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DISSECTING THE MOLECULAR MECHANISMS OF BREAST CANCER BONE METASTASIS FOR THERAPEUTIC TARGETING

by

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Those who take a leap of faith, collaborate and adapt,
are difficult to stop.

Sirkku Pollari
Dissecting the molecular mechanisms of breast cancer bone metastasis for therapeutic targeting
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ABSTRACT

Breast cancer that has metastasized to bone is currently an incurable disease, causing significant morbidity and mortality. The aim of this thesis work was to elucidate molecular mechanisms of bone metastasis and thereby gain insights into novel therapeutic approaches. First, we found that L-serine biosynthesis genes, phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1) and phosphoserine phosphatase (PSPH), were up-regulated in highly bone metastatic MDA-MB-231(SA) cells as compared with the parental breast cancer cell line. Knockdown of serine biosynthesis inhibited proliferation of MDA-MB-231(SA) cells, and L-serine was essential for the formation of bone resorbing osteoclasts. Clinical data demonstrated that high expression of PHGDH and PSAT1 was associated with decreased relapse-free and overall survival and with features typical of poor outcome in breast cancer. Second, RNA interference screening pointed out heparan sulfate 6-O-sulfotransferase 2 (HS6ST2) as a critical gene for transforming growth factor β (TGF-β)-induced interleukin 11 (IL-11) production in MDA-MB-231(SA) cells. Exogenous heparan sulfate glycosaminoglycans heparin and K5-NSOS also inhibited TGF-β-induced IL-11 production in MDA-MB-231(SA) cells. Furthermore, K5-NSOS decreased osteolytic lesion area and tumor burden in bone in mice. Third, we discovered that the microRNAs miR-204, -211 and -379 inhibited IL-11 expression in MDA-MB-231(SA) cells through direct targeting of the IL-11 mRNA. MiR-379 also inhibited Smad-mediated signaling. Gene expression profiling of miR-204 and -379 transfected cells indicated that these microRNAs down-regulate several bone metastasis-relevant genes, including prostaglandin-endoperoxide synthase 2 (PTGS2). Taken together, this study identified three potential treatment strategies for bone metastatic breast cancer: inhibition of serine biosynthesis, heparan sulfate glycosaminoglycans and restoration of miR-204/-211/-379.

Keywords: breast cancer, bone metastasis, L-serine, IL-11, heparan sulfate glycosaminoglycan, microRNA

Sirkku Pollari
Rintasyövän luustoetäpesäkkeiden mekanismien selvittäminen lääkekehitystä varten
Farmakologia, lääkekehitys ja lääkehoito, Turun yliopisto; Lääkekehityksen biotekniikka, VTT ja Turun biotekniikan keskus; Suomen Lääketutkimuksen tohtoriohjelma, Lääkekehityksen jaos, Turku
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TIIVISTELMÄ

Syövän etäpesäkkeet luustossa lisäävät rintasyövästä johtuvaa sairastavuutta ja kuolleisuutta. Parantavaa hoitoa ei ole vielä pystytty kehittämään. Tämän väitöstyön tavoitteena oli selvittää rintasyövän luustoetäpesäkkeiden muodostumisen mekanismia uusien lääkintäkeinojen kehittämistä varten. Tutkimustuloksemme osoittivat, että L-seriinin biosynteesiin osallistuvien entsyymien, PHGDH:n, PSAT1:n ja PSPH:n, ilmentyminen oli korkeampi tehokkaasti luustoetäpesäkkeitä muodostavissa MDA-MB-231(SA)-soluissa alkuperäiseen MDA-MB-231-rintasyöpäsolulinjaan verrattuna. MDA-MB-231(SA)-solujen kasvu on riippuvainen L-seriinin biosynteesistä, ja L-seriini on välttämätön luuta hajottavien osteoklastien erilaistumiselle. Kliinisissä rintasyöpänäytteissä PHGDH:n ja PSAT1:n korkea ilmentyminen liittyy nopeaan syövän uusiutumiseen, lyhentyneeseen elinaikaan ja huonon ennusteen ominaisuuksiin. Toisessa osatyössä käytimme RNA-interferenssiseulontaa ja osoitimme, että heparaanisulfaattia muokkaava entsyymi HS6ST2 säätelee TGF-β-kasvutekijän indusoimaa interleukiini 11:n (IL-11) tuottoa MDA-MB-231(SA)-soluissa. Myös kasvuliuokseen lisätyt heparaanisulfaatit hepariini ja K5-NSOS vähensivät TGF-β:n indusoimaa IL-11:n tuottoa. Kokeet rintasyövän luustoetäpesäkkeiden muodostumista mallintavilla hiirillä osoittivat, että K5-NSOS vähentää syövän kasvua ja luun hajoamista. Kolmannessa osatyössä havaitsimme, että mikroRNA:t miR-204, -211 ja -379 vähentävät IL-11:n ilmentymistä MDA-MB-231(SA)-soluissa sitoutumalla IL-11:n lähetti-RNA:han. Lisäksi miR-379 estää Smad-viestinvälitystä. MiR-204 ja -379 vaimentavat useiden rintasyövän luustoetäpesäkkeiden muodostumista säätelevien geenien, kuten PTGS2, ilmentymistä rintasyöpäsoluissa. Tämä tutkimus johti täten kolmen rintasyövän luustoetäpesäkkeiden muodostumiseen liittyvän, mahdollisesti terapeuttisesti hyödynnettävän mekanismin tunnistamiseen. Mahdollisia uusia lääkintäkeinoja ovat seriinin biosynteesin estäminen, heparaanisulfaatit sekä miR-204, -211 ja -379.

Avainsanat: rintasyöpä, luustoetäpesäke, L-seriini, IL-11, heparaanisulfaatti, mikroRNA

TABLE OF CONTENTS

ABBREVIATIONS

ActRIIA	activin receptor type IIA
ADAM	a disintegrin and metalloproteinase
AP-1	activator protein 1
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CASR	calcium-sensing receptor
CCL2	chemokine (C-C motif) ligand 2
CDC42	cell division cycle 42
cDNA	complementary DNA
c-Fms	M-CSF receptor
CGH	comparative genomic hybridization
COX-2	cyclooxygenase 2
CTX	C-telopeptide of type I collagen
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
DKK1	dickkopf 1
DMP1	dentin matrix acidic phosphoprotein 1
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial to mesenchymal transition
ER	estrogen receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
FDA	Food and Drug Administration
GO	Gene Ontology
GPNMB	glycoprotein nonmetastatic B
HER2	human epidermal receptor 2
HIF	hypoxia-inducible factor
HMGA2	high mobility group AT-hook 2
HOXD10	homeobox D10
HRE	hypoxia-response element
IGF-2	insulin-like growth factor 2
IL	interleukin
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor
MET	mesenchymal to epithelial transition
miRNA	microRNA
MMP	matrix metalloproteinase
mRNA	messenger RNA
MTA1	metastatic tumor antigen 1

NF-κB	nuclear factor κ B
OPG	osteoprotegerin
PAI	plasminogen activator inhibitor
PAK1	p21 protein-activated kinase 1
PCNA	proliferating cell nuclear antigen
PGE_2	prostaglandin E2
PgR	progesterone receptor
PHGDH	phosphoglycerate dehydrogenase
PI3K	phosphoinositide-3 kinase
PP_i	inorganic pyrophosphate
PSAT1	phosphoserine aminotransferase 1
PSPH	phosphoserine phosphatase
PTGS2	prostaglandin-endoperoxide synthase 2
PTHrP	parathyroid hormone-related protein
RANK	receptor activator of nuclear factor κB
RANKL	receptor activator of nuclear factor κB ligand
Re-186	rhenium-186 hydroxyethylidenediphosphonate
RhoA	Rho GTPase A
RhoC	Rho GTPase C
RISC	RNA-induced silencing complex
RKIP	Raf kinase inhibitory protein
RNAi	RNA interference
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDF1	stromal-derived factor 1
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLC1A4	solute carrier family 1, member 4
Sm-153	samarium-153 lexidronam
SPARC	secreted protein acidic and rich in cysteine
Sr-89	strontium-89 hydrochloride
TAK1	TGF-β-associated kinase 1
TF	Thomsen–Friedenreich
TGF-β	transforming growth factor β
TIAM1	T lymphoma invasion and metastasis 1
TRACP	tartrate-resistant acid phosphatase
TTP	tristetraprolin
uNTx	N-telopeptide of type I collagen
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VCAM1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
ZEB	zinc finger E-box-binding homeobox

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I–III. In addition, some unpublished data are presented. The original publications have been reproduced with the permission of the copyright holders.

I **Pollari S**, Käkönen S-M, Edgren H, Wolf M, Kohonen P, Sara H, Guise T, Nees M, Kallioniemi O. Enhanced serine production by bone metastatic breast cancer cells stimulates osteoclastogenesis. *Breast Cancer Res. Treat.* (2011) 125:421–430.

II **Pollari S**, Käkönen R, Mohammad K, Rissanen J, Halleen J, Wärri A, Nissinen L, Pihlavisto M, Marjamäki A, Perälä M, Guise T, Kallioniemi O, Käkönen S-M. Heparin-like polysaccharides reduce osteolytic bone destruction and tumor growth in a mouse model of breast cancer bone metastasis. *Mol. Cancer Res.* In press.

III **Pollari S**, Leivonen S-K, Perälä M, Fey V, Käkönen S-M, Kallioniemi O. Identification of microRNAs inhibiting TGF-β-induced IL-11 production in bone metastatic breast cancer cells. Submitted for publication.

1 INTRODUCTION

Breast cancer is the most common malignancy and the leading cause of cancer-related deaths in women. World-wide, nearly 1.3 million women are diagnosed with breast cancer each year (Global Cancer Facts & Figures 2nd Edition 2011). Most of the morbidity and mortality is due to metastatic spread rather than the primary tumor. Bone is the most common organ for breast cancer metastasis: approximately 70% of patients with hematogenous spread of breast cancer have bone metastases (Coleman 2006). Median survival after diagnosis of bone metastasis is 2–4 years. Patients with bone metastases suffer from pathologic fractures, spinal cord compression, bone pain and hypercalcemia. Pathologic fractures, which occur in approximately 60% of breast cancer patients with bone metastases, often require surgical intervention. Vertebral fractures cause spinal deformity and may also lead to spinal cord compression and paralysis (Lipton 2010).

Currently, breast cancer patients with bone metastases are treated with systemic therapy selected based on the characteristics of the primary tumor. In addition, agents tailored to the treatment of bone metastasis, such as bisphosphonates or antibodies against the receptor activator of nuclear factor κB ligand (RANKL), are used. These agents predominantly act in bone and especially bisphoshphonates accumulate into the areas of high bone turnover, including the metastatic sites. Minimal systemic exposure reduces but does not completely eliminate toxic side effects (Bamias *et al.* 2005, Markowitz *et al.* 2003). None of the currently used therapeutic schemes is able to prevent skeletal metastasis or result in regression of the established metastatic disease. In order to develop more curative and specific treatment strategies, knowledge on the critical molecular mechanisms of bone metastatic progression is needed. Ideally, successful identification of breast cancer patients most likely to benefit from the bone-targeted therapies would enable prevention of bone metastasis and improve patient survival.

Each of the sequential steps in the metastatic cascade requires coordinated spatio-temporal expression of proteins and cellular processes and is a potential target for therapeutic intervention. Metastasis is initiated when tumor cells invade from the local mammary tissue into the blood circulation or lymphatic veins. The cells then need to evade the immune system, survive in the circulation until they reach the bone marrow, extravasate and start metastatic growth in the bone microenvironment. The aim of this thesis work was to apply comprehensive genomic and functional tools to elucidate the molecular mechanisms of breast cancer bone metastasis and thereby gain insights into novel therapeutic approaches.

2 REVIEW OF THE LITERATURE

2.1 Molecular mechanisms of breast cancer metastasis to bone

2.1.1 Intrinsic properties of the primary tumor

Specific immunohistochemical markers, estrogen receptor (ER), progesterone receptor (PgR) and human epidermal receptor 2 (HER2), have long been used to define breast cancer subtypes with different clinical outcomes. Furthermore, over the past decade, large-scale microarray-based gene expression profiling studies have led to the classification of molecular subtypes of breast cancer that are defined by distinct transcriptional signatures (Hu *et al.* 2006, Perou *et al.* 2000, Sorlie *et al.* 2001, Sorlie *et al.* 2003). The luminal subtypes A and B contain ER+ cases and are distinguished by the expression of genes regulated by the ER signaling pathway. Recent studies, however, have suggested that the distribution of luminal cases, rather than two distinct categories, may be a better classification scheme (Reis-Filho *et al.* 2010, Wirapati *et al.* 2008). The molecular ERBB2+ subtype generally – although imperfectly – corresponds to the immunohistochemically defined HER2+ tumors (Perou *et al.* 2000) while the basal-like subtype largely overlaps with the triple-negative (ER–, PgR–, HER2–) cases (Carey *et al.* 2007, Nielsen *et al.* 2004). The normal-like subtype resembles normal epithelial tissue (Perou *et al.* 2000). In an alternative scheme, two ER– subtypes are defined by different expression levels of the androgen receptor, while HER2+ cases do not form a separate group but rather cluster according to their ER status (Guedj *et al.* 2011). Other ER– molecular subtypes, such as the claudin-low subtype and the molecular apocrine class, have also been reported (Farmer *et al.* 2005, Hennessy *et al.* 2009).

