required to support the growth of some GPNMB-negative tumors? If so, what type of stromal cells are expressing GPNMB? GPNMB overexpression has been observed in M2-polarized macrophages that had been treated with tumor cell conditioned media [217] and in tumor endothelial cells [215], which, if killed by CDX-011 could compromise blood flow to the tumor cells, resulting in tumor cell death. Or does the presence of GPNMB-expressing stromal cells

allow CDX-011 to become localized to the tumor in high enough concentrations, such that some toxin is liberated from the antibody and is able to non-specifically enter surrounding cells?

Recent evidence using SGN35, an antibody-drug conjugate (ADC) (CD30-targeted antibody conjugated to MMAE) supports this hypothesis. Okeley *et. al.* found that the ADC can be taken up by cells expressing CD30 at their cell surface, but once inside the cells, MMAE is cleaved and can be effluxed [329]. Due to its membrane permeability, MMAE can then enter and kill neighboring CD30-negative cells. This previously undescribed characteristic of antibody drug conjugates provides a unique benefit over naked antibody based therapies, in that ADCs may be more effective at killing cells that lack target expression in a heterogeneous tumor. Breast cancer in particular, is known to be highly heterogeneous in terms of gene expression - both across tumors and within individual tumors. Indeed, we found that GPNMB expression is heterogeneous within breast tumors [171]. Moreover, in our analysis only 10% of tumors expressed GPNMB positive epithelium, whereas nearly 70% of tumors expressed GPNMB within the stromal compartment. While we found that GPNMB expression in the tumor epithelium - but not the tumor stroma - is associated with recurrence [171], GPNMB-positive stroma may still represent a valid target for therapeutic intervention. Indeed, results from an on-going Phase I/II clinical trial investigating the utility of CDX-011 in the treatment of breast cancer, showed that patients with GPNMB positive tumors had improved response (16.6-17.3 weeks PFS, 20-33% ORR) compared to the entire unselected patient population (9.1 weeks PFS, 12% ORR). This GPNMB-associated benefit from CDX-011 was similar among patients with GPNMB-positive tumor epithelium and GPNMB-positive stroma.

In our *in vivo* studies with CDX-011, we found that a single dose of CDX-011 was able to cause tumors to regress to less than 50% of their original volume, and that this regression was sustained for a period of at least 6 weeks. Upon inspection of GPNMB expression in untreated MDA-MB-468 tumors by IHC, we noted that far fewer than 50% of the tumor cells expressed GPNMB (data not shown). Together these findings, albeit preliminary, in concert with the recent characterization of ADC-based mechanisms of cell killing from Okelely *et. al.* are strongly supportive of the idea that GPNMB-expressing breast tumor stroma represents a valid target for therapeutic intervention with CDX-011. Thus, CDX-011 may belong to a group of cancer therapeutics, including bisphosphonates and anti-angiogenic agents, whose functionality is primarily attributed to their effects on the tumor stroma.

5.6 Summary

The work described in this thesis represents the first characterization of GPNMB as novel molecular mediator of breast tumor progression. We built upon our initial identification of GPNMB as metastasis-promoting gene in a mouse model of breast cancer by confirming its ability to promote migration and invasion of human breast cancer cells. We went on to show that GPNMB expression in human breast tumors correlates with poor outcome and that it is most commonly expressed in the basal-like or triple-negative sub-type. This work provided the rationale for investigating the clinical utility of CDX-011 as novel therapeutic for the treatment of metastatic breast cancer, which has now advanced to Phase II trials. In our most recent paper we began to characterize the functional domains required for GPNMB function, while providing the

first evidence that GPNMB is also involved in promoting breast tumor growth and angiogenesis. In summary, we have characterized a role for GPNMB in a number of molecular processes that are critical for tumor progression. However, the specific mechanisms by which GPNMB regulates these processes still remain elusive, and future work aimed at characterizing these mechanisms will be invaluable towards understanding breast cancer progression and for optimizing GPNMB-targeted therapies for the treatment of metastatic breast cancer.

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5.8 Figures and Legends

Figure 1: Functional roles for GPNMB in tumor progression. **A)** GPNMB promotes tumor cell invasion. GPNMB has been reported to promote invasion of glioma, hepatoma and breast cancer cells (BCC). We found that in breast cancer cells GPNMB expression was associated with MMP-3 induction and Rich et. al found that inhibition of MMP-3 was sufficient to abrogate GPNMB-induced invasion of glioma cells. Our unpublished observations using mutational analysis have revealed that both the extracellular RGD domain and the cytoplasmic tail of GPNMB are required for GPNMB-induced invasion – suggesting that cell surface-bound GPNMB may interact with integrins, leading to

activation of its hemITAM and subsequent pro-invasive signals which may include MMP-3 induction. **B)** GPNMB induces angiogenesis. Ectopic GPNMB expression in breast tumor cells enhances angiogenesis *in vivo*. GPNMB ectodomain is constitutively shed from breast cancer cells in and ADAM-10 dependent manner. Shed GPNMB is detectable in conditioned media from breast cancer cells, in the serum of mice harboring GPNMB-expressing tumors, and in serum from patients with melanoma. Shed GPNMB is capable of inducing endothelial cell (EC) migration, potentially via its interaction with integrins on the cell surface. The functional role of the remaining cytoplasmic tail following ectodomain shedding is unknown, but it may be capable of mediating intracellular signalling via an activated hemITAM. **C)** GPNMB inhibits anti-tumor immunity to facilitate tumor growth. It has been reported that GPNMB promotes primary melanoma tumor growth, in a manner that is entirely dependent on the presence of T-cells. We found that GPNMB-dependent increases in beast tumor growth rate are still present, albeit to a lesser degree, in mice that lack functional T-cells. The effects of GPNMB have been well characterized by the Ariizumi group. GPNMB interacts with syndecan-4 (SD4), which is expressed on a subset of activated T-cells. This interaction leads to decreased T-cell production of the inflammatory cytokines IFN-gamma and Il-2, which are capable of activating resting T-cells. Thus, the GPNMB/SD4 interaction results in a dampening of the anti-tumor response. It is unclear whether this SD4 interaction occurs with cell surface bound GPNMB, shed GPNMB, or by GPNMB released in exosomes.

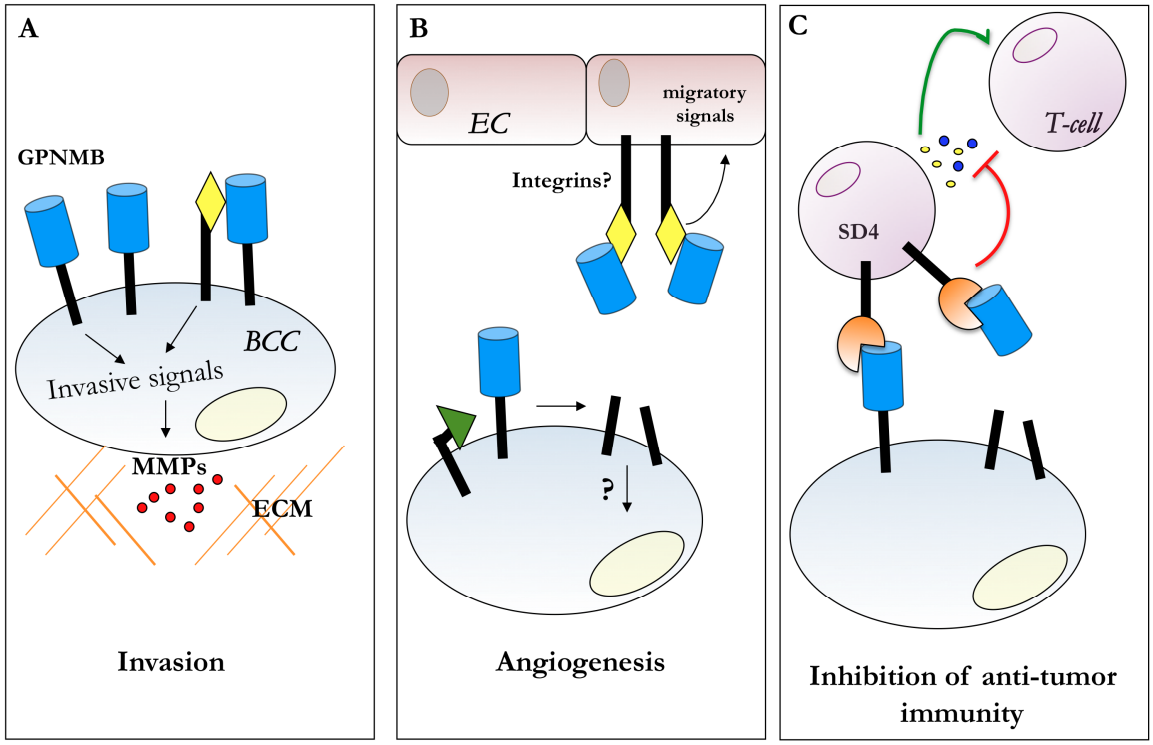
Figure 2: Gene expression changes induced by GPNMB in both mouse and human breast cancer cells. A) Immunoblot analysis showing GPNMB expression in two