Clinical reports have shown associations between breast tumor subtypes and preferred sites of metastasis. ER– tumors preferentially metastasize to soft tissues and lungs, liver, brain and other viscera while ER+ tumors exhibit an increased rate of bone metastases (Hess *et al.* 2003). Metastases in bone are also often observed in luminal A and B and, to a lesser extent, in ERBB2+ cases. Brain metastases, in turn, are relatively common in basal-like and ERBB2+ as well as in immunohistochemically defined HER2+ cases (Kennecke *et al.* 2010, Smid *et al.* 2008). These associations, however, have recently been questioned due to evidence showing that the receptor status can change during metastatic progression and as a consequence of anticancer treatment (Amir *et al.* 2008, Holdaway and Bowditch 1983, Kuukasjarvi *et al.* 1996).

Metastasis has traditionally been considered to be due to the accumulation of mutations during cancer progression, leading to subclones of cells with metastatic potential. However, alternative models for metastatic progression and site selectivity have also been introduced. According to the contrasting theory, individual cancer cells

forming the primary tumor differ in their metastatic capability. The 'dynamic heterogeneity theory' suggests that the metastatic potential of tumor cells is determined by the rate at which metastatic variants are generated within the primary tumor (Hill *et al.* 1984). The cancer stem cell hypothesis states that metastasis is initiated by a subset of tumor cells that possess stem cell characteristics which may facilitate cancer cell invasion and metastatic progression (Li *et al.* 2007). Genome-wide gene expression profiling of tumor cells has provided some answers to the question of metastatic progression, but interpretation of the data is still controversial.

Supporting the idea of clonal tumor progression toward metastasis, gene expression patterns of primary tumors can be used to classify breast tumors to those with good or poor prognosis (Sorlie *et al.* 2001). Furthermore, several different gene expression signatures that predict the likelihood of later appearance of metastases have been reported (van 't Veer *et al.* 2002, van de Vijver *et al.* 2002). A comparative study of primary adenocarcinomas versus adenocarcinoma metastases revealed a metastatic gene signature that was common to several different tumor types and had significant prognostic value (Ramaswamy *et al.* 2003). It has also been shown that gene expression profiles of metastases are similar to the gene expression profile of the primary breast tumor from the same patient (Perou *et al.* 2000) and that premalignant stages of breast cancer and invasive ductal carcinoma show similar gene expression profiles (Ma *et al.* 2003, Porter *et al.* 2003).

Analyses of bulk tumor samples, however, cannot rule out the existence and possibly critical role of rare variant cells with a high metastatic propensity within the primary tumor. Cells isolated from metastases have been shown to be more metastatic than the bulk population of cells from the primary tumor (Fidler and Kripke 1977, Hart and Fidler 1981). Heterogeneity within the primary tumor has been demonstrated by studies with murine (Fidler and Kripke 1977, Kripke *et al.* 1978, Nicolson *et al.* 1978, Poste *et al.* 1981) and human tumors (Kozlowski *et al.* 1984) as well as by studies with clonal cell lines derived from human cancer cells: highly metastatic variants can be isolated by subcloning or *in vivo* selection and cultured *in vitro* (Montel *et al.* 2005, Urquidi *et al.* 2002).

Alterations in the expression of only a few genes can convert cells from weakly to highly metastatic (Clark *et al.* 2000, Kang *et al.* 2003) without prominently changing the overall gene expression profile of the primary tumor. Therefore, the contrasting models for metastatic progression are not mutually exclusive, and in light of the current evidence, it seems that both global evolution of the primary tumor and rare metastatic variants are potential underlying causes for metastasis (Hynes 2003).

2.1.2 Tumor cell dissemination

In order to become metastatic, the first aspect that cancer cells need to achieve is to detach from the extracellular matrix at the primary tumor site. Normally, detachment leads to apoptosis or anoikis, and therefore, metastatic tumor cells need to overcome

these processes. Epithelial to mesenchymal transition (EMT) is thought to be necessary for tumor cells to develop into invasive and metastatic cells (Thiery 2002). EMT is a cellular program that induces changes in the shape and motility of epithelial cells (Polyak and Weinberg 2009). Cells lose their cell–matrix and cell–cell contacts as well as epithelial polarity and become motile mesenchymal type cells (Thiery 2002). Early stages of the metastatic process are similar to EMT, but at later stages, after extravasation into a metastatic site, cancer cells undergo an inverse differentiation process, mesenchymal to epithelial transition (MET) (Baum *et al.* 2008, Yang and Weinberg 2008). Induction of EMT or MET is stimulated by extracellular cytokines, such as transforming growth factor β (TGF-β), or intracellular mediators, such as Twist and Ras (Kang and Massague 2004, Polyak and Weinberg 2009).

After disconnecting from the extracellular matrix, the next step in the metastatic cascade is to enter the blood circulation. This requires disruption of the endothelial cell layer and degradation of the basement membrane. Tumor cells can also enter the lymphatic system, and subsequent drainage into the blood circulation can lead to bone metastasis. However, the clinical evidence of the prognostic significance of lymph node status to the development of bone metastases is controversial. The data published by Coleman *et al.* indicate that breast cancer patients with at least four positive axillary lymph nodes are likely to have metastases outside the skeleton, and cancers with 1–3 or no axillary lymph node involvement more likely remain confined to the skeleton (Coleman *et al.* 1998). The findings of Kuru *et al.* and Solomayer *et al.* in turn suggest that nodal status does not correlate with the likelihood of skeletal metastases (Kuru *et al.* 2008, Solomayer *et al.* 2000).

The blood circulation is a hostile environment for circulating tumor cells. Most of the cells are eliminated as a result of high shear forces and immune surveillance. Tumor cells may acquire protection against the immune system by a number of mechanisms, such as by attracting platelets (Jurasz *et al.* 2004). Those rare cells that survive in the systemic circulation and reach the low-pressure blood vessels in the bone marrow will then invade across the sinusoidal wall which is lined with non-overlapping endothelial cells and is therefore porous in structure.

2.1.3 Homing to bone

Metastasis to bone is not a random process. The site selectivity of metastasis is partly determined by anatomical and mechanical aspects, such as blood flow, but mostly due to the specific properties of the local microenvironment at the metastatic site (Gupta and Massague 2006). Breast cancer cells home to bone and adhere to the bone marrow endothelium using at least partly the same mechanisms as hematopoietic stem cells homing to bone. Cancer cells express cell surface molecules that can bind to molecules in the extracellular matrix and on cells that reside in the bone microenvironment and thereby mediate cancer cell colonization and survival in bone. For example, high expression of the αvβ3 integrin, which interacts with fibronectin,

vitronectin and osteopontin, in breast cancer cells is associated with higher rates of bone metastasis (Clezardin 2009, Schneider *et al.* 2011).

Breast cancer cells also express the chemokine (C-X-C motif) receptor (CXCR) 4 on their surface, and this is likely to play an important role in breast cancer cell homing to bone because the ligand for CXCR4, CXCL12 (also known as SDF1), is highly expressed by bone marrow stromal cells and osteoblasts. It has been reported that CXCR4 is overexpressed in highly bone metastatic subpopulations of the human breast cancer cell line MDA-MB-231 (Kang *et al.* 2003) and that silencing of CXCR4 in breast cancer cells inhibits metastasis (Liang *et al.* 2005). Ionized calcium, released from the bone matrix as a result of bone resorption, can also be a potent chemoattractant to breast cancer cells, supporting the cancer cell homing to bone (Saidak *et al.* 2009). In addition, interactions between hyaluronan and hyaluronan receptor CD44 as well as interactions between galectin-3 and Thomsen–Friedenreich (TF) antigen can facilitate breast cancer cell adhesion to bone endothelium (Glinskii *et al.* 2005, Glinsky *et al.* 2001, Hill *et al.* 2006).

Cancer cells that have homed to bone marrow may remain dormant without immediate consequences. One third of the patients with localized breast cancer have disseminated tumor cells in the bone marrow at diagnosis (Diel *et al.* 1996, Pantel *et al.* 2003). This reservoir of dormant cells resistant to chemotherapy can survive in the bone marrow for long periods of time. It is known that metastases in bone or other tissues may occur up to ten years after the primary operation and a disease-free period (Aft *et al.* 2010, Braun *et al.* 2000). Presence of disseminated tumor cells in the bone marrow of breast cancer patients is associated with an increased risk of recurrence and cancer-related death (Braun *et al.* 2005).

2.1.4 Metastatic growth in the bone microenvironment

2.1.4.1 Reciprocal interactions and consequences

Bone microenvironment, comprised of mineralized extracellular matrix and specific cell types, is a fertile soil for cancer cells. The major cell types responsible for bone remodeling are osteoclasts and osteoblasts. Osteoclasts are multinucleated myeloid lineage cells that secrete acid and proteases to demineralize bone matrix and degrade bone matrix proteins. Osteoblasts, in turn, are derived from mesenchymal stem cells in the bone marrow and are actively involved in bone formation (Crockett *et al.* 2011). Breast cancer cells secrete proteins that directly or through the effects on other resident cells in the bone microenvironment stimulate the differentiation and modulate the activity of osteoclasts and osteoblasts. This disrupts the fine-tuned balance of physiological bone resorption and formation. Growth factors and ionized calcium that are released from the bone matrix during bone resorption as well as factors secreted by the host and transient cells in the bone microenvironment

promote tumor cell growth and production of factors that modulate the activity of host cells (Holen 2010) (Figure 1).

The interplay between tumor cells and the bone microenvironment can lead to distinct types of skeletal lesions, conventionally classified as osteolytic and osteoblastic. Osteolytic lesions are characterized by bone destruction whereas osteoblastic lesions by overt bone formation. In most of the metastatic skeletal sites, the lesions include both of these features, but osteolytic lesions are the most prevalent type in breast cancer patients (Guise *et al.* 2006, Holen 2010).

In addition to the cancer cell effects on osteoclasts and osteoblasts, interactions with immune cells, platelets and myeloid cells in the bone microenvironment can contribute to the development of bone metastases (Boucharaba *et al.* 2004, Yang *et al.* 2008). Furthermore, tumor cells recruit cell types that are required to establish a blood flow (van der Pluijm *et al.* 2001, Winding *et al.* 2000). Tumor-associated macrophages and other cell types express immunosuppressive factors that protect tumor cells from the elimination by the immune system. B and T lymphocytes are important to normal bone homeostasis, and loss of either cell type can lead to osteoporosis (Li *et al.* 2007). T lymphocytes also secrete cytokines that increase osteoclast-mediated bone resorption (Fournier *et al.* 2006, Sato *et al.* 2006, Takayanagi 2009), but their importance in regulating tumor growth in the bone microenvironment remains largely undefined because *in vivo* modeling of bone metastasis is often done using immunocompromised mice.

2.1.4.2 Factors modulating the extracellular matrix

After extravasation, cancer cells must invade through the extracellular matrix in order to reach bone and start the metastatic growth. Several cancer cell-derived proteolytic enzymes have been proposed to facilitate in the process (Woodward *et al.* 2007). The urokinase plasminogen activator (uPA) and its receptor (uPAR) are involved in the conversion of plasminogen to plasmin, resulting in the degradation of vitronectin, fibrin, proteoglycans, laminin and type IV collagen (Blasi and Carmeliet 2002). The uPA/uPAR complex is inhibited by plasminogen activator inhibitors (PAI) 1 and 2. PAI-1 has been shown to prevent MDA-MB-231 breast cancer cell-mediated degradation of non-mineralized bone matrix (Morgan and Hill 2005). Comparative analyses of the expression of uPA and uPAR in nonmalignant breast epithelial cells, primary breast tumors, micrometastatic cells and breast cancer cells in bone metastases suggest an important role for the uPA/uPAR activity in metastatic progression (Fisher *et al.* 2000, Hemsen *et al.* 2003, Pierga *et al.* 2005, Solomayer *et al.* 1997, Togel *et al.* 2001).

Matrix metalloproteinases (MMPs) are produced by both stromal and tumor cells. MMPs break down extracellular matrix components and thereby facilitate tumor cell migration and invasion. In addition, MMPs support metastatic growth and angiogenesis by releasing growth factors from the bone matrix and through the

cleavage of latent growth factors (Woodward *et al.* 2007). MMP-2, for example, can activate TGF-β2, interleukin (IL) 1b, MMP-1 and MMP-13. Cathepsin K is the major proteolytic enzyme secreted by osteoclasts and contributes to the degradation of extracellular matrix proteins by breaking down type I collagen. Cathepsin K has also been shown to be expressed by breast cancer cells (Le Gall *et al.* 2007, Littlewood-Evans *et al.* 1997), but whether cancer cells themselves are able to degrade bone matrix remains undefined.

2.1.4.3 Tumor cell effects on osteoclasts and osteoblasts

Tumor cell-derived parathyroid hormone-related protein (PTHrP) is one of the most studied mediators of bone destruction associated with bone metastases (Guise *et al.* 1996, Yin *et al.* 1999b). PTHrP stimulates osteoblasts and stromal cells to produce RANKL which binds to its receptor RANK on osteoclast precursors and mature osteoclasts, inducing osteoclast formation and activation. RANKL is the primary mediator of osteoclast formation, survival and function (Lacey *et al.* 1998, Yasuda *et al.* 1998). Over 90% of bone metastases that arise from breast cancer express PTHrP (Southby *et al.* 1990), and the expression of PTHrP is higher in breast cancer bone metastases than in the primary tumor or soft tissue metastases (Henderson *et al.* 2006, Southby *et al.* 1990). Interestingly, a large clinical study revealed that PTHrP expression in primary breast cancer cells is associated with decreased metastasis to all sites, including bone (Henderson *et al.* 2006). Furthermore, PTHrP ablation in the mammary epithelium has been shown to delay primary tumor initiation and progression and to reduce metastasis to distal sites in a breast cancer mouse model (Li *et al.* 2011b). These data imply that PTHrP confers on cancer cells a less metastatic phenotype in the early stages of dissemination and only in the bone microenvironment promotes metastatic growth.

Similar to PTHrP, IL-11 and -6 also affect osteoclasts via RANKL (Kozlow and Guise 2005). IL-11 is expressed by breast cancer cells (Kang *et al.* 2003, Singh *et al.* 2006a), and it mediates osteolysis by stimulating osteoclast formation and bone resorption activity (Girasole *et al.* 1994, Girasole *et al.* 1994, Morgan *et al.* 2004, Sotiriou *et al.* 2001). High expression of IL-11 has been shown to correlate with high histological grade and poor survival in breast cancer (Hanavadi *et al.* 2006). The IL-6 family of cytokines, comprised of ten members, is produced by numerous cell types, including cancer cells and osteoblasts. The role of IL-6 cytokines in the pathophysiology of bone metastasis is complex, as they can induce both bone formation and resorption. The net effect of IL-6 cytokines is likely to be determined by the balance between the different types and by interactions with other factors, such as PTHrP and RANKL, in the bone microenvironment (Blanchard *et al.* 2009). IL-8, in turn, induces bone resorption through a mechanism independent of the RANKL pathway, by acting directly on the IL-8 receptor CXCR1 on osteoclasts and osteoclast

precursors (Bendre *et al.* 2003, Bendre *et al.* 2005). Elevated serum levels of IL-8 in breast cancer patients predict early metastatic spread (Benoy *et al.* 2004).
Vascular endothelial growth factor (VEGF) is highly expressed by breast cancer cells at the bone metastatic sites, and VEGF receptors are expressed by osteoclasts, osteoclast precursors and breast cancer cells (Aldridge *et al.* 2005, Clauss *et al.* 1996, Niida *et al.* 1999). VEGF in combination with RANKL stimulates osteoclast differentiation and bone resorption (Aldridge *et al.* 2005, Niida *et al.* 1999).

Cyclooxygenase 2 (COX-2) expression by breast cancer cells has been suggested to mediate development of bone metastasis through the generation of prostaglandin (PGE_2) which increases the expression of RANKL in osteoblasts and stromal cells. COX-2 also induces the production of IL-8 and -11 in breast cancer cells (Singh *et al.* 2006a, Singh *et al.* 2006b).

Breast cancer cells can also stimulate bone resorption through the Jagged 1-Notch pathway. Jagged 1 directly stimulates osteoclast differentiation as well as activates Notch signaling in osteoblasts, resulting in increased IL-6 secretion by osteoblasts (Sethi *et al.* 2011). Furthermore, breast cancer cells secrete factors, such as noggin, activin A and dickkopf 1 (DKK1), that inhibit osteoblast differentiation and activity (Leto 2010, Schwaninger *et al.* 2007, Voorzanger-Rousselot *et al.* 2007).

2.1.4.4 Bone-derived molecules

Osteolytic bone resorption results in the release of growth factors and ionized calcium from the bone matrix. One of the most abundant growth factors in bone is TGF-β. TGF-β is also secreted by tumor cells and resident bone marrow cells. TGF-β stimulates cancer cells with a variety of consequences. It also inhibits T cell proliferation and the activity of natural killer cells and thereby suppresses the immune system (Fournier *et al.* 2006). TGF-β binds to its receptor on tumor cell surface and activates auto-phosphorylation and signaling through the canonical Smad and non-Smad signaling pathways. The non-Smad signaling pathways involve TGF-β-associated kinase 1 (TAK1) and the p38 and Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) pathways, which in turn activate the transcription factors c-Jun and c-Fos of the activator protein 1 (AP-1) complex. Small GTPases, such as CDC42, Ras, Rho and Rac, have also been implicated in the non-Smad TGF-β signaling (Bhowmick *et al.* 2001, Edlund *et al.* 2002, Wilkes *et al.* 2003). Several studies have elucidated the importance of TGF-β signaling to the development of bone metastases. TGF-β has been shown to regulate the expression of PTHrP, IL-11, CTGF, CXCR4, MMP-1, VEGF and Jagged 1 in breast cancer cells (Blagosklonny *et al.* 2009, Donovan *et al.* 1997, Kakonen *et al.* 2002b, Kang *et al.* 2003, Kang and Massague 2004, Sethi *et al.* 2011). Blockade of TGF-β-signaling by a dominant-negative mutant of the TGF-β type II receptor in MDA-MB-231 breast cancer cells suppresses the ability of these cells to form osteolytic bone metastases in mice (Yin *et al.* 1999b). Knockdown of SMAD4 has a similar effect. In addition, immunohistochemical analysis of human breast cancer

bone metastases has provided evidence for active Smad signaling in human bone metastatic lesions (Kang *et al.* 2005a).

In addition to TGF-β, numerous other bone matrix proteins that are released during bone resorption or secreted by bone resident cells have been implicated in breast cancer bone metastasis. These include osteopontin, bone sialoprotein, secreted protein acidic and rich in cysteine (SPARC), periostin, dentin matrix acidic phosphoprotein 1 (DMP1), syndecan 1 (SDC1), decorin and insulin-like growth factors (IGFs), all of which have also been reported to be expressed by breast cancer cells (Araki *et al.* 2009, Bellahcene *et al.* 2008, Bucciarelli *et al.* 2007, Contie *et al.* 2011, Kelly *et al.* 2010, Pederson *et al.* 1999, Sangaletti *et al.* 2008, Sharp *et al.* 2004, Shevde *et al.* 2010).

Bone-derived calcium affects tumor cells through the calcium-sensing receptor (CASR) (Sanders *et al.* 2000). Calcium stimulates proliferation and inhibits apoptosis (Yamaguchi *et al.* 2001) and leads to increased PTHrP secretion by tumor cells (Sanders *et al.* 2000). A clinical study by Mihai *et al.* showed a correlation between high CASR expression in breast cancer cells and occurrence of bone metastases (Mihai *et al.* 2006).

2.1.4.5 Hypoxia and angiogenesis

In addition to cellular and molecular interactions, hypoxic conditions (1–7% O_2) in the bone microenvironment affect metastatic growth (Blagosklonny *et al.* 2009, Hiraga *et al.* 2007). One of the major mediators of hypoxic signaling that regulates adaptation to hypoxia is hypoxia-inducible factor 1α (HIF-1α) (Mendez-Ferrer *et al.* 2010). Under hypoxic conditions, HIF-1α translocates to the nucleus and heterodimerizes with HIF-1β. The heterodimer binds to hypoxia-response elements (HREs) in the promoter regions of target genes, promoting their transcription (Kollet *et al.* 2006). Several genes implicated in bone metastasis, including VEGF, CXCR4 and IGF-2, harbor HREs (Mendez-Ferrer and Frenette 2007), supporting the critical role of hypoxia in metastatic bone disease. HIF-1α has been shown to stimulate angiogenesis and osteoclast differentiation and to inhibit osteoblast differentiation (Hiraga *et al.* 2007). Silencing of HIF-1α in MDA-MB-231 breast cancer cells inhibits the formation of bone metastases in a mouse model of bone metastasis (Blagosklonny *et al.* 2009). TGF-β stabilizes HIF-1α by inhibiting its degradation (McMahon *et al.* 2006).

High vascularity of the bone marrow facilitates tumor cell survival and growth (Chavez-Macgregor *et al.* 2005), but after the arrest in bone, induction of angiogenesis becomes a prerequisite for the metastatic growth and development of clinically significant metastases. Several growth factors and cytokines secreted by tumor cells, such as VEGF and IL-8, are potent stimulators of angiogenesis (Ferrara and Kerbel 2005). Cancer cells are able to induce endothelial cell proliferation, differentiation and migration (van der Pluijm *et al.* 2001, Winding *et al.* 2000). Furthermore, both osteoblasts and osteoclasts can stimulate angiogenesis. Osteoblasts produce VEGF in

response to hypoxia and thereby couple angiogenesis and bone formation (Cackowski *et al.* 2010, Wang *et al.* 2007).

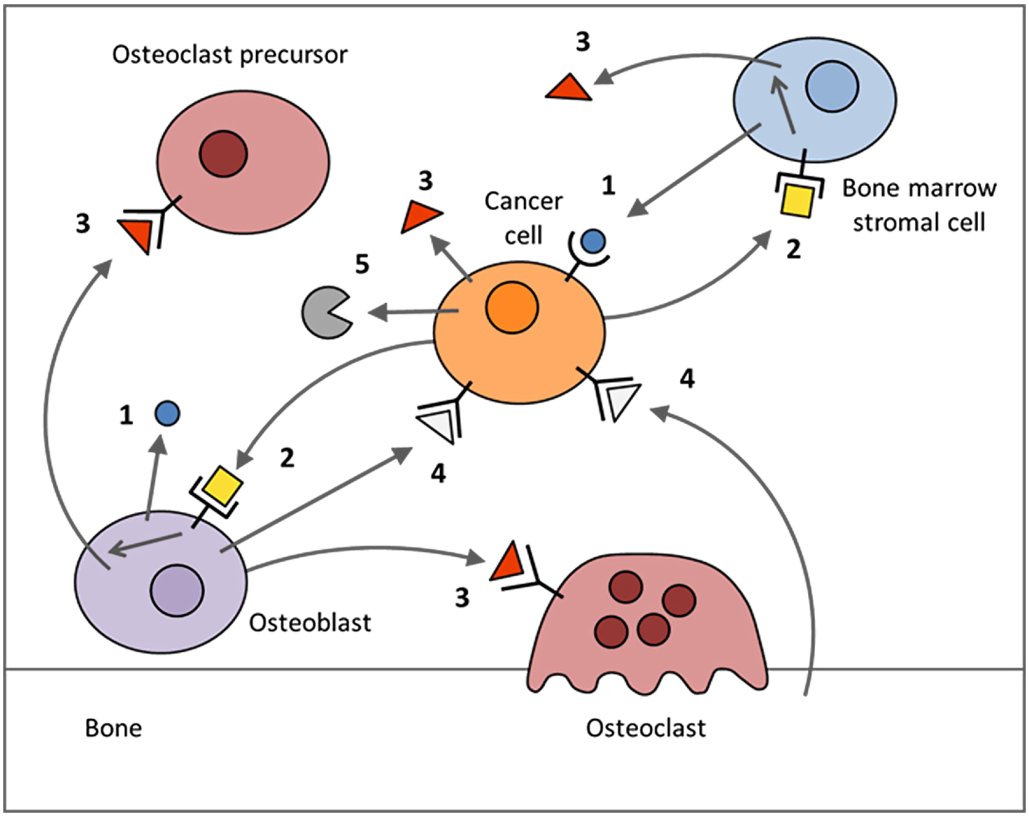

Figure 1. Reciprocal interactions in the bone microenvironment. Stromal cells and osteoblasts produce factors, such as CXCL12, that attract breast cancer cells to the bone microenvironment (1). Breast cancer cells secrete factors, such as PTHrP (2), that modulate the activity of surrounding bone resident cells. PTHrP stimulates osteoblasts and stromal cells to produce RANKL which binds to its receptor RANK on osteoclast precursors and mature osteoclasts (3), inducing osteoclast formation and activation. Factors released from bone during osteoclastic bone resorption or secreted by the resident cells (TGF-β, Ca^{2+}, osteopontin) (4) act on tumor cells to stimulate proliferation or expression of metastatic mediators. Cancer cells release proteolytic enzymes, such as MMPs and cathepsin K, (5) to remodel the extracellular matrix.

2.1.5 microRNAs as regulators of metastasis

Non-coding RNAs, including microRNAs (miRNAs), have emerged as essential regulators of cellular signaling (Alvarez-Garcia and Miska 2005, Kloosterman and Plasterk 2006). Inevitably, altered expression of miRNAs is important to cancer

metastasis as well. MiRNAs are 19–24 nucleotides in size and regulate gene expression in a sequence-specific manner. Following the incorporation into the RNA-induced silencing complex (RISC), miRNAs bind to their target messenger RNAs (mRNAs) through complementarity with the bases 2–7 at the 5′ end of the miRNA, the miRNA seed region (Lewis *et al.* 2003, Lewis *et al.* 2005). Most commonly, this results in either mRNA degradation or inhibition of translation of the target mRNA, but in some cases, miRNAs can also activate gene expression (Orom *et al.* 2008, Stark *et al.* 2007). In addition, miRNAs can directly bind to DNA and thereby regulate gene expression at the transcriptional level (Kim *et al.* 2008). Each miRNA has the potential to bind to several sites on the same mRNA or on hundreds of different mRNAs (Garzon *et al.* 2010). Over 1,000 miRNAs have been identified in humans, but biological roles of only a small fraction of them have been elucidated. Many miRNAs are encoded as genetically linked clusters.

Genome-wide association studies have demonstrated that over 50% of the miRNA genes are located at chromosomal regions linked to cancer, such as fragile sites and regions of deletion or amplification, suggesting that the relevance of miRNAs in cancer might be currently underestimated (Calin *et al.* 2004, McManus 2003). Indeed, it was reported that the expression levels of 217 miRNAs were more effective in cancer classification than the expression levels of more than 16,000 protein-coding genes (Lu *et al.* 2005).

Studies of clinical breast cancer samples have demonstrated that remarkably reduced or completely abolished expression of some miRNAs and overexpression of other miRNAs is associated with metastasis (Martello *et al.* 2010, Valastyan *et al.* 2009). Functional *in vitro* and *in vivo* studies have elucidated metastasis-relevant regulatory functions for miRNAs (Table 1). However, the specific role of miRNAs in the bone metastatic process of breast cancer has only been addressed in a few published studies. Tavazoie *et al.* profiled the expression of 453 miRNAs in MDA-MB-231 human breast cancer cell line variants that were highly metastatic to bone or lung and in the parental MDA-MB-231 cells and subsequently showed that the restored expression of miR-335, miR-206 or miR-126 significantly inhibited formation of bone metastasis in a mouse model of bone metastasis (Tavazoie *et al.* 2008). Another study showed that overexpression of let-7 miRNA in a highly bone metastatic variant of the MDA-MB-231 cell line suppressed bone metastasis (Dangi-Garimella *et al.* 2009). In addition, using a breast cancer mouse model containing human bone and breast tissue, Xia *et al.* profiled miRNA expression in breast cancer cell line variants derived from orthotopic tumors in implanted breast and metastases in implanted bone (Xia *et al.* 2011).

Table 1. Regulatory functions of miRNAs in cancer metastasis.

miRNA	Mechanism	References
EMT / MET		
miR-9	Reduces the expression of E-cadherin and induces EMT and cell invasion *in vitro*.	Ma *et al.* 2010
miR-29a	Suppresses the expression of TTP, induces EMT and promotes breast cancer lung metastasis in a mouse model.	Gebeshuber *et al.* 2009
miR-103, miR-107	Down-regulate miR-200 levels, promote EMT and metastasis *in vivo*.	Martello *et al.* 2010
miR-155	Promotes TGF-β-induced EMT, reduces RhoA expression and disrupts tight junction formation *in vitro*.	Kong *et al.* 2008
miR-200 family	Directly target ZEB1, ZEB2 and TGF-β, which are transcriptional repressors of E-cadherin. Down-regulation initiates EMT. Restored expression promotes MET in later stages of metastatic progression.	Bracken *et al.* 2008, Burk *et al.* 2008, Dykxhoorn *et al.* 2009, Gregory *et al.* 2008b, Park *et al.* 2008
miR-205	Down-regulates ZEB1, ZEB2 and TGF-β. Inhibits EMT *in vitro* and metastasis *in vivo*.	Gregory *et al.* 2008b
miR-448	Forms a positive feedback loop with NF-κB and is involved in TWIST1-induced EMT. Suppression induces EMT *in vitro* and causes lung metastasis *in vivo*.	Li *et al.* 2011c
miR-661	Inhibits SNAI1-triggered EMT and anchorage-independent growth.	Reddy *et al.* 2009, Vetter *et al.* 2010
Cancer cell stemness		
Let-7	Inhibits cancer cell stemness and self-renewal through the silencing of MYC, RAS and HMGA2.	Dangi-Garimella *et al.* 2009, Yu *et al.* 2007
miR-30	Suppression maintains cell self-renewal and stem-like features *in vitro*. Ectopic miR-30 expression reduces metastasis.	Yu *et al.* 2010
miR-200c	Inhibits cell stemness and breast cancer metastasis by targeting ZEBs, TGF-β and BMI1.	Shimono *et al.* 2009
Modification of ECM		
miR-193b	Reduces uPA expression. Inhibits breast cancer metastasis *in vivo*.	Li *et al.* 2009
miR-335	Suppresses the expression of an extracellular matrix component tenascin C and SOX4 as well as several genes whose expression is associated with ECM interactions and risk of distant metastasis.	Tavazoie *et al.* 2008
miR-373, miR-520c	Promote the loosening of ECM connections through the suppression of CD44.	Huang *et al.* 2008

Invasion and migration		
miR-7	Reduces PAK1 expression and inhibits motility and invasiveness of breast cancer cells.	Reddy *et al.* 2008
miR-9	Induces cell invasion *in vitro*.	Ma *et al.* 2010
miR-10b	Promotes cell migration and invasiveness of breast cancer cells through the activation of RhoC by inhibiting HOXD10 and inhibition of Rac by suppressing TIAM1. Induces metastasis *in vivo*.	Bourguignon *et al.* 2010, Edmonds *et al.* 2009, Ma *et al.* 2007, Moriarty *et al.* 2010
miR-31	Inhibits local invasion, early post-intravasation and metastatic colonization.	Valastyan *et al.* 2009, Valastyan *et al.* 2009, Valastyan *et al.* 2010
miR-103, miR-107	Promote migration, invasion and metastasis *in vivo*.	Martello *et al.* 2010
miR-145	Inhibits breast cancer cell motility and invasiveness.	Gotte *et al.* 2010
miR-146	Directly down-regulates the transcription of EGFR, CXCR4 and ROCK1 and inhibits cell migration and invasion.	Bhaumik *et al.* 2008, Bhaumik *et al.* 2008, Hurst *et al.* 2009
miR-155	Induces cell migration and invasion.	Kong *et al.* 2008
miR-193b	Inhibits breast cancer cell migration and invasion *in vitro* and breast cancer metastasis *in vivo*.	Li *et al.* 2009
miR-200 family	Regulate cell migration and EGF-driven invasion.	Dykxhoorn *et al.* 2009, Uhlmann *et al.* 2010
miR-206	Inhibits cancer cell migration.	Song *et al.* 2009
miR-335	Inhibits cell migration.	Tavazoie *et al.* 2008
miR-373, miR-520c	Stimulate cancer cell migration and invasion *in vitro* and *in vivo*.	Huang *et al.* 2008
miR-661	Inhibits cell motility and invasion. Reduces the expression of MTA1.	Reddy *et al.* 2009
Angiogenesis		
miR-9	Down-regulates E-cadherin and thereby activates β-catenin signaling, leading to increased expression of VEGF and tumor angiogenesis.	Ma *et al.* 2010
miR-126	Represses negative regulators of the VEGF pathway and thereby regulates angiogenic signaling and vascular integrity.	Fish *et al.* 2008, Harris *et al.* 2008
miR-132	Enhances neovascularization *in vitro* and *in vivo*.	Anand *et al.* 2010
miR-205	Directly targets and inhibits the expression of VEGF.	Wu *et al.* 2009

Abbreviations: CXCR4: chemokine (C-X-C motif) receptor 4, ECM: extracellular matrix, EGF: epidermal growth factor, EGFR: epidermal growth factor receptor, EMT: epithelial to mesenchymal transition, HMGA2: high mobility group AT-hook 2, HOXD10: homeobox D10, MET: mesenchymal to epithelial transition, MTA1: metastatic tumor antigen 1, NF-κB: nuclear factor κ B, PAK1: p21 protein-activated kinase 1, RhoA: Rho GTPase A, RhoC: Rho GTPase C, ROCK1: Rho-associated, coiled-coil containing protein kinase 1, TGF-β: transforming growth factor β, TIAM1: T lymphoma invasion and metastasis 1, TTP: tristetraprolin, uPA: urokinase plasminogen activator, VEGF: vascular endothelial growth factor, ZEB: zinc finger E-box-binding homeobox.

2.2 Therapeutic intervention of bone metastatic breast cancer

2.2.1 Current treatment

Bone metastases cause severe morbidity and mortality. The treatment of breast cancer patients with bone metastases aims to prevent further progression and skeletal-related events, relief bone pain and improve the patient's quality of life. Current therapeutic options include surgical intervention, targeted radiotherapy, radioisotope treatment, bisphosphonates and the RANKL antibody denosumab. Surgical management reliefs bone pain, improves neurological functions and inhibits local tumor growth (Kurth 2010). Radiotherapy is used to relieve bone pain, but it can also induce tumor regression and bone healing (Koswig and Budach 1999). Treatment with bisphosphonates decreases bone resorption and may improve disease-free survival (Coleman 2008). Denosumab, which was recently approved by U.S. Food and Drug Administration (FDA) for the treatment of patients with bone metastases from solid tumors, suppresses bone resorption and delays the time to the first skeletal-related event in breast cancer patients with bone metastases (Lipton *et al.* 2007, Stopeck *et al.* 2010).

A multimodal approach, including collaboration between radiologists, pathologists, hemato-oncologists, radiation oncologists, nuclear medicine and orthopedic oncologists, is a prerequisite for the effective management of patients with bone metastases (Kurth 2010). The decision to use surgery, radiation and/or bone-targeted agents, alone or in combination, as well as the treatment response depend on the extent of the systemic disease, symptoms, overall prognosis and the life expectancy of the patient at the time of treatment. Most patients with a metastatic bone lesion without a fracture can be managed without surgery, but surgical intervention is often necessary in the case of pathologic fractures, imminent risk of fracture, or to stabilize the weakened bone (Kurth 2010, Zou *et al.* 2008).

2.2.2 Bisphosphonates

In the 1960's, endogenous inorganic pyrophosphate (PP_i) was found to inhibit ectopic calcification (Fleisch and Bisaz 1962). Subsequently, PP_i analogues resistant to hydrolysis, bisphosphonates, were developed and shown to inhibit bone resorption *in vivo* (Fleisch *et al.* 1969). Bisphosphonates have a strong affinity to bone hydroxyapatite and surfaces undergoing active remodeling. Bone-resorbing osteoclasts internalize bisphosphonates, leading to multiple consequences in osteoclasts. Early generation, non-nitrogen containing (pyrophosphate-like) bisphosphonates (clodronate, etidronate) are converted into methylene-containing ATP analogs and cause mitochondrial dysfunction and osteoclast apoptosis (Hughes *et al.* 1995, Roelofs *et al.* 2006, Selander *et al.* 1996). Nitrogen-containing bisphosphonates (alendronate, risedronate, zoledronic acid) inhibit farnesyl pyrophosphatase, an enzyme responsible for the prenylation of GTPases that are essential for osteoclast function, structural integrity and prevention of apoptosis (Luckman *et al.* 1998, Roelofs *et al.* 2006, van Beek *et al.* 1999). Inhibition of farnesyl pyrophosphatase also results in the accumulation of isopentenyl diphosphate which is incorporated into a cytotoxic nucleotide metabolite, ApppI (Monkkonen *et al.* 2006).

Besides the effects on osteoclasts, there are indications for direct effects of bisphosphonates on cancer cells. Bisphosphonates have also been suggested to activate γδ-T cells, modulate tumor-associated macrophages and inhibit angiogenesis (Kunzmann *et al.* 1999, Melani *et al.* 2007, Wood *et al.* 2002). For example, zoledronic acid induces cancer cell apoptosis, reduces vascular invasion and inhibits visceral metastases (Woodward *et al.* 2005). However, higher concentrations of bisphosphonates are required to induce cancer cell apoptosis as compared to the concentrations required for osteoclast apoptosis. Ebert *et al.* showed that zoledronic acid inhibits proliferation and induces apoptosis in human mesenchymal stem cells. These effects depend on the duration of bisphosphonate exposure (Ebert *et al.* 2009). In addition, *in vitro* and *in vivo* studies have shown that sequential administration of chemotherapeutic drugs followed by bisphosphonates enhances cancer cell apoptosis and blocks tumor growth at the metastatic site (Daubine *et al.* 2007, Ottewell *et al.* 2008).

Bisphosphonates are generally well tolerated and only show mild side effects, such as the acute phase reaction which is detected in approximately 10–50% of patients treated for the first time with nitrogen-containing bisphosphonates (Adami and Zamberlan 1996, Saag *et al.* 2007). The most serious adverse effect is osteonecrosis of the jaw. Whether there is an association between the use of bisphosphonates and the risk of esophageal cancer is still controversial (Cardwell *et al.* 2010, Green *et al.* 2010). Due to the strong avidity of bisphosphonates to bone matrix and long half-life in bone, bisphosphonates and other diphosphonate molecules can be used to target other therapeutic compounds to the bone matrix (Erez *et al.* 2008).

2.2.3 Inhibition of RANKL

Given the fundamental role of the RANK–RANKL system in the maturation and function of osteoclasts and thereby in the development of bone metastasis, inhibition of this system has been extensively investigated as a therapeutic tool for the treatment of osteolytic metastasis. The newly FDA-approved agent denosumab is a human monoclonal antibody against RANKL. Denosumab binds to RANKL and thereby prevents the interaction between RANK, which is expressed by osteoclasts, and RANKL produced by osteoblasts. Clinical trials in breast cancer patients with bone metastases have demonstrated that denosumab reduces skeletal-related events and bone resorption, as shown by the reduced levels of a bone turnover marker N-telopeptide of type I collagen (uNTx) (Body *et al.* 2006, Lipton *et al.* 2007). Furthermore, denosumab suppresses bone resorption markers in a fashion independent of prior treatment with bisphosphonates, also in patients who had responded poorly to bisphosphonate treatment (Body *et al.* 2010). A phase III clinical study that compared denosumab and zoledronic acid in the treatment of breast cancer patients with bone metastases, revealed that denosumab was more effective in delaying or preventing skeletal-related events. The overall incidence of skeletal-related events, the adverse effects of renal toxicity and osteonecrosis of the jaw as well as the overall survival of the patients were similar between the two treatment groups (Stopeck *et al.* 2010).

2.2.4 Radioisotope treatment

Internal systemic radiotherapy with bone-seeking radioisotopes is an alternative treatment tool to external radiotherapy for palliation of bone pain caused by bone metastases. Radioisotopes can be particularly useful when painful bone lesions occur at several anatomical sites and in cases where cumulative toxicity from previous radiotherapy is critical. Several different bone-seeking radionuclides have been evaluated in clinical studies (Bauman *et al.* 2005, Paes and Serafini 2010, Roque *et al.* 2003), and to date, three radionuclides have been approved by regulatory agencies in Europe and/or United States: Strontium-89 hydrochloride (Sr-89), samarium-153 lexidronam (Sm-153) and Rhenium-186 hydroxyethylidenediphosphonate (Re-186). However, the majority of clinical evidence arises from trials in prostate cancer patients. Breast cancer patients have participated in randomized controlled trials comparing different radionuclides and different doses of Sm-153, but strong clinical evidence for the efficacy of radionuclides in relieving pain caused by breast cancer bone metastasis is still lacking (Christensen and Petersen 2011).

2.2.5 Emerging therapeutic targets

The currently available therapeutic schemes reduce morbidity but do not prevent bone metastases or result in regression of the established disease. Therefore, inhibitors and antibodies against molecular targets that have been implicated in the bone metastatic process have been developed and are being investigated in preclinical studies and clinical trials. The major types of molecular treatment strategies whose potential in the treatment of bone metastases is currently being investigated are summarized in the Table 2.

Table 2. Current and emerging molecular treatment strategies for bone metastatic breast cancer.

Target	Expression / source	Therapeutic agents	Method	Mechanism / effect	Development stage	References
αvβ integrins	Breast cancer cells	GLPG0187, cilentigide, ATN-161, S247, IMGN388, L-000845704	Antibodies, antagonists	Inhibit cancer cell adhesion to bone marrow endothelium and bone extracellular matrix, inhibit angiogenesis.	Clinical trial	Bauerle *et al.* 2011, Clezardin 2009, Murphy *et al.* 2005
Activin A	Breast cancer cells	ACE-011	Extracellular domain of ActRIIA linked to the Fc portion of human IgG1	Stimulate bone formation and block the formation of osteolytic lesions.	Preclinical	Chantry *et al.* 2010
Calcium receptor	Breast cancer cells		Anti-CaR antibody, CaR silencing by RNAi	Inhibit CaR-induced PTHrP expression and cell growth.	Preclinical	Sanders *et al.* 2000
Cathepsin K	Breast cancer cells, osteoclasts	Odanacatib	Inhibitors	Inhibit collagen type I degradation and bone resorption activity of osteoclasts.	Clinical trial	Le Gall *et al.* 2007
CCL2 (MCP-1)	Breast cancer cells, osteoblasts, stromal cells		Antibody	Inhibit cancer cell migration and osteoclast differentiation.	Preclinical	Craig and Loberg 2006
PTGS2 (COX-2)	Breast cancer cells		Inhibitors	Inhibit cancer cell production of PGE_2, IL-8 and IL-11.	Preclinical	Hiraga *et al.* 2006, Singh *et al.* 2006a, Singh *et al.* 2007

CXCR4	Breast cancer cells, hematopoietic stem cells	Plerixafor, CTCE-9908	Antibody, inhibitors	Block the CXCL12-CXCR4 interaction, reduce breast cancer cell migration and invasion.	Clinical trial	Liang *et al.* 2005, Mehta *et al.* 2007, Richert *et al.* 2009
Galectin-3 / TF antigen	Breast cancer cells, bone marrow endothelial cells		Antibodies, T antigen-masking and -mimicking compounds	Inhibit cancer cell adhesion to the bone endothelium.	Preclinical	Glinskii *et al.* 2005, Glinsky *et al.* 2001
GPNMB	Breast cancer cells, osteoblasts, osteoclasts	Glembatumumab vedotin	Antibody linked to auristatin E (tubulin destabilizer)	Induce cell cycle arrest and apoptosis of cancer cells.	Clinical trial	Tse *et al.* 2006
M-CSF/ c-Fms pathway	Breast cancer cells	Ki20227	c-Fms inhibitor	Decrease the number of osteoclasts and suppress cancer cell-induced osteolysis.	Preclinical	Ohno *et al.* 2006
MET, VEGFR2	Breast cancer cells	Cabozantinib	Inhibitor	Reduce cell invasion *in vitro*. Decrease tumor and endothelial cell proliferation, induce apoptosis and inhibit tumor growth *in vivo*.	Preclinical	Yakes *et al.* 2011
MMPs, ADAM, uPA	Breast cancer cells		Inhibitors	Prevent degradation of the bone matrix.	Preclinical	Morgan and Hill 2005, Winding *et al.* 2002
PGE / PGE receptor	Breast cancer cells		EP4 antagonist	Inhibit the binding of PGE_2 to osteoblasts.	Preclinical	Ohshiba *et al.* 2003
PI3K and MAPK pathway	Breast cancer cells	SB202190	Inhibitors	Inhibit TGF-β-induced MMP-9 activity and uPA production in cancer cells, decrease cancer cell motility.	Preclinical	Suarez-Cuervo *et al.* 2004

PTHrP	Breast cancer cells		Antibody	Induce osteogenesis.	Preclinical	Saito *et al.* 2005
RANKL	Osteoblast precursors	Denosumab	RANKL antibodies and soluble RANK antagonists, Fc-OPG	Prevent RANKL from binding to RANK and hence inhibit osteoclast differentiation and activation.	Approved	Jones *et al.* 2006
Sclerostin	Osteocytes	Scl-AbII	Neutralizing antibody	Inhibit the binding of sclerostin to the LRP5 receptor. Anabolic effects on bone formation.	Preclinical	Ellies *et al.* 2006
Src	Breast cancer cells, osteoclasts	Dasatinib, bosutinib, saracatinib, XL-999, KX2–391, XL-228	Inhibitors	Affect osteoclast morphologic integrity (cytoskeleton organization, ruffled border formation) and bone resorption activity. Reduce tumor cell proliferation, motility, invasion and resistance to apoptotic signals.	Clinical trial	Araujo and Logothetis 2009, Finn 2008, Wheeler *et al.* 2009
TGF-β signaling pathway	Breast cancer cells, bone-resident cells, bone matrix	Trabedersen	Anti-TGF-β agents	Inhibit TGF-β-induced PTHrP expression, inhibit the expression of osteoclastogenic factors, reduce osteoclast activity, enhance osteoblast activity, suppress breast cancer cell migration and invasion.	Preclinical	Kakonen *et al.* 2002b, Yang *et al.* 2002, Yin *et al.* 1999b

Abbreviations: ActRIIA: activin receptor type IIA, ADAM: a disintegrin and metalloproteinase, CaR: calcium-sensing receptor, c-Fms: M-CSF receptor, CCL2: chemokine (C-C motif) ligand 2, CXCL12: chemokine (C–X–C motif) ligand 12, CXCR4: cxc-chemokine receptor 4, COX-2: cyclooxygenase-2, GPNMB: glycoprotein nonmetastatic B, IL: interleukin, MAPK: mitogen-activated protein kinase, MCP-1: monocyte chemotactic protein-1, M-CSF: macrophage colony-stimulating factor, MMP: matrix metalloproteinase, PGE_2: prostaglandin E2, PI3K: phosphoinositide-3 kinase, OPG: osteoprotegerin, PTHrP: parathyroid-hormone-related peptide, PTGS2: prostaglandin-endoperoxide synthase 2, RANK: receptor activator of nuclear factor kappa B, RANKL: RANK ligand, RNAi: RNA interference, siRNA: small interfering RNA, TF: Thomsen–Friedenreichantigen, TGF-β: transforming growth factor β, uPA: urokinase-type plasminogen activator, VEGFR2: vascular endothelial growth factor receptor 2.

3 AIMS OF THE STUDY

The overall goals of this thesis work were to identify molecular mechanisms of bone metastasis in breast cancer, explore molecular and cellular interactions and thereby gain insights into potential novel therapeutic approaches. The specific aims of the three subprojects were:

1. To systematically characterize genes and pathways in highly bone metastatic breast cancer cells and identify mechanisms that may contribute to the metastatic phenotype.
2. To identify genes that regulate TGF-β-induced IL-11 production in breast cancer cells using functional RNA interference screening and to inhibit the development of bone metastases in a mouse model.
3. To identify and characterize miRNAs that inhibit TGF-β-induced IL-11 production in breast cancer cells.

4 MATERIALS AND METHODS

Detailed description of the materials and methods is available in the original publications (I–III) as referred below.

Cells

The MDA-MB-231 cell line (used in I, II and III), originally derived from the pleural effusion of an ER– basal type of breast adenocarcinoma patient in 1973, was obtained from the American Type Culture Collection (ATCC). The MDA-MB-231(SA) variant (used in I, II and III) was spontaneously derived from MDA-MB-231 cells during a long *in vitro* culture. Human osteoclast precursors (CD34 positive hematopoietic stem cells) (used in I and II) were obtained from Lonza (Walkersville, MD, USA).

Mouse model of breast cancer bone metastasis (I, II)

Female nude mice (Balb/c nu/nu, Harlan, Indianapolis, IN, USA) 4–5 weeks of age were inoculated with 100,000 MDA-MB-231(SA) cells into the left cardiac ventricle. The proper inoculation site was determined by drawing a line between orientation points on the thorax and sternum of each mouse. The needle was placed on the midpoint of this line and offset slightly to the left on the ribcage. The needle was then inserted until pulsating blood entered the syringe chamber as a clear indication that the tip of the needle was inside the left cardiac ventricle. The mice were maintained in a pathogen-free environment and monitored daily for clinical signs, such as symptoms of cachexia (curved spine, dry skin and significant loss of weight), overall activity, consumption of feed and water, possible diarrhea and signs of hematoma on the site of compound administration.

***In silico* gene expression analysis of clinical samples**

The GeneSapiens database (www.genesapiens.org) was utilized in the *in silico* gene expression analyses. This database contains Affymetrix microarray data collected from various publically available sources such as Gene Expression Omnibus and ArrayExpress. All data were re-annotated and normalized with a custom algorithm (Kilpinen *et al.* 2008).

Antibodies

Antigen	Species	Supplier	Used in
Primary			
β-actin	Mouse	Sigma	I
Hsc70	Rat	Stressgen	I
PHGDH	Mouse	Abnova	I
PSAT1	Chicken	US Biological	I
PSAT1	Rabbit	Strategic Diagnostics Inc.	I
HRP-conjugated			
Chicken IgY	Donkey	Jackson ImmunoResearch	I
Rat IgG	Goat	GE Healthcare	I
IRDye 800-conjugated			
Rabbit IgG	Goat	Rockland Immunochemicals	I
Alexa Fluor 680-conjugated			
Mouse IgG	Goat	Invitrogen	I

Microarrays

Microarray	Supplier	Used in
Comparative genomic hybridazation		
Human Genome CGH 244K oligonucleotide microarray	Agilent	I
Gene expression		
GeneChip U133 Plus 2	Affymetrix	I
HumanHT-12 Expression BeadChip	Illumina	III
microRNA expression		
Microarray platform v1	Agilent	III

siRNAs

Target gene	ID	Supplier	Used in
AllStars negative control	1027281	Qiagen	II, III
HS6ST2	SI00442232	Qiagen	II
IL-11	SI00013475	Qiagen	II, III

In addition, a library consisting of 193 siRNAs (Qiagen) was used in a cell-based screen (II: Supplementary Table 2).

Other reagents and kits

Reagent	Supplier	Used in
Acid Phosphatase Leukocyte Kit	Sigma	I, II
Amersham ECL Western Blotting Detection Reagents	GE Healthcare	I
Anti-miR miRNA inhibitors	Amgen	III
BoneTRAP Assay	Immunodiagnostic Systems Ltd	I, II
Bovine bone slices	Nordic Bioscience Diagnostics	I, II
CellTiter-Blue Reagent	Promega	I, II, III
Chromogenic IL Test Heparin assay kit	Instrumentation Laboratory	II
Cignal Smad Reporter Assay kit	SABiosciences	II, III
CrossLaps for Culture ELISA kit	Nordic Bioscience Diagnostics	I, II
Cysteine protease inhibitor E64	Sigma	II
Dalteparin (fragmin)	Pharmacia	II
Dual-Glo Luciferase Assay System	Promega	II, III
DuoSet ELISA Development System for Human IL-11	R&D Systems	II, III
Heparin	LEO Pharma	II
High Capacity cDNA Reverse Transcription kit	Applied Biosystems	I, II, III
Hoechst 33258	Sigma-Aldrich	II
IL Test APTT Lyophilized silica kit	ILS Laboratories Scandinavia	II
IL-11 3' UTR reporter constructs	SwitchGear Genomics	III
Pre-miR miRNA precursors	Amgen	III
Recombinant human TGF-β	R&D Systems	II, III
RNeasy Mini kit	Qiagen	I, II, III
shRNA lentiviral transduction particles	Sigma	I
siLentFect Lipid Reagent	Bio-Rad Laboratories	II, III
Soybean trypsin inhibitor	Sigma-Aldrich	II, III
TRITC-conjugated WGA lectin (L-5266)	Sigma	II

Methods

Method	Used in
Array-CGH	I
Bioinformatic analysis of clinical samples	I
Breast cancer cell culture	I, II, III
Gene expression profiling	I, III
Histological analysis	I, II
Immunoassays	I, II, III
In vitro coagulation tests	II
Luciferase reporter assays	II, III
MiRNA expression profiling	III
MiRNA target prediction	III
Osteoclast culture	I, II
Cell viability assay	I, II, III
Reverse transcription PCR	I, II, III
Reverse transfection	II, III
RNA extraction	I, II, III
Statistical analysis	I, II, III
Western blotting	I

5 RESULTS AND DISCUSSION

5.1 *In vivo* and *in vitro* characterization of highly metastatic MDA-MB-231(SA) breast cancer cells (I, III)

5.1.1 Bone metastatic capability in a mouse model of bone metastasis

The MDA-MB-231 breast cancer cell line is commonly used to model breast cancer bone metastasis *in vivo*. When these cells are inoculated into the blood circulation of immunodeficient mice by intracardiac inoculation, osteolytic lesions are formed (Yin *et al.* 1999a). The MDA-MB-231(SA) variant is known to be highly bone metastatic and has been used in several studies (Bendre *et al.* 2005, Kakonen *et al.* 2002a). Because comparative studies on this variant and the parental MDA-MB-231 cell line had not been previously reported, we compared the *in vivo* bone metastatic properties of these cell types. MDA-MB-231(SA) or parental MDA-MB-231 cells were inoculated into the left cardiac ventricle of nude mice. The mice were sacrificed when they became cachectic, or paraplegia was observed. Development of bone metastases in living mice was monitored by x-rays. The heart, lungs, spleen, kidneys, liver, kidneys and adrenal glands were collected for histology at sacrifice in order to determine possible soft tissue metastasis.

The mice inoculated with MDA-MB-231(SA) cells were sacrificed on or before day 26 after inoculation (survival 23 ± 4.2 days, mean ± SD). The mean body weight of the mice in the MDA-MB-231(SA) group was significantly lower already on day 21 as compared to the parental MDA-MB-231 group (I: Figure 1a). The first mouse in the parental MDA-MB-231 group was sacrificed on day 58, and the mean survival time was 76.5 ± 19.1 days (mean ± SD) in this group (I: Figure 1b). Osteolytic lesions were observed in six out of seven mice in the MDA-MB-231(SA) group two weeks after inoculation, and the lesions were severe at sacrifice (I: Figure 1c, Online Resource 2). Histology revealed no soft tissue metastasis. Only one mouse in the parental MDA-MB-231 group had an evident osteolytic lesion.

5.1.2 Gene copy number and gene and microRNA expression profiles

In order to identify molecular mechanisms that could explain the differences in the metastatic potential of the MDA-MB-231(SA) variant and the parental cells, we compared the molecular profiles of these cell types using array-based methods. First, comparative genomic hybridization (CGH) indicated that the genetic aberrations in the two cell lines were highly similar (I: Online Resource 3). Second, as shown by genome-wide gene expression profiling of the MDA-MB-231(SA) variant and the parental cell line, 315 genes (1.7 %) of all genes were over 2.5-fold up-regulated and

198 genes (1.1 %) over 2.5-fold down-regulated in the highly metastatic variant. Third, we measured the expression of 455 miRNAs and found that 16 (3.5 %) of the miRNAs were over 3-fold differentially expressed between the MDA-MB-231(SA) variant and the parental MDA-MB-231 cells. Of these, five miRNAs were down-regulated and eleven up-regulated in the MDA-MB-231(SA) cells (III: Table S1). The miR-200 family was strongly represented: three of the five family members, miR-200b, -200a and -429, were among the four most down-regulated miRNAs in the MDA-MB-231(SA) cells. These miRNAs have previously been reported to regulate EMT (Gregory *et al.* 2008a). Interestingly, miR-210, whose high expression has previously been associated with increased likelihood of distant metastasis (Foekens *et al.* 2008) and poor clinical outcome (Rothe *et al.* 2011) in breast cancer, was 6.5-fold down-regulated in the MDA-MB-231(SA) cells. Taken together, our data from these extensive profiling studies indicated that the MDA-MB-231(SA) variant was clonally closely related to the parental cell line, suggesting that a limited number of genetic alterations and expression changes were sufficient to cause a major difference in the metastatic propensity.

5.2 Importance of serine to breast cancer cells and osteoclasts (I)

5.2.1 Expression of L-serine biosynthesis and transport genes in MDA-MB-231 variants

Analysis of the gene ontology (GO) and pathway associations of the genes up-regulated in MDA-MB-231(SA) as compared to the parental cells indicated that the most significantly enriched pathway was L-serine metabolism (I: Online Resource 4 and 5). All the three enzymes involved in the phosphorylated pathway of L-serine biosynthesis (I: Figure 2), phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1) and phosphoserine phosphatase (PSPH), were up-regulated in the MDA-MB-231(SA) cells. Of note, there was a remarkably strong, 648-fold induction of PHGDH expression. Furthermore, a transporter for serine, alanine, cysteine and threonine, SLC1A4 (solute carrier family 1 (glutamate/neutral amino acid transporter), member 4), was 3.4-fold up-regulated in the MDA-MB-231(SA) cells (I: Figure 3). These findings were further validated by the gene expression profiling data on twelve *in vivo* selected MDA-MB-231 subpopulations with different bone metastatic abilities. PHGDH expression was significantly higher in the strongly versus weakly metastatic cells, and PSAT1 expression was highest in one of the most highly bone metastatic subpopulations. Therefore, L-serine biosynthesis has a potentially critical functional role in the causation of the bone metastatic activity of MDA-MB-231 cells. Whether L-serine biosynthesis specifically influences bone metastatic capability or general metastatic properties of breast cancer cells should be investigated by testing how knockdown of L-serine biosynthesis in breast cancer cells affects their

metastatic behavior in several different mouse models, including an orthotopic model, of breast cancer metastasis.

5.2.2 Knockdown of serine biosynthesis inhibits MDA-MB-231(SA) cell proliferation

The phosphorylated pathway of L-serine biosynthesis is the primary source of L-serine in mammalian tissues (Snell 1984). In order to see whether this pathway is critical to the proliferation of MDA-MB-231(SA) cells, we silenced PSAT1 expression in MDA-MB-231(SA) cells by shRNA and cultured the PSAT1 knockdown and scrambled shRNA control cells as well as parental MDA-MB-231 cells in serine-free conditions and in varying concentrations of L-serine. Cell proliferation rates of all cell types were significantly lower in the absence of serine than in L-serine containing medium, but proliferation of the parental MDA-MB-231 cells as well as MDA-MB-231(SA) cells whose PSAT1 expression had been silenced was significantly more dependent on L-serine than that of the MDA-MB-231(SA) control cells. However, as low as 10% of the physiological serum concentration of L-serine was sufficient to compensate for the PSAT1 knockdown (I: Figure 4). L-serine concentrations may be rate-limiting in the microenvironment of the breast cancer cells *in vivo*, such as in the bone marrow or other phases of the metastatic process, and hence enhanced expression of serine biosynthesis genes in metastatic cell clones may provide a selective advantage for cancer progression.

5.2.3 L-serine stimulates osteoclastogenesis

Ogawa *et al.* suggested that L-serine is essential for the osteoclastogenesis of the RAW264 mouse monocyte/macrophage cell line which is commonly used as a model for osteoclast differentiation (Ogawa *et al.* 2006). We tested whether L-serine is needed for the formation of human osteoclasts capable of resorbing bone. Human osteoclast precursors were cultured on bone slices in the absence and presence of L-serine or phospho-L-serine. Osteoclast formation was quantified by measuring the activity of an osteoclast specific enzyme, tartrate-resistant acid phosphatase (TRACP) 5b, in the culture medium after seven days of culture. On day 11, the cells were stained with TRACP, and bone resorption was quantified by measuring the concentration of the degradation products of C-telopeptides of type I collagen (CTX) in the medium. Our results demonstrated that in the absence of L-serine and phospho-L-serine, the cells remained mononuclear and the TRACP 5b activity was very low. Addition of L-serine or phospho-L-serine significantly increased osteoclastogenesis (I: Figure 5). Taken together, our data suggest that enhanced L-serine production by metastatic breast cancer cells is likely to contribute to bone destruction in metastatic lesions.

5.2.4 Expression of L-serine biosynthesis and transport genes in clinical samples

Clinical significance of PHGDH, PSAT1, PSPH and SLC1A4 was assessed using previously published gene expression data on clinical samples. First, we analyzed the correlation of PHGDH, PSAT1, PSPH and SLC1A4 expression to time to relapse and to overall survival time in breast cancer. This analysis demonstrated a significant association between high PHGDH expression and shorter time to relapse as well as overall survival time. Also, high PSAT1 expression was associated with shorter relapse-free and overall survival (I: Figure 6a and b). High expression of PSPH correlated significantly with overall survival time but not with time to relapse. Second, we studied whether differential expression of PHGDH, PSAT1, PSPH or SLC1A4 correlated with clinically relevant features in a set of 251 breast cancer samples. High expression of PHGDH and PSAT1 was associated with several features typical of poor outcome: ER and PgR negative status, mutated p53, high grade, basal subtype, high expression of proliferating cell nuclear antigen (PCNA) and Ki-67, which are used as proliferation markers, and high ERBB2 expression (I: Figure 6c). A similar but not as strong and significant association was seen between these clinical parameters and high PSPH expression.

We extended our bioinformatic analysis to comprehensive exploration of the expression of L-serine biosynthesis genes in 19,000 clinical non-malignant and cancer samples. These data indicated that PHGDH and PSAT1 are highly expressed in several other tumor types beyond breast cancer (Figures 2 and 3). PHGDH showed the highest expression in glioma, astrocytoma, squamous cell carcinoma, Burkitt's lymphoma and plasma cell leukemia. PSAT1 expression was highly elevated in astrocytoma, glioma, glioblastoma and ovarian clear cell carcinoma. Our finding on the high expression of PSAT1 in the clear cell type of ovarian carcinoma is in line with a recently published proteomic analysis which revealed PSAT1 as a histology-specific biomarker for this subtype (Toyama *et al.* 2012). Among the healthy tissue samples, both PHGDH and PSAT1 are highly expressed in brain (corpus callosum) and spinal cord, consistent with the previously reported functions for L-serine in the central nervous system (de Koning *et al.* 2003). Interestingly, PHGDH and especially PSAT1 are also highly expressed in mesenchymal stem cells, suggesting an association to the stem cell phenotype.

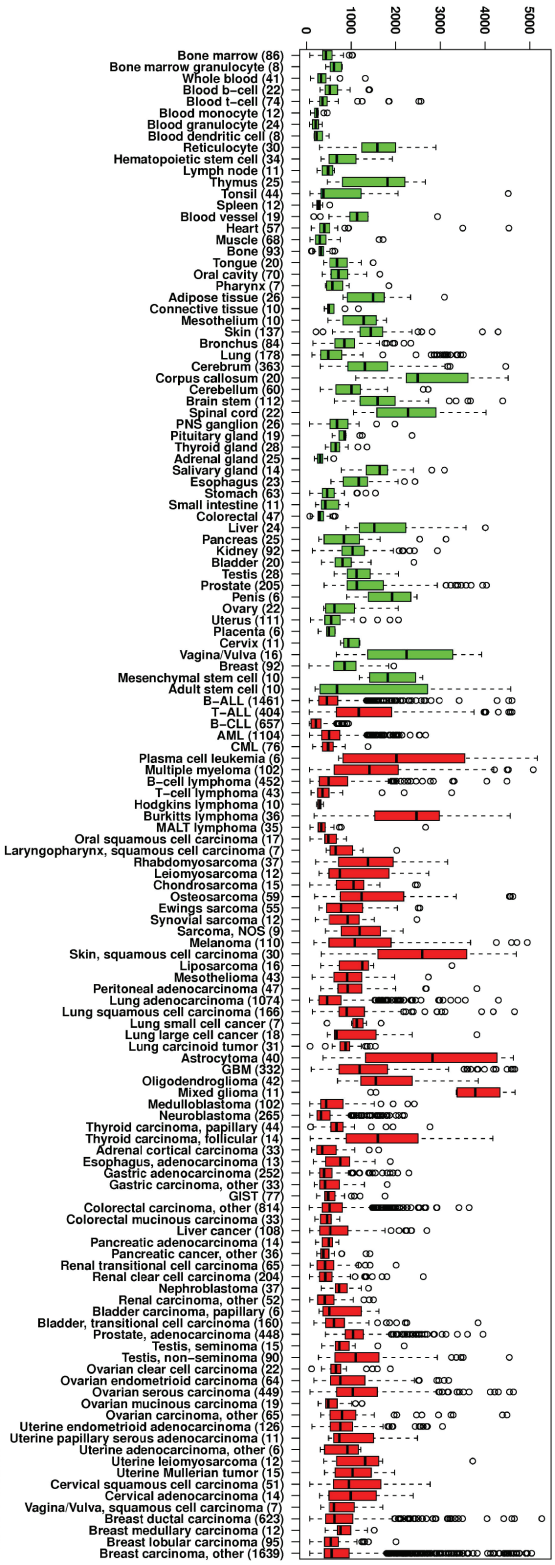

Figure 2. PHGDH mRNA expression in clinical non-malignant (green) and cancer (red) samples. The number of samples is given in the parentheses.

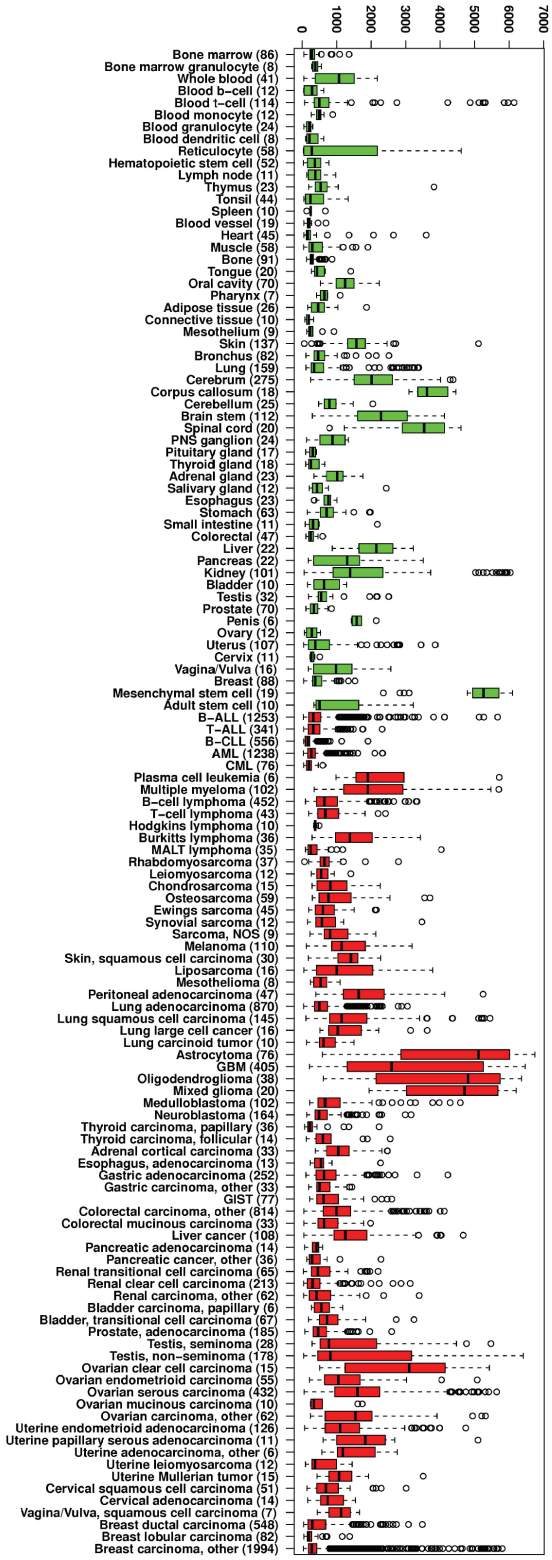

Figure 3. PSAT1 mRNA expression in clinical non-malignant (green) and cancer (red) samples. The number of samples is given in the parentheses.

Overall, our findings are in line with previous observations suggesting that the phosphorylated pathway of L-serine biosynthesis is important for cell proliferation (de Koning *et al.* 2003) and is enhanced in neoplastic as compared to normal tissues (Snell 1984). Our finding on the association of PHGDH expression to the basal subtype, triple-negative (ER–, PgR–, ERBB2–) status and high grade in breast cancer was confirmed by two subsequent studies (Locasale *et al.* 2011, Possemato *et al.* 2011). Immunohistochemical analysis of human breast tumor samples indicated that PHGDH protein expression was elevated in approximately 70% of ER– breast tumors (Possemato *et al.* 2011). These studies (Locasale *et al.* 2011, Possemato *et al.* 2011) also showed that the chromosomal region where PHGDH is located is frequently amplified in breast cancer as well as in melanoma samples and that amplification correlates with protein overexpression. This suggests that PHGDH may be selected for during breast cancer progression and that this may sometimes be the critical rate-limiting step. However, the fraction of ER– breast cancers with elevated PHGDH expression at the mRNA and protein level is higher than the fraction with PHGDH amplification at the DNA level, and PHGDH protein levels are not only elevated in cell lines with PHGDH amplification but also in MT3 and Hs578T (Possemato *et al.* 2011) as well as in MDA-MB-231(SA) cells that do not harbor PHGDH amplification. Therefore, additional mechanisms beyond the increase in the gene copy number must exist for boosting PHGDH expression.

The phenotypic consequences of enhanced L-serine biosynthesis and the mechanism how it promotes tumorigenesis / metastasis remain largely undefined. In addition to our finding on the stimulatory effect of L-serine to breast cancer cell proliferation and osteoclast differentiation, it has been shown that ectopic expression of PHGDH in mammary epithelial cells disrupts acinar morphogenesis and preserves the viability of matrix-deprived cells to survive in an anchorage-independent fashion (Locasale *et al.* 2011). Due to the high frequency of PHGDH amplification and elevated expression of PHGDH and PSAT1 in several types of cancer, additional phenotypic effects and mechanisms are likely to be discovered.

5.3 Anti-bone metastatic properties of heparin-like glycosaminoglycans (II)

5.3.1 Inhibition of TGF-β-induced IL-11 production in MDA-MB-231(SA) cells

We noted an increased TGF-β-induced IL-11 secretion in the highly bone metastatic MDA-MB-231(SA) variant as compared to the parental MDA-MB-231 cell line (II: Supplementary Figure 1). Given the important roles of TGF-β and IL-11 in the bone metastatic process, we aimed to identify critical mediators of the TGF-β induction of IL-11 among the genes that were highly overexpressed in the MDA-MB-231(SA) variant. A cell-based screen was conducted using a library of 193 siRNAs targeting

107 genes that were most highly overexpressed in MDA-MB-231(SA) as compared to the parental cells. One of the most potent inhibitors of TGF-β-induced IL-11 secretion in MDA-MB-231(SA) cells was an siRNA targeting heparan sulfate 6-O-sulfotransferase 2 (HS6ST2) (II: Figure 1A and B, Supplementary Table 2). HS6ST2 encodes an enzyme that attaches sulfate groups to C-6 of glucosamine residues in heparan sulfate (HS) glycosaminoglycans. Overexpression of HS6ST2 in MDA-MB-231(SA) as compared to the parental cells was confirmed by quantitative reverse transcription PCR (RT-PCR) (II: Supplementary Figure 3A). We also studied the effects of other enzymes that attach sulfate groups to glucosamine residues in HS glycosaminoglycans and found that similar to HS6ST2, silencing of HS6ST1 inhibited TGF-β-induced IL-11 production. Conversely, an enzyme responsible for the sulfation of the C-3 of glucosamine residues, HS3ST1, slightly increased TGF-β-induced expression of IL-11 (II: Supplementary Figure 2B).

Because TGF-β induction of IL-11 has been shown to be mediated by the Smad pathway (Gupta *et al.* 2011, Kang *et al.* 2005b), we tested whether silencing of HS6ST2 affected Smad2/3/4-mediated transcriptional activity. This was done using a luciferase reporter construct containing a functional Smad binding site. HS6ST2 silencing suppressed the TGF-β-induced luciferase signal, indicating a decrease in Smad binding to its response element (II: Figure 1C).

Previous studies have indicated that HS glycosaminoglycans can bind several growth factors and cytokines. These interactions critically depend on the amount and positions of the sulfate groups in HS glycosaminoglycans (Fernig and Gallagher 1994, Lyon and Gallagher 1994, Nakato and Kimata 2002). TGF-β has also been shown to bind to highly sulfated HS glycosaminoglycans *in vitro*. Binding potentiates the biological activity of TGF-β (Lyon *et al.* 1997, McCaffrey *et al.* 1989, McCaffrey *et al.* 1994). We therefore hypothesized that exogenous highly sulfated HS glycosaminoglycans could interfere with the physiological interactions between TGF-β and cell surface HS glycosaminoglycans and thereby inhibit TGF-β signaling. Indeed, our data showed that heparin and a chemically sulfated bacterial polysaccharide K5-NSOS inhibited TGF-β-induced IL-11 production in MDA-MB-231(SA) cells. Fragmin did not have a similar effect which might be due to its lower molecular weight and consequently reduced interaction capacity (II: Figure 2).

5.3.2 K5-NSOS and fragmin reduce osteolytic lesions and tumor burden in mice

Therapeutic potential of K5-NSOS and fragmin was evaluated in a mouse model of breast cancer bone metastasis. The mice that had been inoculated with MDA-MB-231(SA) cells were treated with fragmin, K5-NSOS or vehicle once a day. Only 18% of the fragmin-treated mice and 29% of the K5-NSOS-treated mice were cachectic as compared to 86% of the vehicle-treated mice at sacrifice, and the body weights of the fragmin- and K5-NSOS-treated mice at sacrifice were significantly higher as compared

to the vehicle group (II: Figure 3A). Treatment with fragmin or K5-NSOS did not significantly affect the size of the bone area or the number of osteoclasts per mm tumor-bone interface, but osteolytic lesion area, as measured by radiographs, was smaller in the fragmin- and K5-NSOS-treated groups than in the vehicle group (II: Figure 3B and C). Histomorphometric examination showed a significant reduction in tumor burden in bone in both fragmin- and K5-NSOS-treated group as compared to the vehicle group (II: Figure 3D and E). The total tumor area per mouse was significantly smaller in both treatment groups than in the vehicle group (II: Figure 3E). These data extend previously published observations that have implicated anti-metastatic effects for heparin and HLGAGs in animal model systems (Borgenstrom *et al.* 2007, Borsig *et al.* 2001, Kragh *et al.* 2005, Poggi *et al.* 2002, Szende *et al.* 2005, Yee *et al.* 2008). Furthermore, clinical studies have shown that heparin and HLGAGs may prolong survival of cancer patients with solid tumors (Hettiarachchi *et al.* 1999, Kakkar *et al.* 2004, Klerk *et al.* 2005).

5.3.3 Effects of K5-NSOS and fragmin on human osteoclasts

We also investigated the effects of K5-NSOS and fragmin on osteoclasts. Human osteoclast precursors were cultured on bovine bone slices, and after the seven-day differentiation period, fragmin or K5-NSOS was added to the culture medium. To determine the number of osteoclasts, TRACP 5b activity was measured from the medium on day 7. The amount of CTX released from bone was quantified on day 10. As determined by the resorption index CTX/TRACP 5b, K5-NSOS had a dose-dependent inhibitory effect on the resorption activity of osteoclasts, whereas fragmin did not show any significant effect (II: Figure 4A). Multinuclear TRACP-positive osteoclasts were visualized by staining the nuclei and the TRACP content of the cells. Osteoclast activity was also determined by a pit formation assay: resorption pits were visualized using TRITC-conjugated WGA lectin. Neither K5-NSOS nor fragmin had toxic effects on osteoclasts but K5-NSOS treatment reduced the size and the depth of the resorption pits (II: Figure 4B).

The inhibitory effect of K5-NSOS on osteoclast activity was surprising in light of previously reported findings: heparin has been shown to promote bone resorption by increasing the activity and number of osteoclasts (Muir *et al.* 1996, Nishiyama *et al.* 1997, Shaughnessy *et al.* 1995). Furthermore, long-term or high-dose heparin therapy has been reported to cause osteoporosis (Douketis *et al.* 1996, Hawkins and Evans 2005, Nelson-Piercy 1998). The unexpected anti-resorptive effect of K5-NSOS warrants further studies in non-tumor bearing animals. In addition, the effects of K5-NSOS on osteoblasts should be investigated.

5.3.4 Anticoagulant properties of K5-NSOS, fragmin and heparin

Heparin and other highly sulfated HS glycosaminoglycans are commonly used to prevent venous thromboembolism, which is a common complication in cancer patients as well. However, the anticoagulant activity of heparin and HLGAGs limits the applicability of these compounds in cancer therapy. We compared the anticoagulant properties of K5-NSOS to those of fragmin and heparin by measuring the antithrombin (anti-IIa) and antifactor Xa activity (anti-Xa), which are specific coagulation parameters typically determined for heparin-like molecules. Fragmin and heparin showed the expected anti-IIa and anti-Xa activity whereas only moderate anti-IIa and anti-Xa activities were observed for K5-NSOS. In addition, the effects of fragmin and K5-NSOS on activated partial thromboplastin time (APTT) were tested. K5-NSOS had a weak prolonging effect on APTT, similar to that of fragmin, indicating a weak influence on global plasma coagulation (II: Table 1). These properties of K5-NSOS make it substantially more applicable, as compared to heparin and other anti-coagulants, as a cancer therapeutic agent.

5.4 MiRNAs that inhibit TGF-β-induced expression of IL-11 (III)

5.4.1 Identification of miRNAs that regulate IL-11 production

We hypothesized that miRNAs might be responsible for the increased basal and TGF-β-induced IL-11 expression in the MDA-MB-231(SA) as compared to the parental MDA-MB-231 cells. In order to test this, we performed overexpression screens with miRNA precursors in MDA-MB-231(SA) cells and measured IL-11 secretion levels with and without added TGF-β in the medium. We chose to screen for miRNAs that were down-regulated in the MDA-MB-231(SA) cells and those that were predicted to target IL-11 according to the miRanda, PicTar or TargetScan prediction algorithm. This combined selection resulted in 55 miRNAs. We performed overexpression screens with miRNA precursors in MDA-MB-231(SA) cells and measured the effects of miRNAs on IL-11 secretion in the absence and presence of added TGF-β in the medium. Analysis of the data from the replicate screens pointed out miR-204, -211 and -379 as the most potent inhibitors of IL-11 secretion (III: Figure 1). Our follow-up studies indicated that miR-204, -211 and -379 reduced not only IL-11 secretion (III: Figure 2A) but IL-11 mRNA levels as well (III: Figure 2B). Furthermore, inhibition of miR-204 and -211 by specific Anti-miR inhibitors increased IL-11 secretion in the parental MDA-MB-231 cells (III: Figure 2C). In regard to miR-204, our finding is in line with a previous report showing reduced expression of IL-11 protein in miR-204-transfected human trabecular meshwork cells (Li *et al.* 2011a). To our knowledge, miR-211 or -379 have not been previously linked to IL-11 production or breast cancer metastasis.

5.4.2 MiR-204, -211 and -379 bind to the IL-11 3' UTR

We studied whether the inhibitory effect of miR-204, -211 and -379 on IL-11 expression was due to direct targeting of the mRNA. First, we used miRNA target prediction algorithms to investigate the potential of miR-204, -211 and -379 binding to the 3' untranslated region (UTR) of IL-11 mRNA. Four algorithms, miRanda, DIANA-microT, miRDB and PITA all predicted two binding sites in the IL-11 3' UTR for miR-204 and -211 (III: Table S2). These two miRNAs share the same seed sequence and only differ in two nucleotides on positions 17 and 18. MiR-379 was not predicted to target IL-11 according to MiRanda, PicTar, TargetScan, DIANA-microT or miRDB, but the PITA tool showed 17 6-mer seed sites and one 7-mer seed site for miR-379 in the IL-11 3' UTR (III: Table S2). Luciferase reporter assays were then performed to determine whether miR-204, -211 or -379 bind to the IL-11 3' UTR sequence. Binding to the 3' UTR was assayed in two parts: base pairs 1–450 and 451–1,618. A luciferase reporter construct containing one of the two fragments was transfected into MDA-MB-231(SA) cells together with an miRNA precursor and a Renilla luciferase control plasmid. Our results indicated that miR-204, -211 and -379 could bind to the IL-11 3' UTR (III: Figure 3). Our finding on the miR-379 binding of to the IL-11 3' UTR was not predicted by the commonly used miRNA target prediction algorithms, revealing the lack of sensitivity of bioinformatic prediction of miRNA targeting and insufficient knowledge of miRNA binding patterns.

5.4.3 Gene expression changes induced by miR-204 and -379

Given the multi-gene regulatory capacity of miRNAs, miR-204, -211 and -379 are likely to down-regulate the expression of IL-11 through additional mechanisms beyond direct targeting. In order to delineate the regulatory functions of these miRNAs, the gene expression changes caused by miR-204 or -379 overexpression in MDA-MB-231(SA) cells were analyzed using genome-wide gene expression profiling. In addition, MDA-MB-231(SA) cells transfected with IL-11 siRNA were profiled. Using 1.5-fold difference to the negative control siRNA or Pre-miR as a cut-off, the only common gene between the IL-11 siRNA, Pre-miR-204 and Pre-miR-379-treated cells was IL-11, and there was an overlap of only 15 genes between the genes that were over 1.5-fold differentially expressed in response to miR-204 and -379, suggesting that miR-204 and -379 largely engage in distinct regulatory pathways (III: Figure 4, Tables S3 and S4). Interestingly, both miR-204 and -379 down-regulated the expression of prostaglandin-endoperoxide synthase 2 (PTGS2, also known as COX-2). Based on previous publications, TGF-β stimulates PTGS2 expression in breast cancer cells (Hiraga *et al.* 2006) and silencing of PTGS2 decreases IL-11 expression in MDA-MB-231 cells (Singh *et al.* 2006a, Stasinopoulos *et al.* 2007). Therefore, in addition to direct targeting, the inhibitory effects of miR-204 and -379 on IL-11 expression may in part be an indirect consequence of reduced PTGS2 activity. TGFBR2 was also down-

regulated in response to miR-204 which is in line with a previously published finding (Wang *et al.* 2010). This is an additional mechanism likely to contribute to the attenuated TGF-β induction of IL-11 by miR-204. Furthermore, miR-204 decreased the expression of CDC42, which has been implicated in non-Smad TGF-β signaling (Edlund *et al.* 2002, Edlund *et al.* 2004), and IL-6, which a transcriptional target of TGF-β (Franchimont *et al.* 2000). MiR-379, in turn, down-regulated the expression of SERPINE1, another transcriptional target of TGF-β (Westerhausen *et al.* 1991).

The genes whose expression was decreased over 1.5-fold in response to miR-204 or -379 were compared against the gene signatures in the Molecular Signatures Database (MSigDB). MiR-204 showed a significant overlap with the MSigDB set of genes containing a 3' UTR binding motif for miR-204 (III: Table S5), and the genes that were down-regulated in response to miR-379 correlated significantly with the MSigDB miR-379 gene set. In addition, there was a highly significant correlation between the genes down-regulated by miR-379 and a gene set up-regulated in the basal subtype of clinical breast cancer samples and a gene set down-regulated in luminal-like breast cancer cell lines as compared to the mesenchymal-like ones (III: Table S6). This underlines the functional relevance of the gene signature induced by miR-379 and also suggests associations of clinical relevance in regard to EMT and breast cancer subtypes.

5.4.4 MiR-379 inhibits Smad signaling

Because miR-204 and -379 decreased the expression of not only IL-11 but also other genes that have been shown to be regulated by TGF-β, such as PTGS2 (miR-204 and -379) and SERPINE1 (miR-379), we investigated whether miR-204, -211 or -379 affected Smad-mediated transcriptional activity. This was done using a luciferase reporter construct containing a functional Smad binding site. Our results showed that miR-379 inhibited the TGF-β-induced binding of the Smad complex to its response element whereas miR-211 slightly increased the signal (III: Figure 5). The inhibitory effect of miR-379 on Smad signaling indicates an additional contributing mechanism for the IL-11 inhibition by miR-379. This finding is also consistent with the gene expression changes induced by miR-379 that show a highly significant correlation to the genes up-regulated in the basal subtype of breast cancer samples and genes down-regulated in luminal-like breast cancer cell lines as compared to the mesenchymal-like ones.

6 CONCLUSIONS

The MDA-MB-231(SA) cell line variant, which shows an enhanced bone metastatic ability but is closely related in terms of genetic alterations and gene and miRNA expression profiles to the parental MDA-MB-231 breast cancer cell line, was used as a model to study the molecular mechanisms of bone metastasis. In reference to the aims of this study, the thesis work led to the following findings:

1. The three enzymes that catalyze L-serine biosynthesis, PHGDH, PSAT1 and PSPH, are up-regulated in MDA-MB-231(SA) cells as compared to the parental cell line. As demonstrated by our functional studies, knockdown of serine biosynthesis inhibits proliferation of MDA-MB-231(SA) cells. In addition, L-serine is essential for the formation of human osteoclasts that actively resorb bone. High expression of PHGDH and PSAT1 is associated with decreased relapse-free and overall survival and several malignant phenotypic features of breast cancer.

2. HS6ST2, an enzyme responsible for adding sulfate groups to glucosamine residues in heparan sulfate glycosaminoglycans, is critical for TGF-β-induced IL-11 production in MDA-MB-231(SA) cells. In addition, exogenous highly sulfated heparan sulfate glycosaminoglycans heparin and K5-NSOS inhibit TGF-β-induced IL-11 production in MDA-MB-231(SA) cells. K5-NSOS and a low-molecular weight heparin, fragmin, decrease osteolytic lesion area and tumor burden in bone in a mouse model of breast cancer bone metastasis.

3. MiR-204, miR-211 and miR-379 inhibit IL-11 production in MDA-MB-231(SA) cells through direct targeting of IL-11 mRNA. MiR-379 also inhibits Smad-mediated transcriptional activity. As indicated by the gene expression profiles of miR-204 and miR-379 transfected MDA-MB-231(SA) cells, these miRNAs regulate several genes implicated in the development of bone metastasis, including PTGS2. In addition, the genes down-regulated by miR-379 are significantly overlapping with a set of genes up-regulated in the basal subtype of breast cancer, underlining the functional and clinical relevance of the gene signature induced by miR-379.

Taken together, this study highlighted three distinct mechanisms as potential novel key regulators of breast cancer bone metastasis (Figure 4). These findings may be therapeutically exploitable for the management of patients with this difficult-to-treat form of metastatic breast cancer.

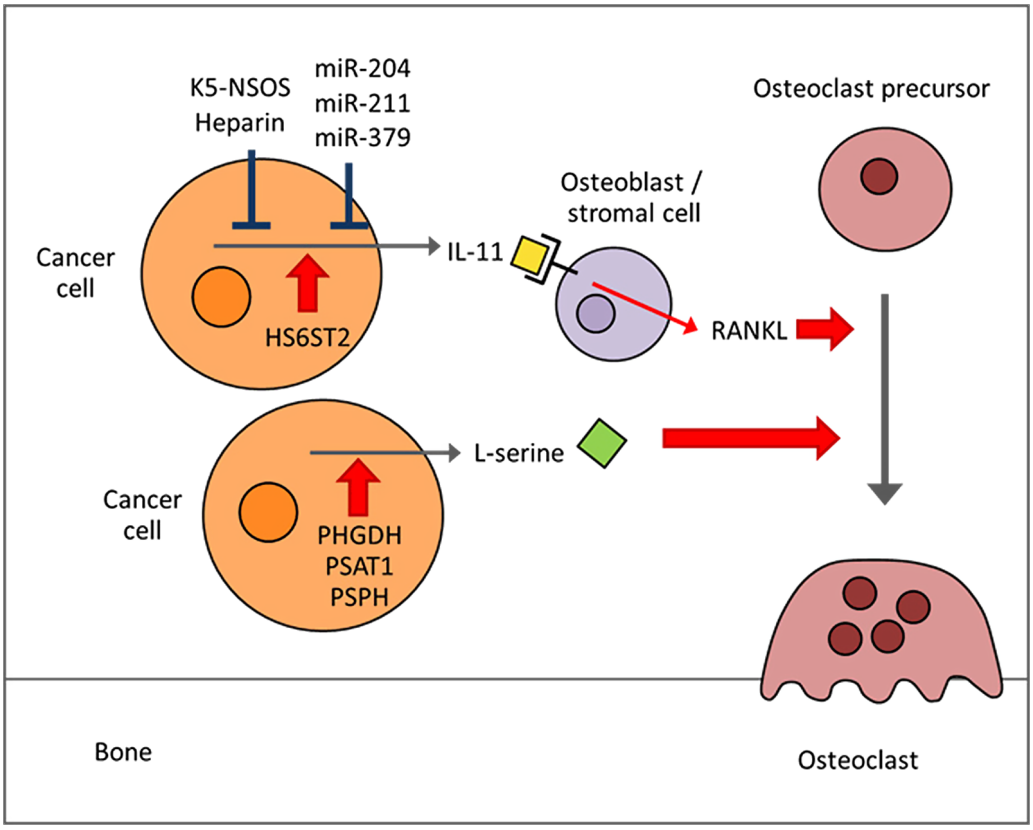

Figure 4. Novel insights into the molecular mechanisms of breast cancer bone metastasis. Breast cancer cells produce L-serine which stimulates osteoclast differentiation and cancer cell proliferation. HS6ST2, an enzyme responsible for adding sulfate groups to glucosamine residues in heparan sulfate glycosaminoglycans, is critical to the TGF-β-induced expression of IL-11 in breast cancer cells. Exogenous highly sulfated glycosaminoglycans K5-NSOS and heparin as well as microRNAs miR-204, -211 and -379 inhibit IL-11 expression in breast cancer cells.

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