independent mouse (66cl4) and human (BT549) breast cancer cell lines used for microarray analysis. An immunoblot for α-Tubulin is included as a loading control. **B)** Whole genome 44K Agilent gene expression arrays were hybridized with RNA from both 66cl4 and BT549 cells (two GPNMB expressing lines and two VC lines for both cell models). Differentially-expressed genes in GPNMB-expressing versus vector control (VC) cells were filtered on a fold change of 2 or greater and a p value of < 0.05. In this way, 17 genes were found to be commonly differentially expressed in both mouse and human cells in response to GPNMB overexpression. (C) The list of the 17 genes with the corresponding fold change in GPNMB vs VC cells in both the 66cl4 and BT549 cell models is shown. Red denotes genes overexpressed and green text indicates genes that are underexpressed in GPNMB-expressing cells.

Figure 3: GPNMB ectodomain is insufficient to promote breast cancer cell invasion. **A)** Schematic representation of wild type (WT) GPNMB, a truncation mutant encoding only the extracellular domain (ECD) and a mutant with the PKD domain removed (PKD). **B)** BT549 breast cancer cells were transfected with a V5-tagged pEF1(neo) expression vector containing empty vector (VC), WT, ECD, or PKD mutants. Transfected cells were subjected to selection under 1mg/ml G418 and surviving colonies were screened for GPNMB expression using a V5 antibody. Each cell population represents a pool of 3 GPNMB expressing clonal populations. **C)** Invasive capacity of BT549 cells was tested and analyzed using the same protocols described in Chapter 3 of this thesis. All experiments were performed at least thrice. Statistical significance of differences in invasion were determined by comparing the indicated GPNMB-expressing cells to VC cells using a T-test: *; P=0.004 **; P=0.014.

Figure 4: GPNMB ectodomain stimulation induces gene-expression changes in HPMEC endothelial cells. $1x10^5$ HPMEC endothelial cells were plated in each well of a 6 well plate, allowed to attach to the plastic and serum starved overnight. The next day, media was replaced with serum free media containing 10ug/mL V5-tagged, recombinant human GPNMB ECD or an equal volume of vehicle. Each stimulation experiment was performed twice. RNA was isolated 6 hours post-treatment and subjected to RT-qPCR using proprietary TAQMAN primers for MMP-3, NRP-1 and GAPDH. Arbitrary values for MMP-3 and NRP-1mRNA levels are normalized to the housekeeping gene GAPDH. qRT-PCR experiments were performed in triplicate wells. Statistical significance of differences in gene expression between vehicle and ECD stimulated cells was assessed with a T-test: *; P = 0.006, **; P = 0.049.

Figure 1:


A
GPNMB
BCC
Invasive signals
MMPs
ECM
Invasion
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migratory signals
Integrins?
?
Angiogenesis
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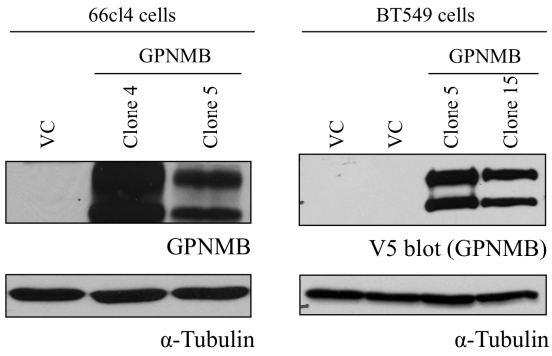
T-cell
SD4
Inhibition of anti-tumor immunity

Figure 2:

A



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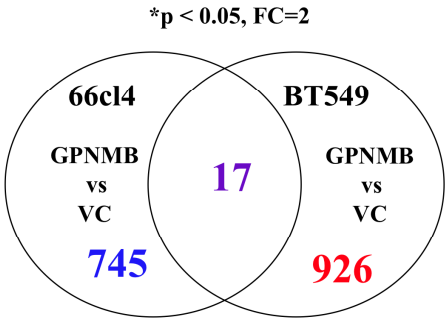
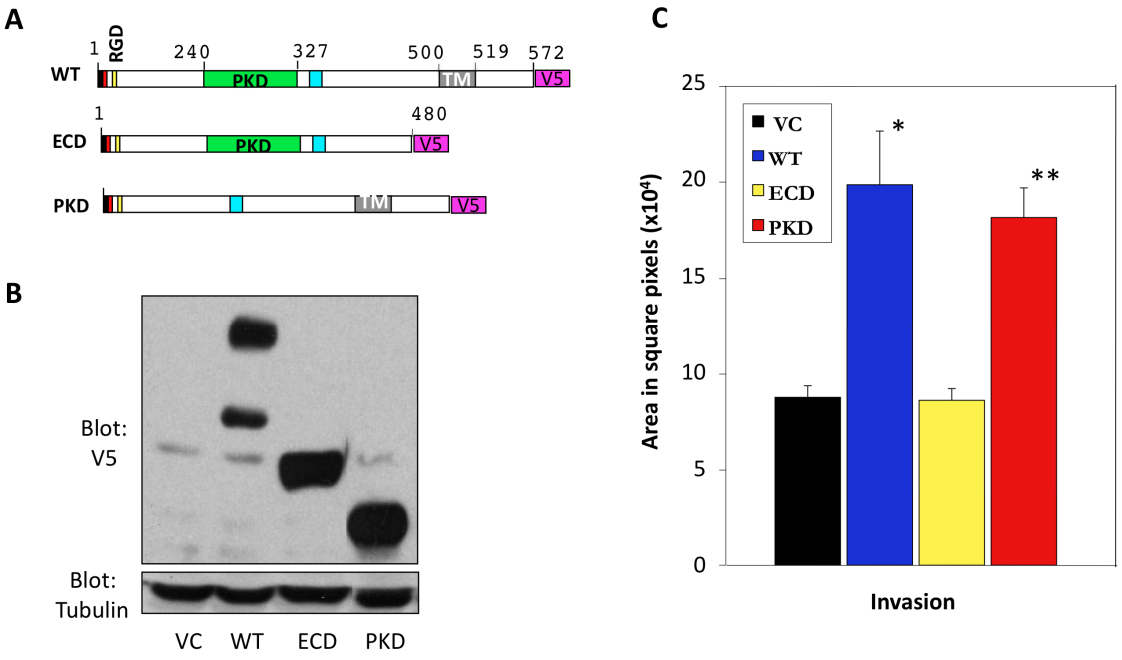


C

Gene Name	66cl4 Fold Change GPNMB vs VC	BT549 Fold Change GPNMB vs VC
Aryl hydrocarbon receptor (AHR)	2.7	2.8
Ankyrin 1 (ANK1)	2.4	5.5
Ca2+/Calmodulin-dep. kinase 4 (CAMK4)	3.2	2.1
CCAAT/enhancer binding protein, delta (CEBPD)	4.9	3
Cadherin 18 (CDH18)	2.5	2.7
Procollagen, type-IV, alpha 6 (COL4A6)	2.1	6.6
Clusterin (CLU)	6.9	2.5
Dedicator of cytokinesis 10 (DOCK10)	2	2
Ectonucleotide pyrophosphatase/phosphodisterase 5 (ENPP5)	6	27.6
Eph receptor A3 (EPHA3)	4	13.7
Neuropilin 1 (NRP1)	2.1	9.6
Pro-rich nuclear coactivator receptor1 (PNRC1)	2.1	2.1
Protease nexin-1 (Serpine2)	71.8	7.5
SH3-domain containing GEF (SGEF)	0.5	0.34
Monoglyceride lipase (MGLL)	0.15	0.45
Reticulon 1 (RTN1)	0.24	0.03
Neuroligin 1 (NLGN1)	0.26	0.18

Figure 3: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1
RGD
240
327
500
519
572
WT
PKD
TM
V5
1
480
ECD
PKD
V5
PKD
TM
V5
B
Blot:
V5
Blot:
Tubulin
VC
WT
ECD
PKD
C
25
20
15
10
5
0
VC
WT
ECD
PKD
*
**
Area in square pixels (x10⁴)
Invasion

Figure 4: