DIFFERENTIAL CONTRIBUTIONS OF HOST-DERIVED MATRIX METALLOPROTEINASES IN MAMMARY TUMOR GROWTH IN THE BONE MICROENVIRONMENT

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LIST OF ABBREVIATIONS

ADAM: a distintegrin and metalloprotease

bFGF: basic fibroblast growth factor

BMP: Bone morphogenic protein

BV/TV: Bone volume/tissue volume

ECM: Extracellular matrix

EGF: Epithelial growth factor

GPI: GlycosylPhosphatidylInositol

ICTP: Carboxy-terminal telopeptide of type I collagen

IGF: Insulin growth factor

IGFBP: Insulin growth factor binding protein

IGF-1R: Insulin growth factor receptor type I

IL: Interleukin

LAP: latency binding protein

LTBP: Large TGF-β binding protein

M-CSF: macrophage colony-stimulating factor

MMP: Matrix metalloproteinase

MMPI: Matrix metalloproteinase inhibitor

MMTV-PyMT: Murine mammary tumor virus- polyoma middle T antigen

NAO: nodulosis, arthropathy and osteolysis

OPG: Osteoprotegerin

OPN: Osteopontin

PDGF: Platelet-derived growth factor

PSA: prostate-specific antigen

PTH: Parathyroid hormone

PTHrP: Parathyroid hormone-related peptide

RANKL: Receptor Activator for Nuclear Factor κ B Ligand

sRANKL: Soluble RANKL

TGF-β: Transforming growth factor-β

TIMP: Tissue inhibitor of MMP

TNF-α: Tumor Necrosis factor-α

TRAcP: Tartrate resistant acid phosphatase

VEGF: Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Breast cancer

One in three American women is predicted to develop breast cancer in the course of her lifetime [1, 2]. Patients suffering from breast cancer do not usually die from the growth of the tumor at the primary site but rather from the spreading of the cancer cells to distant organs [3, 4]. A study on more than 500 patients presenting with advanced breast cancer showed that 69 % of these women had evidence of bone metastases [5]. Bone metastasis causes severe complications such as pain, pathological fractures, nerve compression syndromes and hypercalcemia which greatly affect the quality of life of the patients [6]. Furthermore, once breast to bone metastases are actively growing, only palliative treatments can be offered to the patient as no cure is currently available.

Physiology of the bone

The fact that breast cancer displays significant osteotropism was first observed by Stephen Paget in 1889 when he noted that "in cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone" [7]. Why do breast cancer tumor cells have such a significant affinity to the skeleton? What makes the bone such a preferential environment for breast tumor cells? Despite the increasing numbers of studies conducted, our knowledge on the molecular mechanisms underlying breast to bone metastasis remains limited. Since metastatic tumor cells hijack the normal

bone remodeling process to their own benefit, we will first explore the normal physiological bone turn-over process.

Normal bone constituents

The skeleton has two main functions; 1) to support and to protect all vital organs and bone marrow and to be an anchoring point for muscles allowing for locomotion and 2) to serve as a reservoir for calcium, phosphates, hematopoietic progenitor cells and growth factors such as transforming growth factor-β (TGF-β) or insulin growth factors (IGFs) [8]. Two types of bone are found in the normal skeleton: cortical and trabecular bone (Figure 1). Although macroscopically and microscopically different, these two types of bone present the same chemical composition. Cortical bone is dense, compact, mainly calcified and represents 80% of the skeleton; its main function is to support the body weight and to provide protection of all the internal organs [8]. Although representing only 20% of the body mass, trabecular bone is 80% of the bone surface inside the long bones, the vertebrae, the pelvis and the large flat bones [8]. Trabecular bone is less dense, presents a higher turn-over rate than the cortical bone and exhibits mainly a metabolic function. The bone matrix is primarily composed of type I collagen and non-collagenous proteins such as osteocalcin, bone sialoprotein and osteopontin (OPN). Crystals of calcium compounds (hydroxyapatite crystals) are also found on and within the collagen fibers as well as in the matrix and these crystal structures give strength to the bone matrix [9].

Osteoblasts are the cells responsible for the production of the bone constituents and are derived from the mesenchymal stem cells which can also give rise to fibroblasts,

chondrocytes, myoblasts and adipocytes [10] (Figure 1). Osteoblasts are typically found in clusters lining the bone matrix being formed. Bone formation occurs in multiple steps 1) the synthesis of the extracellular matrix (ECM) or osteoid, 2) the maturation of the osteoid and 3) the mineralization of the osteoid. Osteoblasts are also responsible for the production and deposition of growth factors in the osteoid matrix such as TGF-β, IGFs, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and bone morphogenetic proteins (BMPs) [11]. Therefore, bone serves as a reservoir of growth factors, readily accessible via osteolysis when needed. Furthermore, these growth factors can regulate the activity of osteoblasts upon their release from the bone matrix, providing a feedback loop that ensures the proper coupling between bone formation and resorption. During osteoid synthesis a small subset of osteoblasts are entrapped in the newly forming bone and undergo terminal differentiation into osteocytes [12].

Osteocytes form a highly complex canicular network in the bone (Figure 1). The canicules contain osteocytic processes enabling the osteocytes to sense any alteration in the integrity of the bone as well as making contact with the osteoblasts and osteoclasts lining the bone surface[13, 14]. It has been proposed that microfractures and microcracks in the bone are detected by osteocytes which in turn induce the activation of the bone remodeling process [15]. Furthermore, osteocytes can suppress bone formation by secreting factors such as sclerostin [16].

Osteoclasts are the third major cell constituents of the bone and are responsible for osteolysis (Figure 1). They are derived from myeloid stem cells which give rise to monocytes, macrophages and dendritic cells [17]. Mature osteoclasts are giant

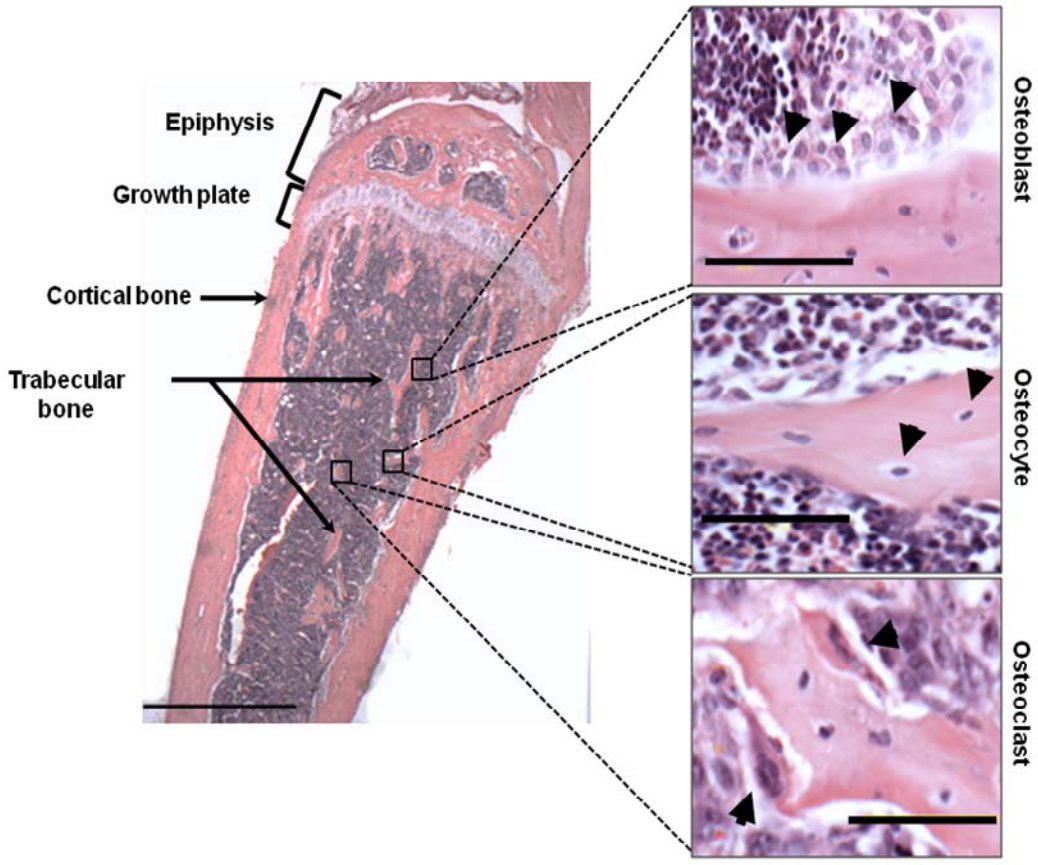


Figure 1. Histology of mouse tibia.

Representative hematoxylin and eosin photomicrograph of a wild type mouse tibia (50X, scale bar is 50µm). Higher magnification images show the three main bone cell types : osteoblasts, osteocytes and osteoclasts. Scale bar is 50µm.

multinucleated cells resulting from the fusion of activated monocytes [18]. Lining the surface of the bone, mature osteoclasts adhere to the bone matrix via integrins such as $\alpha_V\beta_3$ which induce the reorganization of their cytoskeleton to form a sealing zone isolating the extracellular compartment from the bone resorption process [17]. Underneath the sealing zone, osteoclasts induce acidification of the resorption lacunae via an electrogenic proton pump (H^+-ATPase) and a Cl^- channel [19]. The acidification of the milieu allows the mobilization of the mineralized components of the bone matrix, exposing the type I collagen which is degraded by acidophilic collagenases secreted by osteoclasts, the main protease being cathepsin K [17]. Osteoclast functions are regulated by a number of cytokines such as receptor activator of NF-κB ligand (RANKL), interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), macrophage colony-stimulating factor (M-CSF), growth factors (IGF-I, PDGF) and hormones (parathyroid hormone (PTH), insulin) [10].

Normal bone remodeling process

Bone remodeling is a complex process by which old bone is replaced by new tissue. Osteoblast communication with osteoclasts is crucial for this process (Figure 2). There are several mechanisms that facilitate osteoblast-osteoclast communication. 1) Direct contact through membrane-bound ligand-receptor interactions, allows the activation of intracellular pathways in both osteoblasts and osteoclasts. 2) Osteoblasts and osteoclasts can form gap junctions enabling the diffusion of small water-molecules. 3) Paracrine

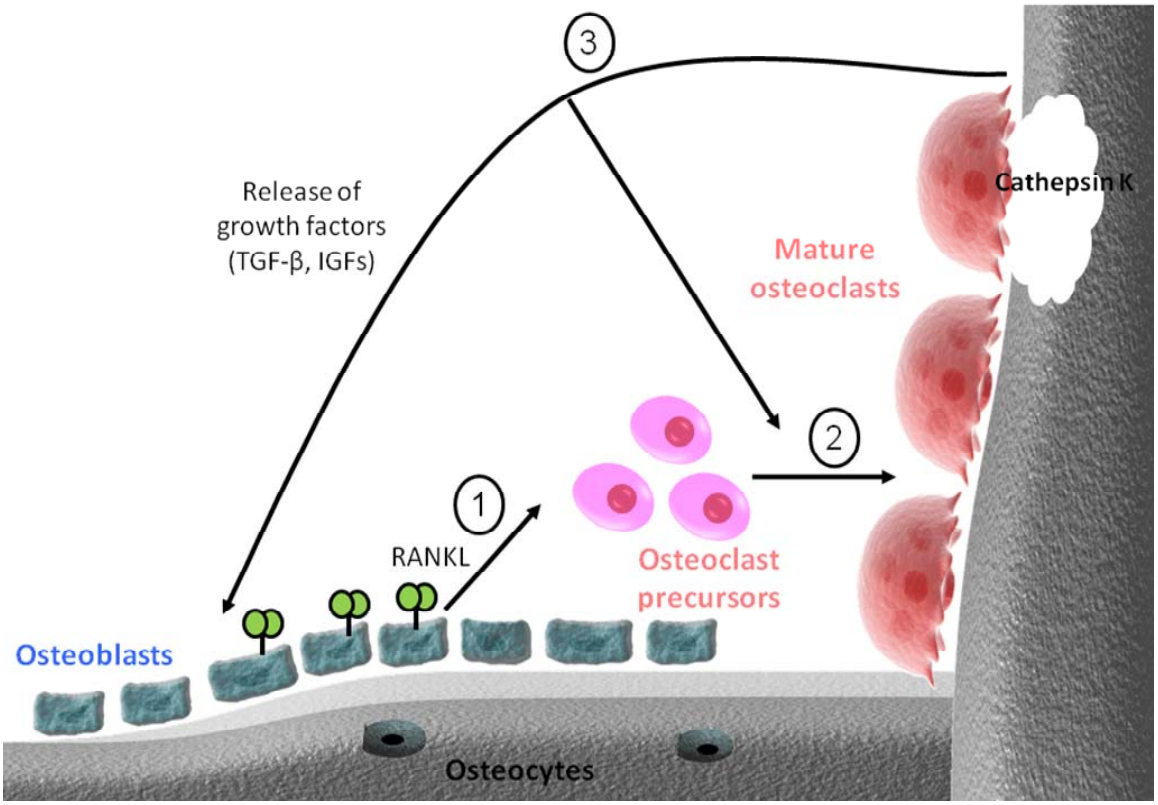


Figure 2. Normal bone remodeling process.

Osteoblasts are responsible for the deposition of the new bone matrix and the secretion of growth factors such as TGF-β and IGFs stored in the newly forming bone. Osteocytes are terminally differentiated osteoblasts, entrapped in the newly formed bone and act as its sentinel. **(1)** Osteoblasts express at their cell surface RANKL, a key factor in the bone remodeling process, which binds to its receptor present at the membrane of osteoclasts precursor cells. **(2)** RANKL interaction with its receptor induces the activation/maturation of osteoclast precursors into multinucleated mature osteoclasts which are the cells responsible for bone resorption. **(3)** Osteoclast-mediated osteolysis induces the release of growth factors like TGF-β and IGFs stored in the bone which are able to signal back to the osteoblasts and osteoclasts and therefore act as a feedback loop to control the extent of the normal bone resorption taking place.

factors such as growth factors, cytokines and chemokines can be secreted by one cell type and act on the other through diffusion. For example, Allan and colleagues demonstrated that PTH and PTH-related peptide (PTHrP) can regulate the level of expression of ephrinB2 in osteoblasts, and that the blockage of the interaction of ephrinB2 with its receptor EphB4 expressed by osteoclasts inhibits osteoblast function [20, 21]. Furthermore, ephrinB2/EphB4 binding inhibits osteoclast differentiation [22]. Local factors such as IL-11, prostaglandin E2, onconstatin M, PTHrP and microdamage sensed by osteocytes induce the expression of RANKL by osteoblasts. RANKL is a member of the TNF family and a key factor in bone remodeling. It has been well established that the interaction of RANKL with its receptor RANK expressed by myeloid osteoclast precursors is essential for osteoclastogenesis [23]. Osteoblasts also expressed osteoprotegerin (OPG), which acts as a decoy receptor for RANKL, thus regulating bone resorption by competing for RANK-binding at the surface of osteoclasts [24]. In addition to RANKL, oteoblasts secrete macrophage colony-stimulating factor (M-CSF) which is required for monocytic/macrophage precursor cell survival [25]. RANKL/RANK interaction induces the fusion of osteoclast precursor cells leading to the formation of multinucleated mature osteoclasts [26]. It has been well established that the cysteine proteinase cathepsin K is the principal enzyme responsible for the degradation of the demineralized bone matrix [27]. Mice deficient for cathepsin K showed an accumulation of demineralized collagen fibers in the subosteoclastic resorption lacunae demonstrating the predominant role of this enzyme in bone degradation [28]. Cathepsin K cleaves type I collagen releasing the triple helix which becomes more susceptible to proteolytic degradation by other collagenases [29]. In addition to cathepsin K, studies conducted by

Everts and colleagues demonstrated that treatment of calvarias with matrix metalloproteinase (MMP) inhibitors (MMPIs) leads to a significant amount of demineralized bone matrix accumulating in the resorption lacunae, demonstrating the importance of MMPs in regulating bone resorption [30-32]. However, whether these effects are due to direct or indirect activity of MMPs remains unknown.

Matrix Metalloproteinases

Classification and structure

The metzincin superfamily of proteolytic enzymes is characterized by a conserved zinc binding motif HEXXHXXGXXHZ where the histidine (H), glutamic acid (E) and glycine (G) are invariant and the 3 histidines are responsible for the binding of the catalytic Zn^{2+} ion [33]. Of this superfamily of proteases, MMPs form a subfamily of 23 human endopeptidases where the Z residue is a serine in the zinc motif in all but few MMP family members. In addition to the zinc motif, MMPs share some sequence homologies, conferring to this family a conserved overall structure. Thus, they are often classified according to their domain structure into several groups including the collagenases, the gelatinases, the stromelysins and the membrane-type MMPs (MT-MMPs) (Figure 3) [34].

The minimal modular domain structure common for all the MMPs consists of a pre domain (for secretion), a pro domain (for latency maintenance) and a catalytic

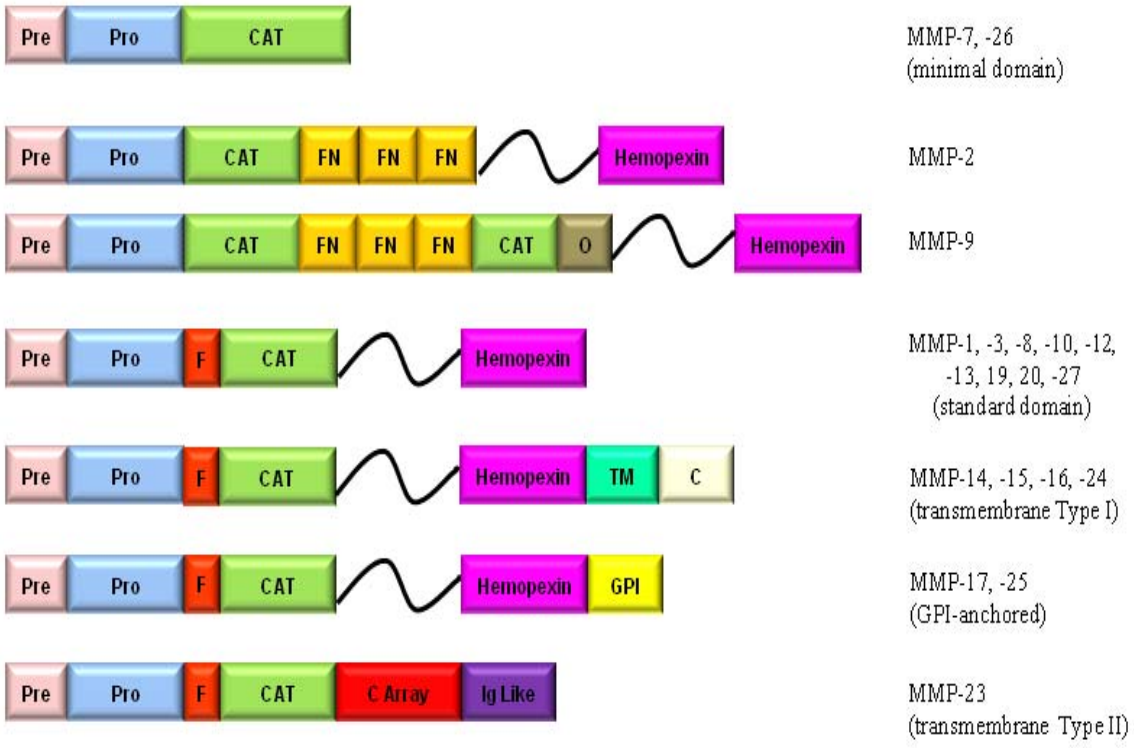


Figure 3. Domain structures of the MMP family.

Pre: pre doman; **Pro**: pro domain; **CAT**: catalytic domain; **hemopexin**: hemopexin domain; **FN**: fibronectin domain; ~ : hinge region; **O**: O-glycosylated domain; **F**: furin cleavage site; **TM**: transmebrane domaine Type I; **GPI**: GPI anchor; **C Array**: cysteine array'; **Ig like**: Ig-like domain.

domain (for proteolytic activity) [35]. All the MMPs are produced as pro-enzymes or zymogens where their latency is ensured by the bond of the cysteine in the pro domain with the $Zinc^{2+}$ ion of the catalytic region. Activation of pro/latent MMPs has been described as a 'cysteine switch'. Cleavage of the pro domain alters this cysteine/$Zinc^{2+}$ interaction and the zinc ion becomes susceptible to hydrophilic attack, allowing for the subsequent interaction of a substrate with the catalytic domain [36]. Most of the MMPs, except MMP-7, MMP-23 and MMP-26, have a hemopexin/vitronectin domain linked to the catalytic domain by a hinge region. This hemopexin domain influences the binding of tissue inhibitors of metalloproteinases (TIMPs), substrate binding, membrane localization and proteolytic activity [37]. MMP-2 and MMP-9 also contain a fibronectin type II repeat within the catalytic domain required for the binding and cleavage of collagen and elastin [38, 39]. Finally, MT-MMPs can be divided in 2 categories: the type I transmembrane MMPs (MMP-14, -15, -16 and -24) presenting a short cytoplasmic C-terminal tail, and the glycophosphatidyl inositol (GPI) MT-MMPs (MMP-17 and -25) possessing a C-terminal hydrophobic tail [34]. These different domains play a crucial role for the localization and the regulation of the activity of the different MMP family members.

Regulation of MMPs

The specific biological function of individual MMPs is mainly dictated by their temporal, spatial and inducible pattern of expression. MMP expression and activation are highly regulated at the transcriptional and post-translational levels [40]. MMP gene expression is

controlled by various growth factors and cytokines (TGF-β, epithelial growth factor/EGF, TNF-α), integrin-derived signals, ECM proteins, phorbol esters, cell stress and changes in cell shape [41]. The majority of these stimuli induce the expression or activation of proto-oncogenes such as c-fos and c-jun, as well as β-catenin and ets transcription factors that mediate the transcription of specific MMPs [37].

MMPs are secreted as pro-enzymes or zymogens and their activation is tightly regulated. MMP-11, MMP-23 and MT-MMPs contain a furin-like enzyme recognition domain between the pro and catalytic domains and can be activated by subtilisin-type serine proteinases such as furin in the intracellular compartment [42]. All other MMPs are activated outside of the cell by either already activated MMPs or by other proteinases [43]. MMP-2 presents an additional unique serine-independent mode of activation involving MT1-MMP and TIMP-2 [40]. TIMP-2 is anchored at the cell surface through the binding of its N-terminal domain to MT1-MMP and acts as a receptor for MMP-2 via the binding of its C-terminal domain to MMP-2 hemopexin domain. Subsequently, a neighboring active MT1-MMP can cleave and activate the anchored proMMP-2 when low levels of TIMP-2 are present [44].

Once activated, MMP activity can be closely regulated by endogenous inhibitors such as α2-macroglobulin and TIMPs [45]. α2-macroglobulin is an abundant plasma protein and acts mainly as a major protease and MMP inhibitor in plasma [46]. TIMPs represent a family of 4 secreted proteins expressed mainly in various tissues and fluids. They reversibly inhibit MMP activity in a 1:1 stoichiometric ratio through the interactions of their N-terminal domain with the MMP catalytic site [47]. However, besides their ability to inhibit MMP activity, studies have demonstrated MMP-independent

functions of TIMPs. For example, TIMP-1, -2 and -3 have been shown to promote cell growth [48]. Furthermore, TIMP-1 and TIMP-2 have been demonstrated to inhibit tumor angiogenesis in an MMP-independent fashion [49, 50].*MMP substrates in the bone*

The classical substrates for MMPs are the various proteins, proteoglycans and glycoproteins constituting the ECM (Table 1). In addition, through the processing of signaling molecules such as growth factors, cell surface proteins and cytokines, MMPs contribute to numerous cell functions (Table 1). Thus, MMPs because of their wide array of substrates are involved in a number of biological processes such as mammary gland involution, angiogenesis and modulation of immune reactions [33].

Since bone is composed of over 90% type I collagen, enzymes with collagenase activity are predicted to be important proteases in bone remodeling. Delaisse and colleagues demonstrated that levels of collagenases positively correlate with elevated rates of bone resorption in calvarias [51]. Several lines of evidence have suggested that osteoblast-derived collagenases may play a role in bone resorption initiation as bone resorbing agents induce collagenase expression by osteoblasts[52, 53]. Furthermore, *in vivo* osteoblasts adjacent to activated osteoclasts present high levels of collagenase [54, 55]. It has been proposed that collagenases degrade the non-mineralized organic matrix allowing osteoclast precursors to adhere to the mineralized bone and become activated [56]. Furthermore, collagenases may release molecules or collagen fragments that can stimulate osteoclast activation [57, 58]. In transgenic mice expressing a type I collagen resistant to MMP-dependent collagenase degradation, calvaria lack a bone marrow cavity and are significantly thicker than in the wild type control mice [59]. These studies suggest the importance of collagenases in bone resorption, and that cathepsin K and MMPs may

MMP	ECM substrates	Non-matrix substrates
MMP-1	Collagen I, II, III, VII, X, XI; gelatin, entactin; aggrecan; fibronectin; laminin; tenascin; vitronectin	Perlecan; IGFBP-2, IGFBP-3; pro-TNF-α; α1-AC; α2-MG; α1-PI
MMP-2	Collagen I, III, IV, V, VII, X, XI; gelatin; elastin; fibronectin; laminin; aggrecan; tenascin; decorin; vitronectin	LAP-TGF-β, LTBP-1, TGF-β2; IL-1β; MCP-3; SDF-1; IGFBP-3; IGFBP-5; TNF-α; FGFR-1; α1-PI
MMP-3	Collagen III, IV, V, VII,IX, X, XI; gelatin; elastin; fibronectin; laminin; aggrecan; tenascin; decorin; vitronectin; entactin	Perlecan; IGFBP-3; TNF-α; α1-AC; α2-Mg; α1-PI; HB-EGF; IL-1β; plasminogen; E-cadherin;
MMP-7	Collagen I, IV; gelatin; elastin; fibronectin; laminin; aggrecan; tenascin; decorin; vitronectin; entactin	TNF-α; FasL; α1-PI; HB-EGF; plasminogen; E-cadherin; β_4 integrin
MMP-8	Collagen I, II, III; aggrecan	A2-MG; α1-PI
MMP-9	Collagen IV, X, XIV; gelatin; elastin; laminin; aggrecan; decorin; vitronectin	LAP-TGF-β, TGF-β2; IL-1β;; SDF-1; TNF-α; FGFR-1; α1-PI; IL-2Ra; α2-MG
MMP-10	Collagen III, IV, V; gelatin; elastin; aggrecan; fibronectin	Not known
MMp-11	Not known	IGFBP-1; α1-PI; α2-MG
MMP-12	Collagen I, IV; gelatin; elastin; laminin; entactin	Plasminogen; α1-PI; α2-MG
MMP-13	Collagen I, II, III, IV, IX, X, XIV; gelatin; fibronectin; aggrecan	α2-MG
MMP-14	Collagen I, II, III; gelatin; laminin; aggrecan; fibronectin; vitronectin; entactin	CD44; transglutaminase; α1-PI; α2-MG
MMP-16	Collagen III; fibronectin; gelatin	transglutaminase
MMP-17	Gelatin	TNF-α; α2-MG
MMP-18 (*Xenopus*)	Collagen I	Not known
MMP-19	Collagen I, IV; gelatin; laminin; aggrecan; fibronectin; tenascin; entactin; COMP	
MMP-20	Collagen XVIII; aggrecan; amelogenin; COMP	Not known
MMP-21 (*Xenopus*)	Not known	Not known
MMP-22 (chicken)	Gelatin	Not known
MMP-23	Not known	Not known
MMP-24	Collagen I; geltin; laminin; fibronectin	Not known
MMP-25	Collagen IV; gelatin; fibonectin	Not known
MMP-26	Collagen IV; gelatin; fibronectin	α1-PI
MMP-27	Not known	Not known
MMP-28	Not known	Not known

Table 1. Matrix and non-matrix substrates of MMPs. (Lynch and Matrisian, 2002)

have different roles in intramembranous and endochondral bone. Cathepsin K cleavage of the native triple helix of collagen releases C-terminal (CTX) and N-terminal (NTX) cross-linked telopeptides which have been shown to be good serum markers for osteoporosis [60]. MMPs such as MMP-1, -2, -8, -9, -13 and MT1-MMP have collagenase activity *in vitro* [61]. But in contrast to cathepsin K, MMPs generate a larger cross-linked C-terminal telopeptide of type I collagen (ICTP). The ICTP fragment has been shown to significantly correlate with extent of bone lesions in patients presenting bone metastasis and to act as chemotactic molecules for osteoclast precursor cells [62-64]. Cleavage of type I collagen also leads to the exposure of cell adhesion sites such as binding sites for integrins, especially $\alpha_V\beta_3$ integrin, influencing the attachment/detachment of osteoclasts and ultimately regulating their migration during bone resorption [65]. MMP-3 and -7 have also been shown to process non-collagenous components of the bone matrix such as OPN, resulting in the exposure of integrin binding sites which could regulate cell adhesion to the bone matrix [66].

In addition to processing bone matrix components, MMPs have also been shown to process numerous non-matrix substrates, several of which are pertinent to the bone (Table 2). Therefore, MMPs can regulate cell behavior by altering the activity status of numerous growth factors and cytokines. For that reason, roles for MMPs, independent of their ability to degrade the ECM, are a distinct possibility in the bone microenvironment.

Roles of MMPs in normal bone physiology

To date, roles for only a small number of MMPs have been described in bone based on MMP deficient animals.

Itoh and colleagues first described MMP-2 deficient mice and reported that these mice have a significantly slower growth rate compared to wild type mice [67]. More detailed studies have shown that MMP-2 deficient mice also have abnormal cranio-facial defects mirroring those of the nodulis, arthropathy and osteolysis (NAO) syndrome in humans having a mutation in *mmp2* gene [68, 69]. MMP-2 deficient mice also demonstrate severe osteoporosis and a significant thickening of the calvaria by 55 weeks of age compared to wild type control mice [68]. Inoue and colleagues also reported the expression of MMP-2 by osteoblasts and osteocytes. Calvaria of MMP-2 deficient mice showed a defect in osteocytic canicular network suggesting a contribution of MMP-2 in the maintenance of the canicular network which is important for the development of the skeleton [68]. In addition, studies have shown that MMP-2 mediates osteoblast proliferation and differentiation [69].

MMP-7 deficient mice have no obvious skeletal defects [70]. However, the phenotype of MMP-7 deficient animals becomes apparent during physiological stresses such as disease or injury for example, MMP-7 has been reported to be important for joint destruction in the development of septic arthritis [71]. Haro and coworkers also demonstrated that macrophage-derived MMP-7 was required for the release of TNF-α by macrophages in a model of herniated disc resorption [72].

In the bone environment, MMP-9 has been reported to be largely expressed by multinucleated osteoclasts [73]. However, MMP-9 deficient mice do not present any obvious skeletal defect. Despite no overt phenotype, Vu and colleagues reported a transient phenotype in the hypertrophic zones of long bones [74]. This is primarily due to a delay in vascularization and apoptosis of chondrocytes inducing a lengthening of the growth plate caused by a defect of osteoclast and endothelial cell invasion into the mineralized cartilage [74, 75]. In absence of MMP-9, vascular endothelial growth factor (VEGF) was found to be sequestered in the ECM, which could explain the delay in angiogenesis and apoptosis of chondrocytes [76].

MMP-13 is the murine ortholog of human MMP-1 and is expressed by osteoblasts, osteocytes and mononuclear pre-osteoclast cells [77, 78]. Embryos deficient for MMP-13 present an elongated growth plate that is still evident at 3 months of age [79]. Adult MMP-13 deficient mice show a thickening of the trabecular bone, potentially caused by an abnormal osteoblast proliferation and function rather than a defect in osteoclast activity [61].

Lastly, MT1-MMP is expressed in a number of bone cells but is particularly abundant in osteoclasts [80]. MT1-MMP deficient mice present a severe skeletal phenotype with delayed ossification of the calvaria and incomplete suture closure. By 2 to 3 months after birth, most of the mice die [81-83]. Furthermore, homozygous mutant animals develop a generalized arthropathy with osteoclast-like giant cells in the articular tissues and a loss of osteocyte processes due to a defect in type I collagen degradation [81, 82]. Because of the role of MT1-MMP in MMP-2 activation, some of the skeletal defects observed in MT1-MMP deficient mice could be caused by an absence of active MMP-2. However,

Holmbeck and colleagues reported that impaired osteocyte processes observed in the long bones are associated with the loss of MT1-MMP [82]. MT1-MMP is importance for the maintenance of the osteocytic canicular network in tibias and femurs, but its contribution in osteocyte integrity in the calvaria remains to be determined [82]. Finally, degradation of the embryonic cartilage during skeletal development seems to be due to MT1-MMP collagenolytic activity [84].

The 'vicious cycle' of bone metastasis

Metastasis is a complex process where the tumor cells need to complete a cascade of events before reaching the bone. Breast cancer cells have to break away from for primary tumor (1), invade the blood stream (2), survive in the blood circulation (3), adhere to vascular endothelium of a distant organ (4), extravasate from the blood vessel into the metastatic site (5) and finally proliferate in the secondary site (6) [85]. MMPs have been shown to play multiple roles in the progression of cancer [86]. Given the role of host MMPs in the bone development and physiology, it is clear that they may be relevant to tumor metastases to the bone and tumor-bone microenvironment

After extravasation from the vasculature in the bone marrow cavity, metastatic tumor cells encounter a microenvironment that is metabolically active and rich in growth factors, cytokines and chemokines known to promote tumor growth. Since bone is a hard tissue, breast tumor cells must hijack the normal bone resorption process in order to establish and grow (Figure 4). Studies have demonstrated that metastatic breast tumor cells secrete various factors such as IL -1, -6, -8 and -11 and PTHrP that stimulate

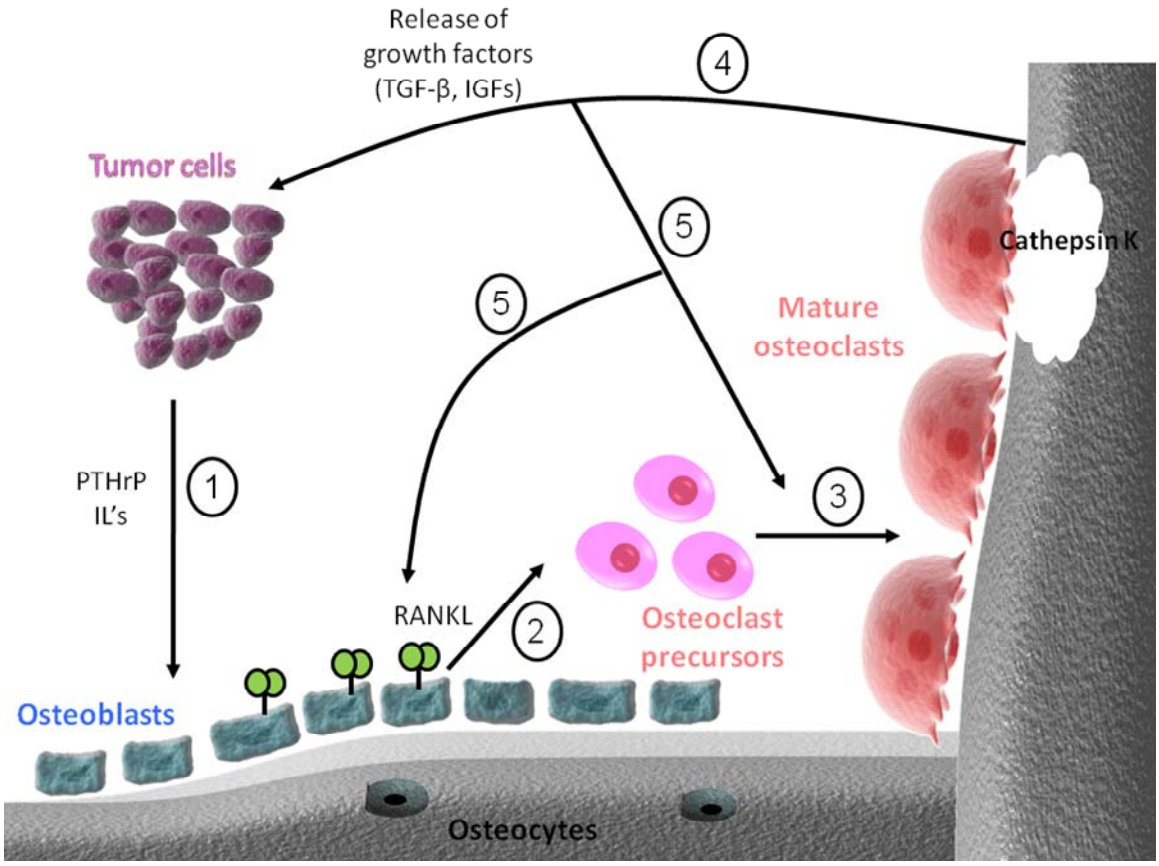


Figure 4. The 'vicious' cycle of bone metastasis. (1) Metastatic tumor cells present in the bone, secrete various factors such as PTHrP and different interleukins which stimulate **(2)** the osteoblasts to promote **(3)** the maturation and activation of osteoclast precursors. This high number of active osteoclasts leads **(4)** to an extensive bone resorption, releasing increasing levels of growth factors such as TGF-β and IGFs. **(5)** Besides promoting tumor growth in the bone, these growth factors have been shown **(5)** to modulate osteoblast and osteoclast functions that promote the continuation of the vicious cycle.

osteoblast expression of osteoclastogenic factors M-CSF and RANKL [87]. It has been established for years that circulating PTH acts as a stimulus for bone remodeling, however PTHrP is a paracrine factor expressed mainly by osteoblast precursor cells and cells present in the bone marrow [88]. Both PTH and PTHrP have been shown to induce osteoblast differentiation and to inhibit osteoblast apoptosis [89]. Furthermore, mice heterozygous for PTHrP present a low level of bone formation and specific deletion of PTHrP expression in osteoblasts induced defective bone matrix synthesis [90, 91]. In addition to its effect on osteoblast differentiation and bone formation, PTHrP indirectly influences osteolysis by inducing osteoblast expression of RANKL, an important regulator of osteoclastogenesis [92]. PTHrP is expressed as a pre form which requires proteolytic cleavage to release the mature active peptide [93]. Furin and prostate serum antigen (PSA), two serine proteases, have been shown to cleave PTHrP inducing osteoblast differentiation [92, 94]. Immunohistochemical studies have demonstrated an increase of PTHrP expression in samples of breast cancer metastases to bone compared to soft tissues or primary breast tumor samples [95, 96]. Intracardiac injection of the human breast tumor cell line MDA-MB-231 overexpressing PTHrP increased the number of metastases in the bone [97]. Neutralizing antibodies to PTHrP injected in tumor bearing mice resulted in a decreased of both osteolytic lesions and tumor growth [97]. Taken together these data demonstrated that PTHrP expression is an important feature utilized by breast tumor cells to induce the vicious cycle of bone metastasis.

Bone is one of the richest reservoirs of growth factors such as TGF-β, IGFs and BMPs in the human body. During bone synthesis, osteoblasts secrete these factors which become embedded in the new bone. During the resorption of the bone, osteoclasts

release these growth factors that in turn provide signals to the osteoblasts to locally control the bone remodeling process. IGFs are abundant growth factors sequestered in the bone matrix [98]. Supernatants of cultures of resorbing neonatal calvarias increased breast tumor proliferation [99]. A neutralizing antibody against IGF-I receptor significantly attenuated the growth effect of these calvarial culture supernatants suggesting that IGF released from the bone promotes tumor cell growth.

In the bone, TGF-β possesses a unique role as it influences both bone resorption and bone formation. TGF-β can influence chemotaxis, proliferation and maturation of osteoblast progenitor cells, and its effects appear to be both stimulatory and inhibitory depending on the differentiation state of the osteoblasts [100-104]. Osteoclast maturation and function are also influenced by TGF-β which has a dual stimulatory/inhibitory role depending on its levels [105-108]. These dual effects of TGF-β are essential for the fine regulation of bone remodeling, TGF-β levels act as a 'sensor' of the extent of bone resorption and formation either stimulating or inhibiting osteoclasts and osteoblasts to ensure locally controlled bone remodeling. TGF-β has been shown to increase the expression and stabilize the mRNA of PTHrP in MDA-MB-231, contributing to the establishment and progression of breast tumor in the bone [97]. TGF-β is expressed by both differentiated osteoblasts and osteoclasts and released upon bone resorption; many studies have demonstrated its effect on normal and tumor cell proliferation, expression of matrix proteins and enzymes that control matrix turn-over such as MMPs [109-111]. Upon intracardiac injection, MDA-MB-231 cells expressing a dominant negative TGF-β receptor induced less osteoclast recruitment and thus induced less osteolysis. Tumor

growth was also reduced which demonstrated the importance of TGF-β in promoting breast tumor progression in the bone [112].

Potential contributions of MMPs to the 'vicious cycle'

As reported in table 1, several non-matrix substrates including factors controlling the vicious cycle, are MMP substrates. Through the release of active growth factors, MMPs could contribute in different steps of the vicious cycle 1) the growth of the tumor 2) osteolysis either by directly acting on osteoclast maturation and function or 3) indirectly by promoting osteoblast differentiation and expression of RANKL or 4) by inducing PTHrP expression by the tumor which can lead to osteoblast differentiation.

PTHrP expression by metastatic tumor cells stimulate RANKL expression by osteoblasts and its subsequent interaction with RANK at the surface of osteoclast precursor cells result in osteoclast maturation and activation [33]. Although, no MMP has yet been shown to process PTHrP, a membrane bound metalloproteinase neprilysin (NEP) is able to modulate the activity of PTHrP [113-115].

Lynch and colleagues reported that RANKL was processed by MMP-3 and MMP-7 [116]. In an animal model of prostate tumor growth in the bone, they demonstrated that MMP-7 expression correlated with the level of tumor-induced osteolysis. Furthermore, they reported that MMP-7 was primarily expressed by mature osteoclasts present at the tumor-bone interface. RANKL-processing mediated by MMP-7 resulted in a soluble form of RANKL which is as efficient and active as full length RANKL with respect to inducing osteoclast activation. This finding was significant as it demonstrated

a novel way of activating osteoclast without the necessity of direct osteoblast-osteoclast contact [116]. This study demonstrated for the first time a direct contribution of a specific host MMP to the progression of the vicious cycle. Besides MMP-3 and MMP-7, MMP-1 and MMP-14 but also members of a disintegrin and metalloproteinase (ADAM) family such as ADAM-17 and ADAM-19 have been shown to generate soluble RANKL [117-119].

TGF-β is maintained in a latent state through its the interaction with a latency associated peptide (LAP) and latent TGF-β binding proteins (LTBP-1-4) [120]. By processing the latent binding proteins that keep TGF-β in an inactive state, MMPs can enhance osteoclast function leading to a more extensive bone resorption [121-124]. This increased osteolysis can promote the release of higher levels of growth factors such as TGF-β itself and also IGFs which in turn could signal back to the osteoclast/osteoblast to induce more bone resorption and tumor growth.

IGFs are expressed as a latent molecule through complexing with IGF binding proteins (IGFBP-1 to -4) [125]. The release of IGFs from IGFBPs has been shown to be mitogenic for various tumor cells such as breast, prostate and colon [126-128]. Furthermore, Wang and colleagues demonstrated that IGF-I is important for normal osteoclast/osteoblast interactions and can regulate osteoclastogenesis by promoting their differentiation [129]. Several MMPs have been shown to induce the release of IGFs via the processing of IGFBPs [130-133]. Therefore by contributing to the activation of IGFs, MMPs can affect osteoclast/osteoblast interactions and influence bone resorption.

A number of other factors that can also be processed by MMPs exist in the tumor-bone microenvironment (Table 2) and therefore, roles for the soluble factors generated by

other MMPs in vicious cycle are possible. Given the potential contributions of MMPs to tumor progression in the tumor-bone microenvironment, through different matrix and non-matrix substrates, MMPs should be an attractive therapeutic target for the treatment of bone metastases.

MMP inhibitors, still a viable treatment for tumor-induced bone lesions?

By the virtue of their numerous substrates, MMPs have been implicated in every step of tumor progression from initiation to metastasis and as a consequence were thought to be ideal targets for drug therapies in the treatment of cancer [134]. However, human clinical trials using MMPIs largely failed to show efficacy due to the dose limiting side effects, mainly arthralgia, the broad spectrum of inhibition of the drugs used and the failure to identify appropriate end-point measures of the efficacy of the treatment [135]. Prolonged treatment with MMPIs, induced the development of musculoskeletal pain and inflammation and the question as of the identity of the MMPs responsible for these side effects arose [135]. Studies have demonstrated that broad spectrum inhibitors reducing 'sheddase' activity of proteases do not seem to induce musculoskeletal side effects [136]. Despite these side effects, MMPIs have been shown to be clinically efficient in the tumor-bone microenvironment. Pre-clinical studies using broad spectrum MMPIs such as BB94 demonstrated that treatment of animal bearing breast or prostate tumor with MMPIs can reduce and prevent tumor growth and the development of bone lesions [137-139].

Substrate		Substrate Function	MMP/References	Product Function	Role in bone microenvironment
Receptor	**FGFR 1**	Receptor for FGF	MMP-2[134, 135]	Soluble FGFR1 that inhibits FGF signaling by binding soluble FGF	Prevention of FGF effect on osteoblast differentiation and bone formation (BF)
	uPAR	Regulates uPA activity at the cell surface and signaling.	MMP-2, -3, -8, 9, 12, 13, 14 [136, 137]	Inactivation and deacrease in cell motility	Inhibition of prostate cancer metastases to the bone by up to 80%
	LRP	Endocytosis/Wnt signaling	MMP-14, -15, -16, -17[138]	Inactivation of LRP attenuates endocytosis and Wnt signaling	Inhibition of osteoblast differentiation and BF
	IL-2Rα	Interluekin receptor	MMP-2, -9[139]	Inactivation and prevention of T-cell proliferation	Generation of immune privileged site at the tumor bone interface
	FAS	Fas ligand receptor	MMP-7[140]	Inacivation of the receptor	Prevention of FasL-mediated osteoblast differentiation BF
	PAR-1	G-protein coupled receptor activated by proteases	MMP-1[141]	Activation of the receptor and enhanced cell migration and invasion	Activation of PAR-1 on tumor cells in the bone promotes migration.
Ctyokine/Growth Factor	**HB-EGF**	Pleiotropic effects on growth differentiation and apoptosis via ErbB-4 and ErbB-1	MMP-3, -7[142-144]	Active HB-EGF	Myeloma progression
	IGF-BPs	Sequester IGF in a latent form	MMP-1, -2, -3, -6,-11[130-133, 145]	Active IGF	Proliferation via activation of the IGF receptors
	FasL	Induces apoptosis by activating Fas	MMP-3, -7[146-149]	Active soluble form of FasL	Selection of apoptosis resistant tumor cells. Apoptosis of invading immune cells
	TNF-α	Pleiotropic effects on immune cells and tumor cells	MMP-1, -2, -3, -7, -9, 12, -14, -15, -17[150-152]	Soluble active form of TNF-α	Proximal and distal effects on cells. Activation of osteoclasts
	IL-1β	Immunce cell activity and infiltration to sites of infection and tumor growth	MMP-1, -2, -3, -9[153-156]	Activation of the pro-form Inactivation of the mature cytokine	Control of osteoclast maturation. Prevention of immune cell infiltration
	Kit Ligand	Maintaining the stem cell niche in the bone	MMP-9[157]	Active soluble Kit ligand that can mobilize HPC's	Angiogenesis of the primary tumor and potential homing to the bone stem cell niche
Cell adhesion molecules	**Cadherins**	Cell :cell contact in epithelial, vascular and neuronal cells	MMP-3, -7, -14[158-161]	Degradation, leading to disassembly of the adherens junctions	Greater tumor cell migration. Enhanced angiogenesis
	Integrins	Cell contact with the basement membrane	MMP-14[162, 163]	Maturation of the integrin and increased cell adhesion migration	Migration of osteoclasts
	CD-44	Cell anchorage to the basement membrane	MMP-14, 15, -16, 18, -24[164, 165]	Degradation of CD-44 results in enhanced cell motility	Increased tumor cells migration
	ICAM-1	Leukocyte infiltration and adhesion	MMP-9[166]	Inactivation of ICAM-1 leading to improper immune cell function	Generation of immune privileged site at the tumor bone interface
Cell surface glyco proteins.	**EMMPRIN**	Regulation of MMP activity and promtes cell migration?	MMP-1, -2, 14, 15[167-169]	Soluble EMMPRIN can enhance MMP expression	Potential induction of MMP expressio
	Mucin-1	Protection of epithelial cell surfaces	MMP-14[170, 171]	Solubilization of Mucin-1 influences tumor progression and invasion	Expression of MUC-1 by tumor cells in the bone and processing by MMPs may allow for immunoevasion
	Sydecans	Cell adhesion to multiple ECM components	MMP-7, -14, -16[172-174]	Soluble syndecans can enhances tumor cell migration	Expressed on tumor cells in the bone milieu and processing by MMPs may promote migration

Table 2. Non-matrix substrates of MMP in the bone.

As a result, bone metastasis appears to be a relevant target for the use of MMPIs, however we need to identify the specific role of individual MMPs in the osteolytic tumor-bone microenvironment [140]. The rationale for using more selective MMPIs in the tumor-bone microenvironment has been shown by Bonfil and colleagues. The authors demonstrated in a model of prostate cancer-induced osteolysis that using an inhibitor with high selectivity for MMP-2 and -9 reduced tumor growth, angiogenesis and the development of bone lesions [141]. Although, the use of prinomastat (an MMP-2 and MMP-9 selective inhibitor) has been shown to still induce joint pain, the execution of more studies focusing on identifying the contribution of individual MMPs in tumor-induced osteolysis should lead to the development of therapies for patients presenting with incurable bone metastases.

Aims of this dissertation

The aims of this dissertation are to increase our understanding on how individual host MMPs contribute to mammary tumor growth and osteolysis.

In a rat model of prostate tumor growth in the bone, Lynch and coworkers assessed the expression of 1200 genes at the tumor-bone interface and tumor area alone. Several genes showed a significant increased in their expression at the tumor-bone interface and interestingly, several MMPs such as MMP-2, -3, -7, -9 and -13 were particularly upregulated in comparison to the tumor area alone [116]. Furthermore, the authors demonstrated that MMP-7 significantly impacted prostate tumor growth-induced osteolysis via the solublization of RANKL which in turn could activate osteoclast

precursor cells [116]. Through the Orthopaedics and Rehabilitation Department at Vanderbilt, we obtained 11 human samples of breast-to-bone metastasis and expression of various MMPs were assessed by immunofluorescent staining (Table 3). MMP-7 and MMP-9 presented a distinct localization pattern, mainly in mature osteoclasts at the tumor-bone interface. Although, other stromal cells appeared to express MMP-7 and -9, breast tumor cells were largely negative for these two MMPs. Human samples of breast-to-bone metastases were also stained for MMP-2, -3 and -13 however, their pattern of expression appeared more diffuse throughout the tumor/stroma compartment.

Since osteoclasts are the main cells responsible for bone resorption, we decided to investigate further the potential contributions of host-derived MMP-7 and MMP-9 in mammary tumor-induced osteolysis. In addition, based on recent studies reporting the importance of host MMP-2 in osteoblast behavior and given the role of osteoblasts in osteoclast activation and function, we also examined the effect of host MMP-2 in mammary tumor growth and mammary tumor-induced osteolysis. Based on these data, this dissertation focused on identifying the contributions of host MMP-2, -7 and -9 in mammary tumor-induced bone lesions. The approach utilized was intratibial injection of mammary tumor cells in syngeneic and immunocompromized MMP deficient mice to investigate the contribution of individual host MMPs in mammary tumor growth and mammary tumor induced osteolysis in the bone.

Sample	MMP-2		MMP-7		MMP-9		MMP-13	
	Tumor	Stroma	Tumor	Stroma	Tumor	Stroma	Tumor	Stroma
2-3-06	+	+	-	+	-	+	+	+
2-08-07	+	+	-	+	-	+	+	+
1-11-07	+	+	-	+	-	+	+	+
3-21-07	-	-	-	-	-	-	-	+
6-10-06	+	+	-	+	-	+	+	+
2-4-06	+	+	-	+	-	+	+	+

Table 3. MMP expression in human breast-to-bone metastatases samples.

MMP expression was assessed by immunofluorescent staining for specified MMP. + indicates positive staining in the specific compartment (tumor or stroma) and – indicates an absence of staining throughout the sample.

CHAPTER II

MATERIALS AND METHODS

Cell culture

Two independent syngeneic FVB mammary tumor cell lines derived from the polyoma virus middle T (PyMT) model of mammary tumorigenesis tagged with a luciferase reporter gene and designated, PyMT-Luc or PyMT-Dsred2 and 17L3C-Luc, were used in all studies [142]. A luciferase tagged 4T1 mammary tumor cell line was kindly provided by Dr. Swati Biswas of the Vanderbilt Center for Bone Biology (Aslakson et al, 1992). COS-7 cells were used for the LTBP-3 overexpression experiment (ATCC #CRL-1651™). All cell lines were cultured in DMEM (Gibco BRL, Long Island, NY) supplemented with 10%FBS (Atlanta Biologicals, Atlanta, GA) and 5 μg/ml puromycin (only for PyMT-Luc and 17L3C-Luc cells, Sigma-Aldrich, St Louis, MO) at 37^{o}C, 5% CO_2.

Generation of PyMT-DsRed mammary tumor cell line

PyMT cells were labeled with DSred Express through transduction with a lentiviral titer made by co-transfection of HEK293-T17 cells with a pPACK Packaging Plasmid Mix (System Biosciences) and a pLenti6-based (Invitrogen) lentivector construct modified to express DSred Express (construct obtained from Dr. Meenhard Herlyn, Wistar Institute).

After 24 hours in 10 % serum, tumor cells were then treated with 20 μg/ml blasticidin (Invitrogen) to select the infected cells. Once selected, PyMT-DsRed cells were maintained in 10 μg/ml blastocidin.

Isolation of primary osteoblasts

Calvaria from wild type or MMP-2 deficient 3 to 4 day-old pups were harvested in cold sterile 1X Phosphate buffered saline buffer (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 KH_2PO_4, pH 7.6) and stripped off the periosteum and sutures using a razor blade, only the parietal bone is used for the remaining of the isolation protocol . Calvaria were then subjected to three digestions (respectively 15 min, 30 min and 1 hour) in digestion buffer (α-MEM and 0.025% trypsin, Gibco BRL, Long Island, NY, 10mg/ml collagenase P, Roche), at 37^{o}C with vigorous shaking every 15 min. Isolated primary cells from the supernatants of the digestion were then maintained in α-MEM and 10% fetal bovine serum (Atlas Biologicals), 100 μg/ml penicillin/streptomycin (Invitrogen) and 250 unit/ml fungizone (Gibco, BRL, Long Island, NY). Primary osteoblasts were plated at a density of $2x10^5$ cells/well in 6 well-plates and 24 h after seeding; cells were cultured in serum starved α-MEM media. After 24 h, conditioned media was collected, centrifuged at 1100 rpm to remove cellular debris and subsequently used for the MTT and soft agar colony formation assays described below.

Isolation of primary osteoclast precursor cells

Bone marrow cells from tibias and femurs were isolated from 6 week-old wild type and MMP-2 deficient mice by flushing the cells with 1 ml of cold 1X PBS using a 25G$^{5/8}$ gauge needle. Isolated cells were centrifuged at 1,000 rpm and rinsed with 1 ml of 1X PBS. CD11b positive cells were then isolated using Macs® Separation columns (Miltenyi Biotech) following the manufacturer's protocol and plated in α-MEM and 10% fetal bovine serum (Atlas Biologicals), 100 μg/ml penicillin/streptomycin (Invitrogen) and 250 unit/ml fungizone (Gibco, BRL, Long Island, NY) for the migration and differentiation assays described below.

Mice and genotyping

All experiments involving animals were conducted after review and approval by the office of animal welfare at Vanderbilt University. Immunocompromised recombinase activating gene-2 (RAG-2) null MMP-7 deficient mice were generated as previously described (Lynch et al, 2005). Wild type and MMP-2 and MMP-9 deficient mice in the FVB/N-Tg background were kindly provided by Dr. Lisa Coussens, Dept. of Pathology, University of California San Francisco. All mice were weaned at 3 weeks of age and genotyped by PCR analysis. Primers designed to identify RAG-2 null and wild type allele were described previously [143]. The PCR conditions for the amplification of mouse RAG-2 or mutant allele were 94^{o}C 4 min for 1 cycle; 94, 58, 72^{o}C 1 min each for 30 cycles; 72^{o}C 1 min for 1 cycle. Primers designed to identify MMP-7 mutant or wild type allele were designed previously in the Matrisian laboratory, wild type allele: forward

primer 5' AAT GAG CTG GCT GTG GAT CTG GT 3', reverse primer 5' GAG GGT TTG ATT TCT ATT TTC AG 3'. Primers for the mutant allele are forward primer 5' CAT CGC CTT CTA TCG CCT TCT TG 3', reverse primer 5' TGC GTC CTC ACC ATC AGT CCA GTA 3'. The PCR cycle conditions for the amplification of mouse wild type MMP-7 or the mutant allele were 95^{o}C 15 min for 1 cycle; 94, 57, 72^{o}C 1 min each for 4 cycle; 91, 57, 72^{o} C 30 sec each for 28 cycles. Double deficient RAG-2 null MMP-7 null mice were used for the animal experiments described in this dissertation. Primers designed to identify mutant MMP-9 allele are forward primer 5' CTA AAG CGC ATG CTC CAG AC 3' and reverse primer 5' GAA GAG GTG ACT GCG ACT CC 3'. Primers used to identify wild type MMP-9 allele were generated previously in the Matrisian laboratory, forward primer 5' GCA TAC TTG CGC TAT GG 3' and reverse primer 5' TAA CCG GTG CAA ACT 3'. The PCR conditions for amplification of mouse wild type or mutant MMP-9 allele were 94^{o}C 4 min for 1 cycle; 94, 58, 72^{o}C 1 min each for 30 cycles; 72^{o}C 1 min for 1 cycle. MMP-2 null mice were genotyped using gelatin zymography. Serum was isolated from wild type and MMP-2 deficient animals. Serum samples mixed with sample buffer (10% SDS, 0.5M Tris-HCl pH 6.8, 30% glycerol, and 0.02% bromophenol blue) were separated on a 8% SDS-PAGE gel containing 3 mg/ml gelatin (Sigma-Aldrich, St Louis, MO). The gel was then rinsed for 15 min in 2.5% Triton X-100 buffer (Sigma-Aldrich, ST Louis MO), then incubated overnight in activation buffer (50 mM Tris-HCl, 10 mM $CaCl_2$) and then stained for 1 hour in staining buffer (0.5 % coomassie in 50 % methanol and 10 % acid acetic). The gel was destained in distilled water until clear bands were visible.

Intratibial injection and in vivo quantitation of tumor growth

PyMT-Luc, 4T1-Luc and 17L3-Luc tumor cells ($2x10^5$) in a 10µl volume of sterile PBS were injected into the tibia of anesthetized immunocompetent or immunocompromised 6 week old mice that were wild type or deficient in MMP-2, MMP-7 or MMP-9. The contralateral limb was injected with 10µl of PBS alone and acted as a sham injected control for the bone remodeling due to the direct injection in the bone. The IVIS™ system (Caliper Life Sciences) was used to detect luminescence from the mammary tumor cells after intratibial injection. Firefly luciferin (120mg/kg in sterile PBS, Gold Biotechnology, Inc.) was delivered retro-orbitally 1 to 2 minutes prior to imaging. Mice were imaged at 24 hours and every 3 days after surgery. Living Image™ software (Calipers Life Sciences) was used to quantify the luminescence intensity in the tumor bearing limb over time. Mice were sacrificed at specified time points post-surgery and both the tumor injected and contralateral control tibiae were harvested. All animal studies were independently repeated at least three times.

Histology

De-identified human samples of frank osteolytic breast to bone metastasis (n=11) were collected by curettage with IRB approval from Vanderbilt University from 2005 to 2008. Fresh human breast-to-bone metastases, tumor and sham injected mouse tibiae were fixed overnight in 10% buffered formalin and decalcified for 3 weeks in 14% EDTA at pH 7.4 with changes every 48-72 hours. Tissues were embedded in paraffin and 5µm thick sections were cut. For MMP-7, MMP-9 and tartrate resistant acid phosphatase (TRAcP)

localization, the following technique was employed. Sections were rehydrated through a series of ethanols and then rinsed in tris buffered saline (TBS; 10mM Tris at pH 7.4, 150mM NaCl) with Tween-20 (0.05%) (TBST). For antigen retrieval, slides were immersed in a 20μg/ml solution of proteinase K according to the manufacturer's instructions for 10 minutes at room temperature. Following washing in TBS, tissue sections were blocked using standard blocking criteria (10 % serum of the appropriate specie in 1X TBS) for 1 hour at room temperature. MMP-7 [144] or MMP-9 (Oncogene, Cambridge, MA) antibodies at a dilution of 1:100 were added in blocking solution overnight at 4^{o}C. Slides were washed extensively in 1X TBST prior to the addition of a species specific fluorescently labeled secondary antibody (Alexafluor 568nm, Invitrogen) diluted 1:1,000 in blocking solution for 1 hour at room temperature. Slides were washed in TBS and then equilibrated in an acetate buffer as described [145]. The ELF97 TRAP stain (Invitrogen) was diluted 1:1,000 in acetate buffer and slides were incubated for 15 minutes at room temperature. Following washing, slides were aqueously mounted in media (Biomeda Corp) containing 2μM DAPI (4′,6 diamidino-2-phenylindole) for nuclear localization. For MMP-2 staining, no antigen retrieval was used, following washing in TBS, tissue sections were blocked using standard blocking criteria for 1 hour at room temperature. MMP-2 (Abcam) antibody at a dilution of 1/150 was added in blocking solution overnight at 4^{o}C. Slides were washed extensively in TBST prior to the addition of a species specific anti-goat biotinylated IgG antibody (Vector Laboratories) diluted 1:1,000 in blocking solution for 1 hour at room temperature. Labeled cells were visualized using an avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories) and 3,3′ –Diaminobenzidine tetrahydrochloride substrate (Sigma, St. Louis,

MO). Sections were counterstained with hematoxylin, dehydrated and permanently mounted.

TRAcP was also detected using a colorimetric kit according to the manufacturer's instructions (Sigma-Aldrich, St Louis, MO). Gross anatomy of the mouse tibiae was assessed by hematoxylin and eosin (H&E) staining using a standard protocol. Proliferation (anti-phospho Histone H3, Upstate, Lake Placid, NY, 1:50 dilution, or Mcm2, Abcam, Cambridge, MA, 1:200 dilution) and apoptosis (anti-Caspase-3, Cell Signaling Technology, Danvers, MA, 1:400 dilution) were assessed by immunohistochemistry as described above.

Micro computed tomography (μCT) and histomorphometric analyses

For gross analysis of trabecular bone volume, formalin fixed tibiae were scanned at an isotropic voxel size of 12μm using a microCT40 (SCANCO Medical). The tissue volume (TV) was derived from generating a contour around the metaphyseal trabecular bone that excluded the cortices. The area of measurement began at least 0.2mm below the growth plate and was extended by 0.12mm. The bone volume (BV) included all bone tissue that had a material density greater than 438.7 mgHA/cm^3. These analyses allowed for the calculation of the BV/TV ratio. The same threshold setting for bone tissue was used for all samples thus allowing for direct comparison between groups.

For histomorphometry of the BV/TV ratio, three non-serial sections of tumor bearing and saline injected hind limbs were H&E stained to assess the ratio of BV/TV or

with TRAcP to assess osteoclast number per mm bone at the tumor bone interface using Metamorph® software (Molecular Devices).

Immunoprecipitation, Immunoblotting and ELISA

Tumor and sham injected tibias from wild type or MMP null animals were harvested at specified time points post-injection and flash frozen in liquid nitrogen. Tissue homogenates were generated by mortar and pestle and total protein was subsequently extracted using a standard protein lysis buffer (0.1 % sodium dodecyl sulfate, 0.5 % sodium deoxycholate, 1 % Triton X100, 20 mM Tris pH 7.5, 150 mM NaCl) containing a complete protease inhibitor cocktail (Roche). Lysates were then centrifuged at 14,000 rpm for 10 min and supernatant was then isolated. Protein concentration in isolated samples was quantitated using a bicinchoninic acid (BCA) assay as per manufacturer's instructions (Pierce). For immunoprecipitation and quantitation of soluble RANKL in the tumor-bone microenvironments, equal concentrations of total protein (1mg) in 1ml of PBS were pre-cleared with 10µl of protein-G-sepharose beads (Amersham Biosciences) for one hour at 4^{o}C. Pre-cleared lysates were then incubated with 2µg of antibody directed to the N-terminus of RANKL (Santa Cruz Biotechnology) for 1 hour at 4^{o}C with rocking prior to the addition of 10µl of protein-G-sepharose beads. Subsequently, 10µl of protein G-sepharose beads were added to the samples and the bead-antibody-protein complexes were allowed to form overnight at 4^{o}C. A nutator was used during all steps for agitation. The complexes were washed extensively (100mM NaCl, 50mM Tris-HCl, pH7.5, 0.5% NP-40) and then boiled in sample buffer (10% SDS, 0.5M Tris-HCl pH 6.8,

30% glycerol, 1% β-mercaptoethanol and 0.02% bromophenol blue) for 10 minutes prior to loading on to a 15% SDS-PAGE gel. Recombinant RANKL (RnD Systems, Minneapolis, MN) or MMP-7 solubilized RANKL (10µg recombinant RANKL incubated with 100ng active MMP-7 (Calbiochem, LaJolla, CA) for 1 hour at 37^{o}C) as previously described (Lynch et al, 2005) were added as positive controls for the molecular weight of MMP solubilized RANKL. Proteins were transferred to nitrocellulose membranes and blocked for 1 hour at room temperature (5% milk powder in 1xTBS; 5mM Tris-HCL pH 7.4). The blots were then panned with an antibody directed to the N-terminus of RANKL (1: 1,000 dilution; Axxora LLC in 5% milk in 1xTBST (TBS with 0.05% Tween 20)) overnight with rocking at 4^{o}C. The following day, blots were washed extensively with 1X TBST prior to the addition of a secondary infra-red labeled anti-mouse antibody (1: 5,000 dilution in 1xTBST, Rockland Inc.) for 1 hour at room temperature. After washing in 1xTBST, blots were developed and bands of interest were quantitated using the Odyssey system (LI-COR Biosciences, Lincoln, NE). For LAP and LTBP-3 immunoblotting, equal amounts of protein were loaded on either 4-12% gradient SDS-PAGE (Invitrogen) or 6 % SDS-PAGE non-denaturing gels respectively. Immunoblotting protocol followed was identical as described above; primary antibodies used were goat anti-human LAP (R&D System, dilution 1:1,000) and rabbit specific anti-mouse LTBP-3 (anti-L3C, dilution 1:1,000, kindly provided by Dr D. Rifkin, Dept of Cell Biology and Medicine, New York University School of Medicine). Secondary infra-red labeled anti-goat or anti-rabbit antibodies (1:5,000, Rockland Inc) were used for detection of the antibody/antigen complex in the dark for 1 hour at room temperature.

ELISA was also used for the quantitation of soluble RANKL and TGF-β in samples according to the manufacturer's instructions (Quantikine, R&D Systems).

MTT Assay

Quantitation of viable PyMT-Luc cells treated with conditioned media from primary osteoblast wild-type or MMP-2 deficient mice was assessed using tetrazolium-based colorimetric assay (MTS, Promega Corporation). Tumor cells were plated in 96-well plates at a density of 1000 cells/well and 24 h after seeding, cells were treated with 100 µl of either serum starved media, conditioned media from primary osteoblasts isolated from either wild type or MMP-2 deficient mice. After 24 and 48h of treatment, 20 µl of MTS was added to each well of cells, and the plate was incubated for 3 h at 37°C. Spectrophotometric absorbance of each sample was measured at 490 nm using a MRX revelation microplate reader (Dynex Technologies). Experiments were performed in quadruplicate.

Soft agar colony formation assay

PyMT-Luc cells were plated at a density of $1.5x10^3$ cells/well in 24 well-plates in soft agar containing α-MEM, 5% fetal bovine serum, 0.7% agarose (Fischer, cat. No. BP164). Tumor cells were treated with 400 µl of 5% serum α-MEM, 5% serum and conditioned media from wild type or MMP-2 null primary osteoblasts or 1ng/ml TGF-β and the media was changed 3 times a week. After 10 days of culture, cells were stained

overnight at 37°C by addition of 0.1mg/ml p-iodonitrotatrazolium (Sigma-Aldrich, St Louis, MO) to the media. Numbers of colonies for each condition were counted on 100X photomicrographs. Experiments were performed in quadruplicate.

Migration assay of osteoclast precursor cells

CD11b positive cells were plated at a density of 10^5 cells/well in the upper well of a transwell (Corning Inc., 5μm pore size) in 500 μl of serum free α-MEM media. Cells were allowed to migrate towards the lower well of the transwell (1 ml of chemotactic gradient (10 % serum-α-MEM) or serum-free media as control) for 5 hours at 37°C. CD11b positive cells that migrated through the membrane were harvested in the lower well and counted. Experiments were performed in triplicate.

Differentiation of osteoclast precursor cell assay

CD11b positive cells isolated from 6 week-old wild type and MMP-2 deficient bone marrow cells were plated in 48 well plate in 10 % serum-α-MEM media at a density of $5x10^5$ cells/well. The following day, cells were treated with 75 ng/ml RANKL (R&D system) and 30 ng/ml M-CSF (R&D system) in 500 μl of 10 % serum-α-MEM media. Media was changed every 3 days for a 15 day period. At the end of the assay, cells were fixed in ice-cold methanol and stained using a colorimetric TRAcP kit (Sigma-Aldrich, St Louis, MO) and counter stained in hematoxylin. Multinucleated (more than 3 nuclei)

TRAcP cells were counted in 8 random field acquired using a 10X microscopic objective for each conditions. Experiments were performed in quadruplicate.

COS-7 cells transfection and LTBP-3 digestion

COS-7 cells were transiently transfected with a full length LTBP-3 cDNA construct and human TGF-β1 cDNA (kindly provided by Dr Rifkin, Dept of Cell Biology and Medicine, New York University School of Medicine) using Superfect (QIAGEN). COS-7 cells were plated at a density of 10^5 cells/well in a 6 well plate the day prior the transfection. Cells were then incubated in transfection mix (30 µl of superfect reagent, 0.5 µg of each constructs and 500 µl of DMEM and 10% FBS) overnight. The next day, transfected COS-7 cells were incubated for 48 hours in serum starved DMEM media. 40 µl conditioned media was then incubated for 3 hours at 37^{o}C in presence of 300 ng of recombinant human MMP-2 (Calbiochem) or for 1 hour in presence of 150 ng of plasmin (Sigman-Aldrich, St Louis, MO). Samples were then analyzed by immunoblotting for LTBP-3 as described above.

Flow cytometry analysis

PyMT-DsRed2 cells were intratibially injected into FVB wild type or MMP-2 deficient mice. After 3 days, tumor injected tibias were flushed with 500 µl of sterile PBS 1X using a 25G$^{5/8}$ gauge needle. Harvested cells were then centrifuged at 2000 g for 5 min and then resuspended in 1 ml of red blood cell lysis buffer (8 mM NH_4Cl, 5 mM $KHCO_3$)

for 5 min at room temperature. Cells were centrifuged at 2000 g for 5 min and resuspended in 1 ml of sterile PBS 1X. 10^6 cells were fixed and permeablized for flow cytometry analysis using BrdU Flow kit protocol (BD Pharmingen™, San Jose, CA). Fixed and permeabilized cells were then incubated in presence of anti-mouse CD16/CD52 (mouse BD Fc Block™, BD pharmingen™, San Jose, CA, 1:20 dilution) for 10 min on ice. Primary antibodies, rabbit anti-mouse cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA, 1:100 dilution) and alexa Fluor® 647 Rat anti-Histone H3 antibody (BD Pharmingen™, San Jose, CA, 1:4 dilution) were added to the samples and incubated for 30 min on ice. Cells were then centrifuged at 2000 g for 5 min and resuspended in 500 µl of staining buffer provided by the BrdU flow kit and incubated in presence of secondary antibody Pacific Blue™ goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, dilution 1:400 dilution) for 30 min. Cells were centrifuged at 2000 g for 5 min and resuspended in 500 ul of 1 % bovin albumin serum in PBS 1X and strain them trough a 35 µm nylon mesh and analyzed using 5-laser BD LSRII flow cytometer.

Statistical analyses

For *in vivo* data, statistical analysis was performed using Anova and Bonferroni multiple comparison tests using GraphPad Prism Inc. Software. *In vitro*, statistical significance was analyzed using a student's t test. A value of $p < 0.05$ was considered significant. Data are presented as mean ± standard deviation (SD).

CHAPTER III

CONTRIBUTIONS OF OSTEOCLAST-DERIVED MMPS TO MAMMARY TUMOR GROWTH IN THE BONE MICROENVIRONMENT

Introduction

Osteoclasts are critical for the completion of the vicious cycle since they are the principal cells involved in the direct resorption of the mineralized bone matrix. Therefore, understanding how osteoclast precursors are recruited to areas requiring bone remodeling and understanding the mechanisms involved in controlling their maturation and activation is key for the development of new therapies that can effectively stop the vicious cycle. Osteoclasts express a variety of proteases including the cysteine protease, cathepsin-K and MMPs [77]. While cathepsin-K activity is critical for bone resorption [28], the role of osteoclast-derived MMPs is less clear. MMPs are often overexpressed by the stromal cells rather than the tumor [86]. In the context of the tumor-bone microenvironment, pre-clinical animal studies have demonstrated the efficacy of broad spectrum MMPIs in preventing tumor growth and tumor induced osteolysis [137-139]. However, the failure of MMPIs in human clinical trials prevents their application for the treatment of lytic bone metastases [135]. A main conclusion derived from these trials was the necessity for defining the precise roles of individual MMPs in disease processes that would allow for the generation of highly selective MMP inhibitors. To this end, we have assessed the expression of MMPs in human clinical samples of osteolytic breast to bone metastasis. While the expression of many MMPs was noted throughout the tumor/stroma, MMP-7

and MMP-9 were highly localized to bone resorbing osteoclasts. Given the importance of osteoclasts in driving the vicious cycle, the current study focused on determining if and how these osteoclast derived MMPs impact tumor-induced osteolysis.

Results

MMP-7 and MMP-9 are expressed by osteoclasts in human breast to bone metastases

Previous observations using an animal model of tumor-bone interaction identified several MMPs as being highly expressed at the tumor-bone interface compared to the tumor area alone, namely MMP-2, -3, -7, -9 and -13[116] (unpublished data). The expression of these MMPs was examined in human cases of frank breast to bone metastasis (n=11). Interestingly, MMP-7 and MMP-9 were largely localized to the majority of mature TRAcP positive multinucleated osteoclasts at the tumor-bone interface in human samples containing areas of osteolysis (10 of 11 samples) (Figure 5A-C). Other cells in the stromal compartment stained positively for MMP-7 and MMP-9 but remarkably, the tumor cells were negative for these metalloproteinases. MMP-2, -3 and -13 were also detected but their expression was diffuse throughout the tumor/stroma compartment (Figure 16 and data not shown). Since osteoclasts are the principal cells involved in bone resorption, we examined whether the ablation of host derived MMP-7 or MMP-9 would impact the vicious cycle in terms of mammary tumor growth and/or mammary tumor induced osteolysis.

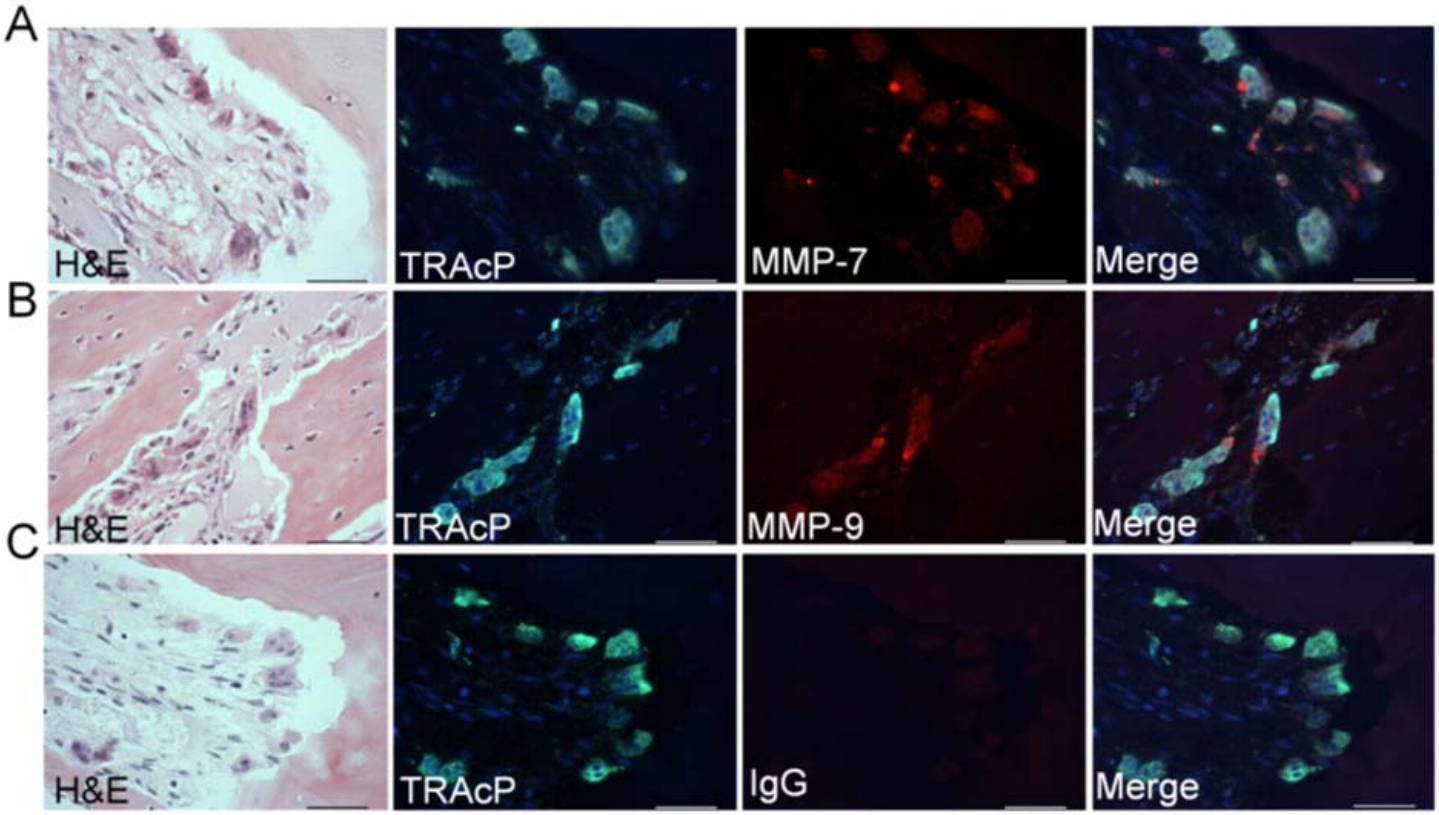
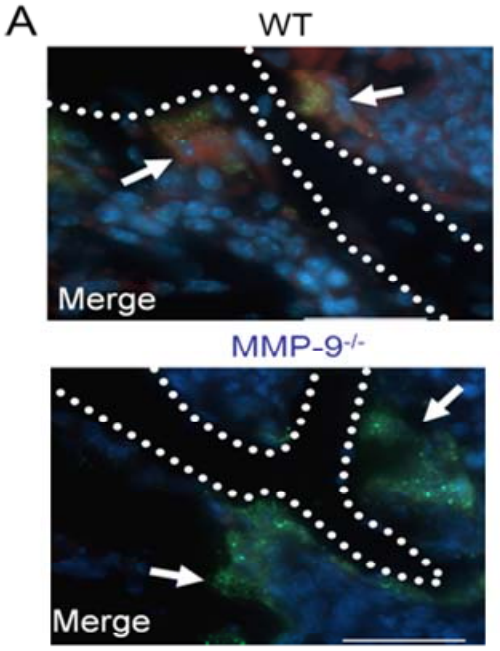


Figure 5. MMP-7 and MMP-9 localization in human breast to bone metastases (n=11).
A-C: Fluorescent TRAcP staining (green) was used to localize osteoclasts (arrows) while immunofluorescence was used to localize MMP-7 and MMP-9 (red). DAPI (blue) was used as a nuclear stain. Murine or rat IgG was used as a negative control. Dashed line represents the tumor-bone interface. Scale bars are 50μm. (Conor Lynch)

Host derived MMP-9 does not contribute to tumor growth or tumor induced osteolysis

MMP-9 has previously been reported to be localized to osteoclasts and MMP-9 null animals have been identified as having a delay in osteoclast recruitment during the development of long bones [75]. Therefore, we initially tested the role of host derived MMP-9 in tumor growth or tumor induced osteolysis. Consistent with our observations in human samples, bone resorbing osteoclasts in wild type mice were positive for MMP-9 expression by immunofluorescent staining while as expected, MMP-9 was not detected in MMP-9 null osteoclasts (Figure 6A). Since MMP-9 null animals have a transient developmental bone phenotype, we determined the baseline trabecular bone volume as a function of tissue volume (BV/TV) in wild type and MMP-9 null animals at 6 weeks of age which was the proposed time-point for introduction of the PyMT-Luc tumor cells. No difference in the BV/TV between the wild-type and MMP-9 null animals was observed (Figure 6B).

To assess the contribution of host MMP-9 in mammary tumor growth in the bone microenvironment, the PyMT-Luc tumor cells, in which MMP-9 expression is undetectable in vivo [146], were injected into the tibia of syngeneic FVB wild-type or MMP-9 null mice. Surprisingly, quantitation of the bioluminescent signal from the tumor cells showed no difference in the tumor growth rate between the MMP-9 null and wild type control mice (Figure 7A). With respect to tumor induced osteolysis, analysis of the BV/TV ratio by high resolution μCT demonstrated that the tumor injected tibias of wild-type and MMP-9 null were significantly lower ($p<0.05$) than their respective sham injected control counterparts (Figure 7B and C). However, a direct comparison of the



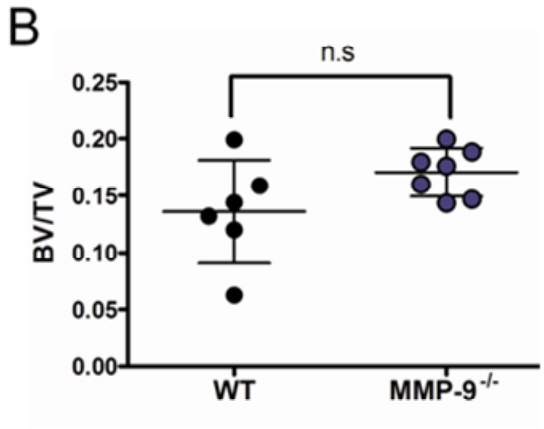


Figure 6. MMP-9 is present in osteoclasts at the tumor-bone interface but does not affect normal trabecular bone volume. A: Representative photomicrographs of MMP-9 (red) localization merged with TRAcP (green) localization in WT and MMP-9-/- animals. DAPI (blue) was used as a nuclear stain. Arrows indicate osteoclasts while dashed line represents the tumorbone interface. Scale bars are 50µm. B:The baseline BV/TV ratio immunocompetent 6 week old non-injected WT (n=6) and MMP-9$^{-/-}$ (n=7) mice on an FVB background was assessed by high resolution µCT scan analysis.

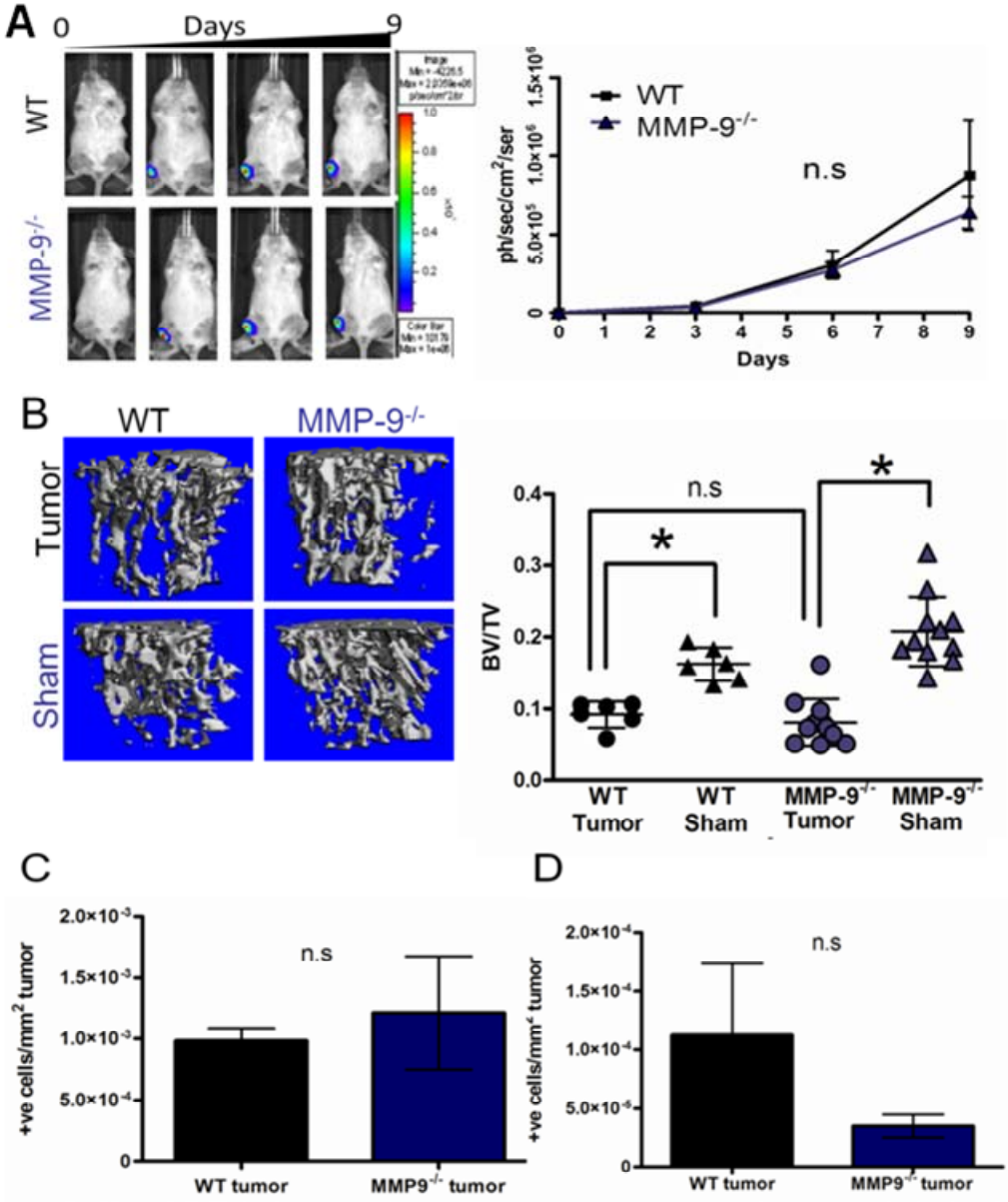


Figure 7. Host MMP-9 does not impact mammary tumor growth or osteolysis in the bone microenvironment. A: PyMT-Luc cells were injected intratibially into syngeneic FVB wild type (WT, n=6) or MMP-9 null (MMP-9-/-; n=11). The contralateral limb received a sham injection of saline. Luminescence was measured over a nine day period. Quantitation of tumor growth in WT and MMP-9-/- animals. B: Representative µCT scans of trabecular bone from tumor bearing and sham injected limbs of WT and MMP-9$^{-/-}$ animals. µCT was also used to calculate the ratio of trabecular bone volume to tissue volume (BV/TV) for tumor injected and sham injected wild type and MMP-9$^{-/-}$ mice. C: The number of proliferative tumor cells, stained for phospho Histone H3 per area of tumor was calculated. D: The number of apoptotic tumor cells per tumor area was assessed by immunohistochemistry for cleaved caspase-3. Data are measn ± SD. N.s indicates a non-significant p-value.

BV/TV ratios between the wild-type and MMP-9 null tumor injected limbs revealed no difference in BV/TV ratios (Figure 7B and C). Furthermore, no difference in tumor growth as assessed by phospho histone H3 for proliferation and cleaved caspase-3 immunohistochemistry for apoptosis (Figure 7DC and E) or trabecular bone volume by histomorphometry was observed between the wild type and MMP-9 null groups (Figure 7B). These experiments, with similar sized groups, were repeated on several occasions with similar results. These results using the intratibial model suggest that host MMP-9 does not contribute to mammary tumor growth in the bone or tumor- induced osteolysis and are consistent with studies examining the role of host MMP-9 in the prostate cancer-bone microenvironment [147].

Host MMP-7 contributes to mammary tumor growth in the bone microenvironment

This is the first report to document the expression of MMP-7 in human breast to bone metastases and in human osteoclasts (Figure 5), although MMP-7 has previously been identified in rodent osteoclasts [116]. Recapitulating observations in human clinical samples, MMP-7 expression was identified in wild type murine osteoclasts and not in MMP-7 null osteoclasts (Figure 8A). Given that MMP-7 expression by osteoclasts is a relatively recent observation, studies into defining roles for MMP-7 in skeletal development have not been explored thus far. Therefore, prior to testing the impact of host derived MMP-7 on the vicious cycle, the trabecular bone volume in non-injected 6 week old immunocompromised wild type and MMP-7 null animals was examined using high resolution μCT. Our results revealed no significant difference in the BV/TV ratio

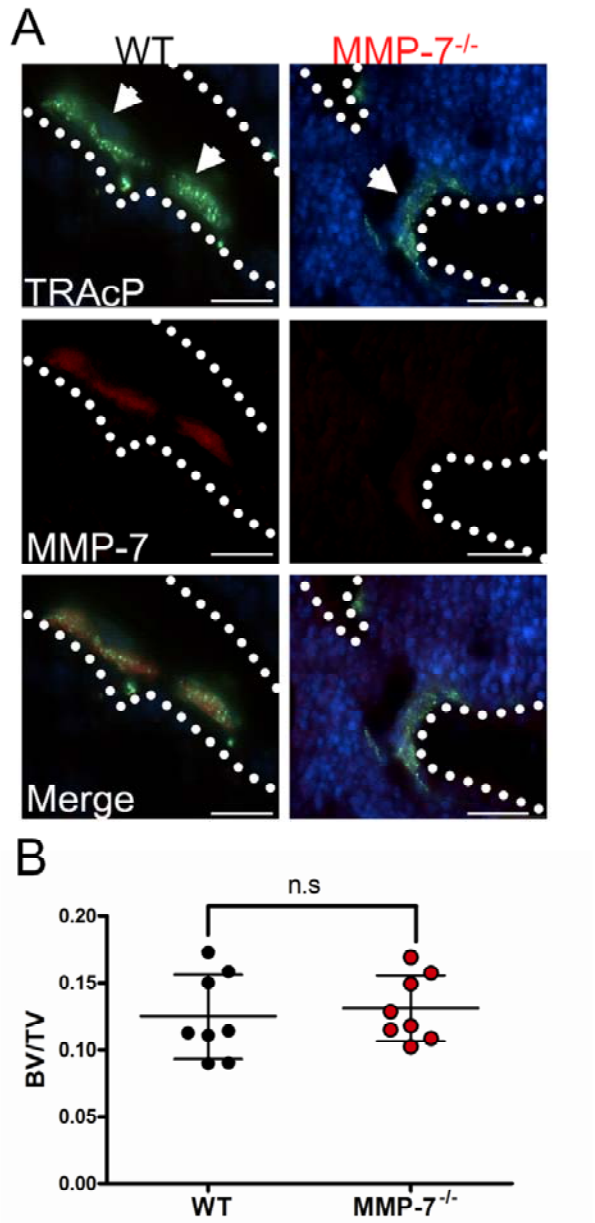


Figure 8. Murine MMP-7 is expressed in mature osteoclast at the tumor-bone interface but does not affect trabecular bone volume baseline in non-tumor bearing mice. A: Representative photomicrographs of MMP-7 (red) immunofluorescent localization merged with TRAcP (green) localization in WT and MMP-7$^{-/-}$ animals. DAPI (blue) was used as nuclear stain. Arrows indicate osteoclasts while dashed line represents the tumor-bone interface. **B:** The bone volume (BV) over tissue volume (TV) from 6 week old non-injected WT and MMP-7$^{-/-}$ animals was determined by µCT scan analysis. N..s indicates $p>0.05$.

between wild type and MMP-7 null animals suggesting that at this time point, MMP-7 null animals do not display an obvious bone phenotype in comparison to the wild type controls (Figure 8B).

To determine the contribution of host MMP-7 to mammary tumor growth in the bone microenvironment, PyMT-Luc cells were injected into 6 week old wild-type or MMP-7 null mice. Quantitation of the bioluminescent signal from the PyMT-Luc cells demonstrated a significant decrease in the tumor growth rate in MMP-7 null mice compared to the wild type controls (Figure 9A). These experiments with similar sized groups in terms of animal numbers were independently repeated on four occasions and similar observations were noted. To further investigate the potential role of MMP-7 in tumor growth, tumor proliferation and apoptosis were assessed by immunohistochemistry for phospho-histone H3 and cleaved caspase-3, respectively, in multiple sections from at least five animals per group (Figure 9B and C). Surprisingly, no difference in tumor proliferation was observed between the wild type and MMP-7 null groups, however, tumor apoptosis was significantly higher in MMP-7 null mice compared to the wild type controls ($p<0.05$). Similar findings with respect to the impact of host MMP-7 on tumor growth using the 4T1-Luc cell line were also observed (Figure 10A-C). These results suggest that host-derived MMP-7 significantly contributes to mammary tumor growth in the bone by enhancing tumor cell survival.

Host derived MMP-7 contributes to mammary tumor induced osteolysis

The vicious cycle of tumor-bone interaction suggests that tumor growth/survival is

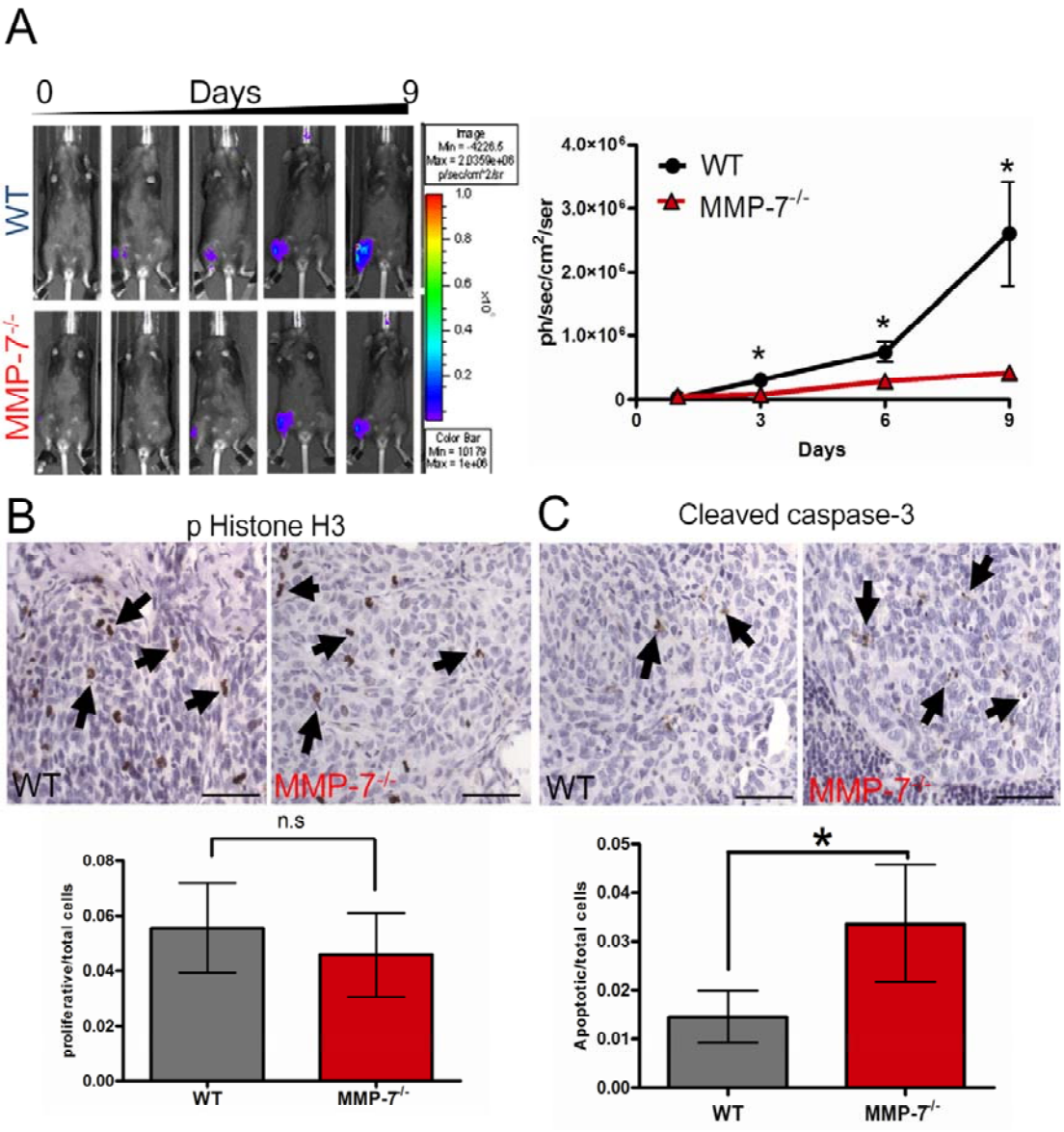


Figure 9. Host MMP-7 contributes to mammary tumor growth in the bone microenvironment. **A:** PyMT-Luc cells were injected intratibially into RAG-2 null (WT; n=5) or MMP-7 null (MMP-7$^{-/-}$; n=10). The contralateral limb received a sham injection of saline. Luciferase activity was measured over a nine day period and used as a measure of tumor growth. **B-C:** Proliferative or apoptotic cells (arrows) in representative sections of WT and MMP-7$^{-/-}$ injected tibias were identified by immunostaining of phospho Histone H3 (pHistone H3) or cleaved caspase-3 respectively. The number of positively stained cells per total number of cells was calculated. Scale bars represent 50μm in all photomicrographs. Data are mean ± SD. Asterisk denotes that $p<0.05$ while n.s. indicates a non-significant p value.

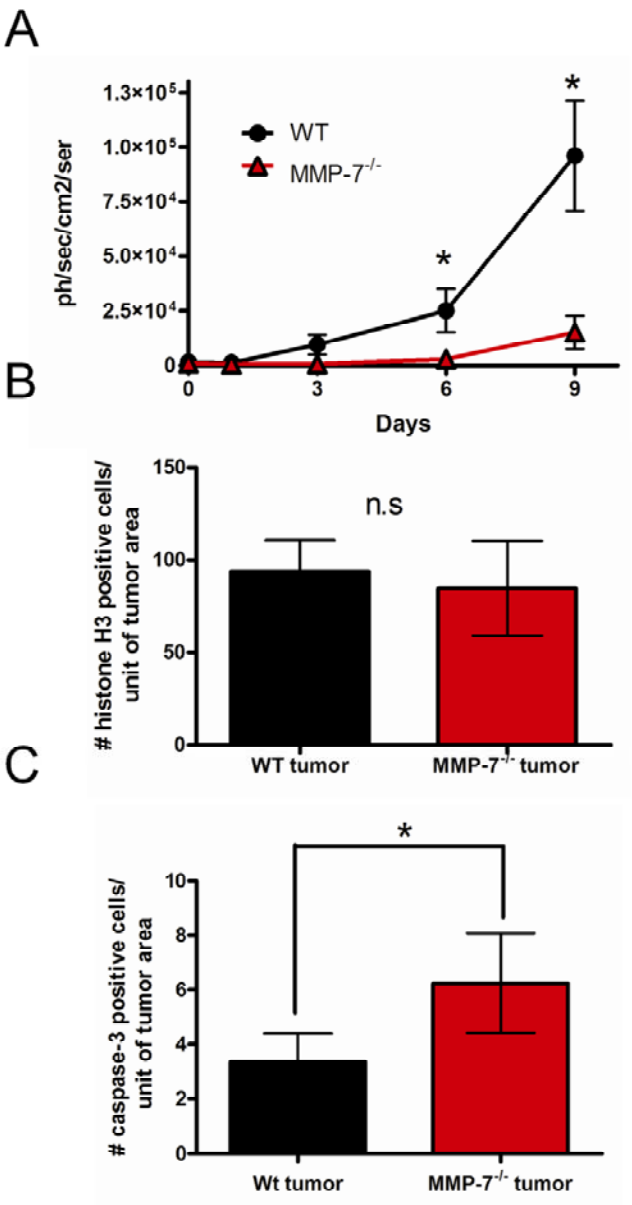


Figure 10. Host MMP-7 impacts 4T1-Luc growth in the bone microenvironment. **A:** 4T1-Luc cells were intratibially injected into RAG-2 wild type (WT; n=6) or MMP-7 null (MMP-7$^{-/-}$; n=6) mice. The contralateral limb received a sham injection of saline and luciferase activity was assessed as a measure of tumor growth in WT and MMP-7$^{-/-}$ animals. These studies were independently repeated with similar numbers of animals and similar results were obtained. **B:** Tumor proliferation was assessed by immunohistochemical staining for phospho histone H3 in tumor bearing tibias of WT and MMP-7$^{-/-}$ deficient mice. **C:** Tumor apoptosis was determined by immunohistochemical staining for cleaved caspase-3 on tumor bearing limbs of WT and MMP-7$^{-/-}$ deficient mice. Data are mean ± SD; n.s. implies a non-significant p value ($p>0.05$).

dependent on osteoclast mediated bone resorption. Since MMP-7 is primarily localized to bone resorbing osteoclasts in the tumor-bone microenvironment, we assessed whether a lack of MMP-7 in osteoclasts impacted tumor induced osteolysis. Analysis of the BV/TV ratios from wild type and MMP-7 null tumor injected tibias using μCT (Figure 11A) and histomorphometry (Figure 11B) revealed that the MMP-7 null group had a significantly higher amount of trabecular bone which is in keeping with our tumor growth data, i.e. less tumor growth in the MMP-7 null animal would lead to less osteolysis. X-ray analysis also revealed a significantly lower tumor volume in the MMP-7 null animals compared to wild type controls (Figure 11C). Studies using the 4T1-Luc cell line also demonstrated that host derived MMP-7 significantly impacted tumor induced osteolysis (Figure 12A and B). These results demonstrate for the first time that host derived MMP-7 significantly impacts mammary tumor induced osteolysis.

MMP-7 mediates RANKL solubilization in the tumor-bone microenvironment

Next, we explored the potential molecular mechanisms through which osteoclast derived MMP-7 was impacting tumor induced osteolysis. Given the acidity of the resorption lacunae (pH<4) and the neutral activity profile of MMP-7, we suggest that MMP-7 does not function in direct bone matrix degradation but in the processing of factors that impact cell-cell communication within the tumor-bone microenvironment. MMP-7 has previously been shown to process a number of growth factors and cytokines to soluble active forms including members of the tumor necrosis factor family (TNF), TNF-α, Fas ligand (FasL) and RANKL [72, 116, 148]. RANKL is essential for osteoclastogenesis and is a

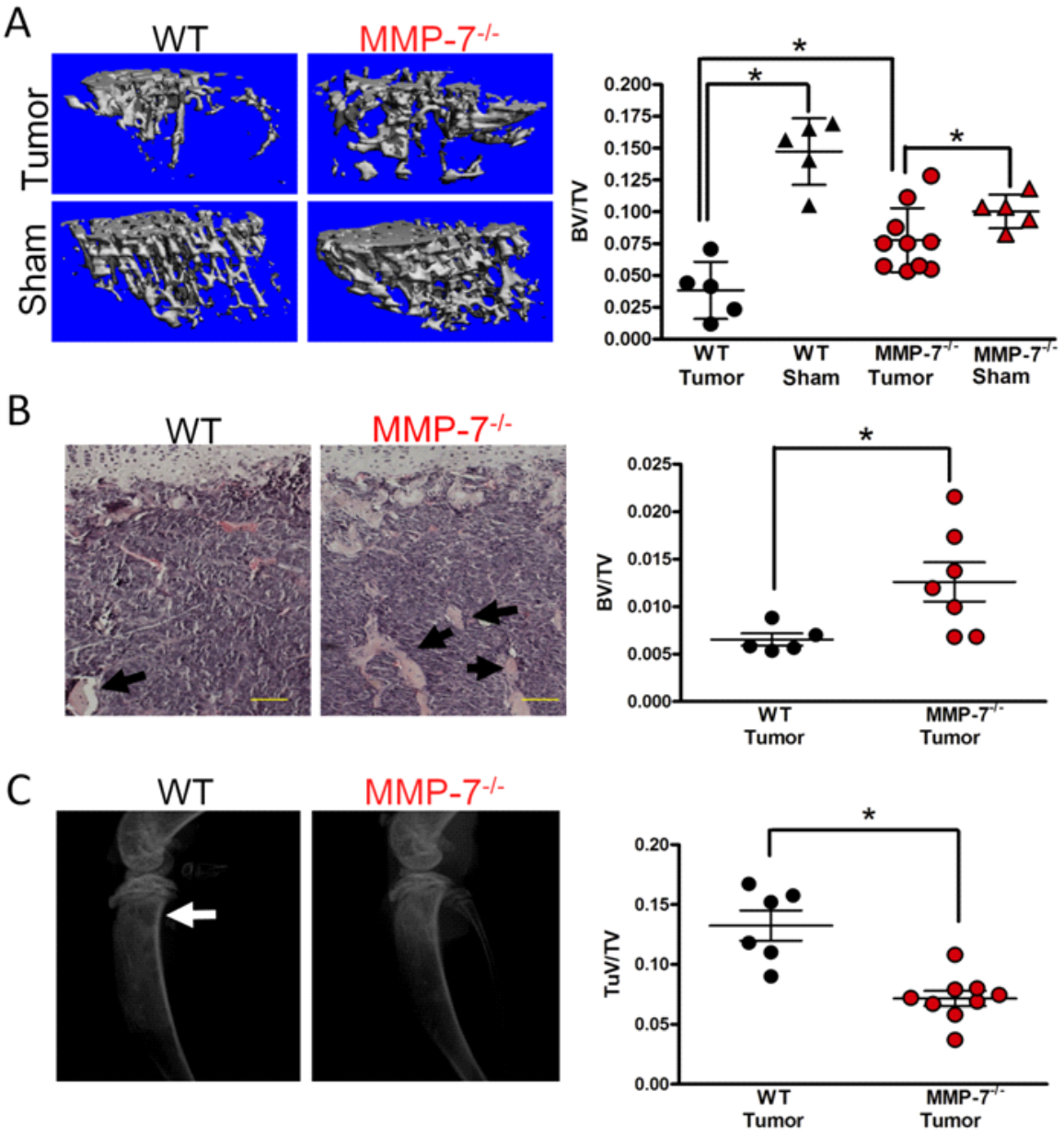


Figure 11. Tumor mediated osteolysis is attenuated in the absence of host derived MMP-7. A: µCT scans of trabecular bone from tumor bearing and sham injected limbs of WT and MMP-7$^{-/-}$ animals allowed for the calculation of the BV/TV ratio. **B**: Representative H&E stained photomicrographs of tumor bearing tibias from WT and MMP-7$^{-/-}$ animals. Arrows indicate trabecular bone. Scale bars are 100 µm. The ratio of trabecular bone volume (BV) to tissue volume (TV) was determined several non-serial sections of tumor injected tibias obtained from WT (n=5) and MMP-7 null animals (n=7). C: Representative radiographic images from tumor injected WT and MMP-7$^{-/-}$ animals at day 9. Arrow indicates lytic tumor lesions in the wild type animals. The tumor volume (TuV) over tissue volume (TV) for tumor injected limbs of WT and MMP-7$^{-/-}$ animals was assessed. Data are mean ± SD. Asterisk denotes that $p<0.05$ while n.s. indicates a non-significant p value.

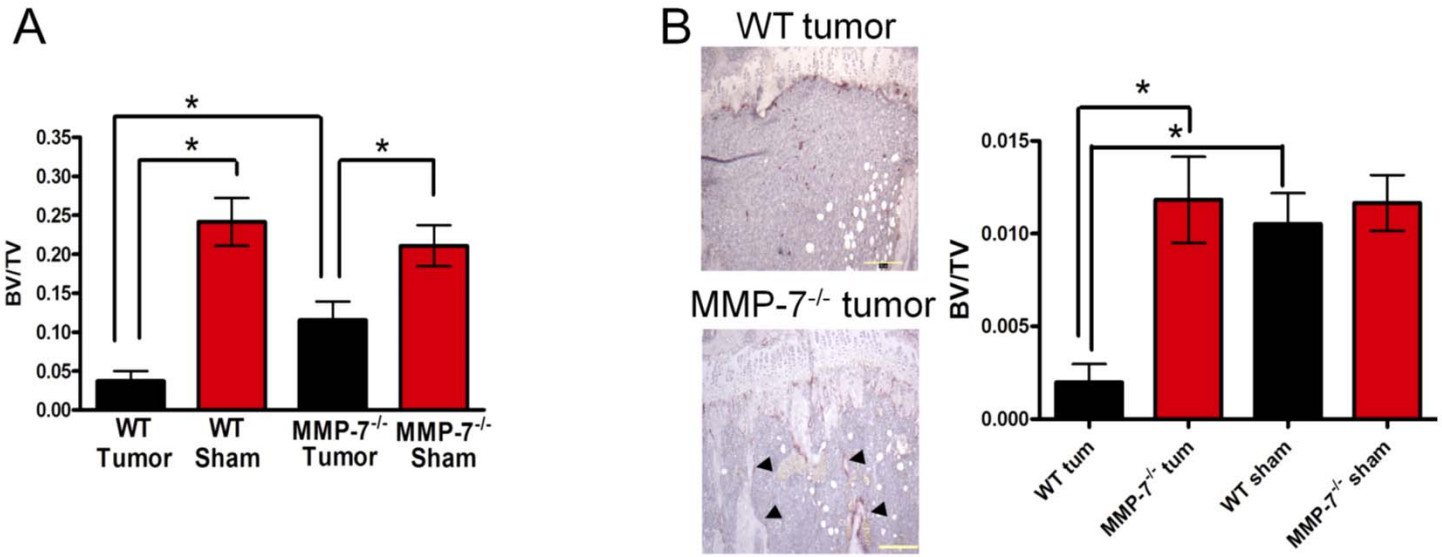


Figure 12. Host MMP-7 contributes to 4T1-Luc tumor induced osteolysis A: µCT scans of trabecular bone from tumor bearing and sham injected limbs of WT (n=6) and MMP-7$^{-/-}$ null mice (n=6) allowed for the calculation of the BV/TV ratio. **B:** Trabecular bone volume in the limbs was assessed by histomorphometry on tumor and sham injected tibias of WT and MMP-7$^{-/-}$ null mice (n=6 for both groups). Full black arrowheads indicate trabecular bone. Scale bars are 100µm. Data are mean ± SD. Asterisk denotes that $p<0.05$.

potent chemotactic molecule for monocytes and osteoclast precursor cells [149, 150]. Therefore, we investigated if MMP-7 solubilization of RANKL was relevant in our model.

ELISA analysis revealed lower levels of total RANKL (membrane bound and soluble) in the tumor injected tibias of MMP-7 null mice compared to wild type control mice (Figure 13A) while no difference was observed in the sham injected control counterparts of each group (data not shown). Similar levels of OPG, a soluble decoy receptor of RANKL, were found in the wild-type and MMP-7 null animals and were not present at a high enough concentration to interfere with the detection of RANKL by ELISA (data not shown). Immunoprecipitation and immunoblotting for soluble RANKL also revealed significantly lower levels of soluble RANKL in PyMT-Luc and 4T1-Luc tumor injected MMP-7 null animals compared to wild type controls as assessed by densitometry (Figure 13B and 14A).

Interestingly, soluble RANKL could still be detected in the tumor bearing limbs of MMP-7 null animals. This suggests that RANKL solubilization is still occurring in the absence of MMP-7. We and others have previously identified that other metalloproteinases such as MMP-1, -3, -14, ADAM-17 and the serine protease cathepsin G are capable of processing RANKL to a soluble active form and therefore, these proteases may also be playing a role in the solubilization of RANKL in our model [116-118, 151]. However, since the levels of RANKL are significantly lower in the MMP-7 null mice, we suggest that MMP-7 is the dominant protease involved in RANKL

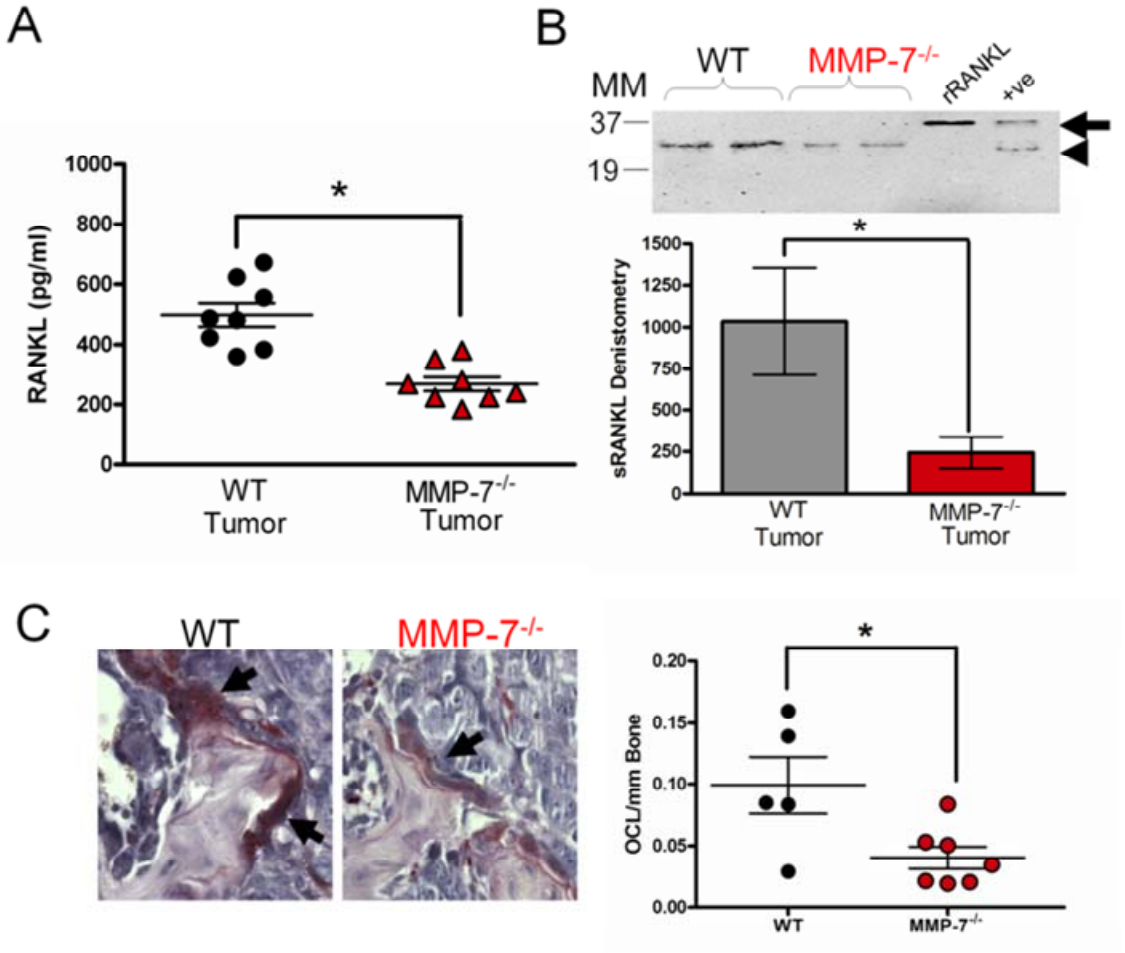


Figure 13. MMP-7 solubilization of RANKL in the tumor-bone microenvironment. A: ELISA analysis of soluble RANKL levels in lysates from tumor injected tibias obtained from WT (n=8) or MMP-7$^{-/-}$ (n=8) animals. B: Immunoprecipitation using antibodies directed toward the N-terminus of RANKL for the detection of soluble RANKL in tumor bearing tibias of WT and MMP-7$^{-/-}$ animals. MM refers to the molecular weight marker in kDa. Unglycosylated full length recombinant RANKL (arrow) was used as a positive control. In addition, MMP-7 solubilized RANKL (arrow head) served as a further positive control (+ve). Densitometry was performed on the level of soluble RANKL in PyMT-Luc bearing limbs derived from wild type (n=11) and MMP-7$^{-/-}$ null (n=12) mice. Data are mean ± SD. Asterisk denotes that $p<0.05$. C: TRAcP (red) positive, multinucleated (blue) osteoclasts (arrows) at the tumor-bone interface in WT and MMP-7$^{-/-}$ animals. The number of osteoclasts at the tumor-bone interface were determined in multiple non-serial sections of tumor injected tibias obtained from WT (n=5) and MMP-7$^{-/-}$ (n=7) animals. Scale bars are 100µm. Data are mean ± SD. Asterisk denotes that $p<0.05$ while n.s. indicates a non-significant p value.

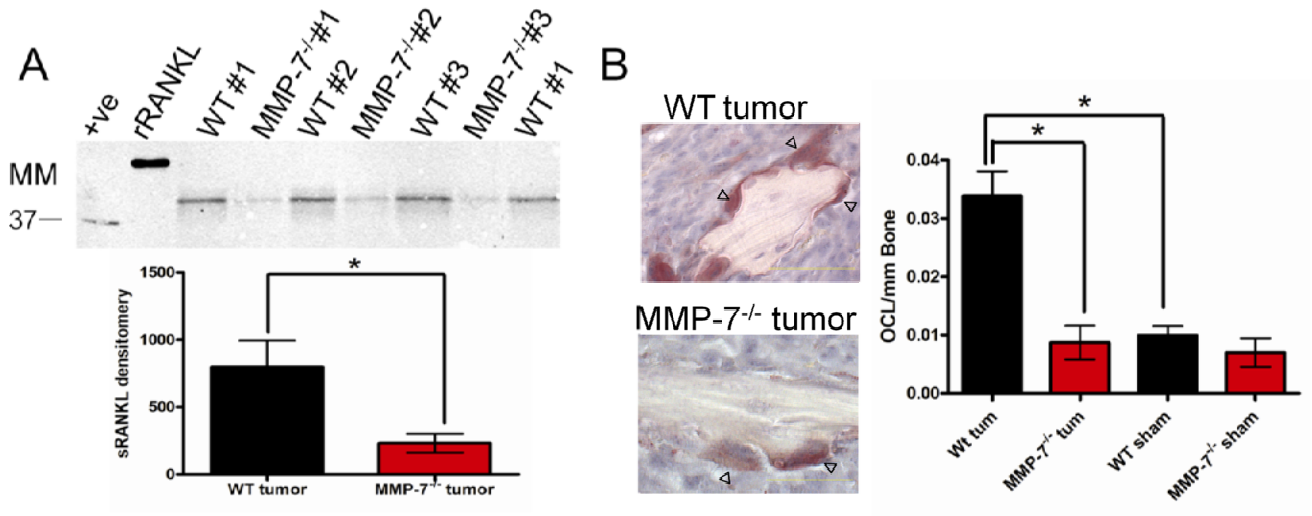


Figure 14. Host MMP-7 contributes to 4T1-Luc tumor induced osteolysis A**:** Immunoprecipitation using antibodies directed toward the N-terminus of RANKL for the detection of soluble RANKL in tumor bearing tibias of WT and MMP-7$^{-/-}$ animals. MM refers to the molecular weight marker in kDa. Unglycosylated full length recombinant RANKL (arrow) was used as a positive control. In addition, recombinant RANKL was incubated with recombinant MMP-7 for 1 hour at 37°C prior to loading on the gel The resultant fragment (arrow head) served as a further positive control (+ve) control for the detection of soluble RANKL in the tumor lysates at approx 25kDa. Densitometry was performed on the level of soluble RANKL in 4T1-Luc bearing limbs derived from wild type (n=14) and MMP-7$^{-/-}$ null (n=13) mice. Data are mean ± SD. Asterisk denotes that $p<0.05$. **D:** Number of mature osteoclasts at the tumor-bone interface was quantified by histomorphometry in tumor and sham injected limbs of WT and MMP-7$^{-/-}$ animals. Empty arrowheads indicate mature multinucleated osteoclasts. Scale bars are 100µm. Data are mean ± SD. Asterisk denotes that $p<0.05$.

solubilization.

Next, since a decrease in the amount of soluble RANKL was detected in the tumor bearing limbs of the MMP-7 null animals, we asked if there was concomitant decrease in the number of osteoclasts in the MMP-7 null tumor-bone microenvironment. We observed significantly lower numbers of TRAcP positive multinucleated osteoclasts per unit length of tumor-bone interface in the MMP-7 null animals compared to the wild type controls (Figure. 13C). Significantly lower numbers of osteoclasts were also recorded in MMP-7 deficient animals injected with 4T1-Luc cells compared to wild type controls (Figure 14B). Given the importance of RANKL in mediating osteoclastogenesis, these data suggest that MMP-7 mediates mammary tumor induced osteolysis by impacting the availability of a key factor for osteoclastogenesis, RANKL.

Conclusions

Understanding the molecular mechanisms that control the vicious cycle is key for the development of new therapeutics that will be effective not only in treating bone metastases but also in curing them. In the current study, we found that in human cases of breast to bone metastasis, osteoclasts were a rich source of MMP-7 and MMP-9. Interestingly, our studies using two unrelated osteolytic inducing tumor cell lines (PyMT-Luc and 4T1-Luc) revealed that only MMP-7 appeared to contribute to mammary tumor growth and tumor induced osteolysis in the bone microenvironment. Furthermore, our data suggests that MMP-7 solubilization of the osteoclastogenic factor RANKL is the principal molecular mechanism underlying these observations. Previously, we have

identified that MMP-7 processing of RANKL results in the generation of an active soluble form that can promote osteoclast maturation and activation [116]. Therefore, in the context of the breast to bone metastases we hypothesize that in the absence of MMP-7 solubilized RANKL, there is a resultant decrease in osteoclast maturation and bone resorption at the tumor-bone interface that in turn results in a decrease in bone derived growth factors that impact tumor growth (Figure. 15). These data suggest that MMP-7 inhibition using a selective approach may be efficient for the treatment of breast to bone metastases.

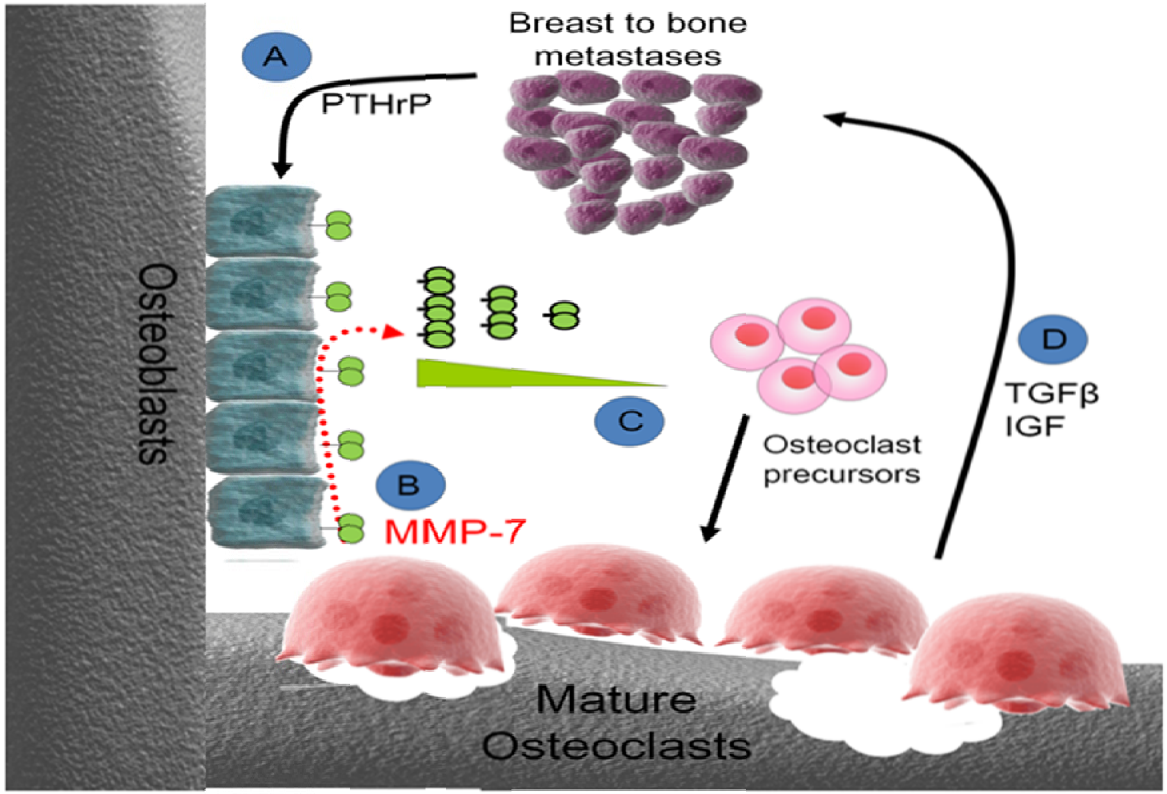


Figure 15. Hypothetical mechanism of osteoclast derived MMP-7 action in the mammary tumor-bone microenvironment. A: Metastatic tumor cells through the secretion of factors such as PTHrP, stimulate osteoblasts to express full length membrane bound RANKL. **B:** Osteoclasts express MMP-7 which can process membrane bound RANKL to a soluble active form. C: Soluble RANKL has been shown to be chemotactic for osteoclast precursors (25). In addition to acting as a potential chemotactic molecule, soluble RANKL stimulates can stimulate the maturation and activation of osteoclast precursors. **D:** Activated osteoclasts in turn execute bone resorption leading to the release of growth factors such as TGF-β and IGFs that promote tumor growth in the bone microenvironment.

CHAPTER IV

CONTRIBUTIONS OF OSTEOBLAST-DERIVED MMP-2 TO MAMMARY TUMOR GROWTH-INDUCED BONE LESIONS

Introduction

Osteoblasts are the cells responsible for the synthesis of the bone and the deposition of growth factors such as TGF-β and IGFs in the bone matrix [11]. These factors are complexed in latency proteins and therefore, the proteases that govern their release and activation are of great importance. TGF-β and IGFs are crucial players in the vicious cycle as they have been shown to promote the differentiation of osteoclast precursor cells into multinucleated mature osteoclasts [23]. By activating osteoclasts, osteoblasts promote osteolysis and the release of growth factors in the tumor-bone microenvironment [87]. Many studies on breast tumor growth in bone have focused on the osteoclast and how to inhibit osteoclast function in a bid to halt bone resorption. However, with the exception of tumor effect on the expression of osteoclastogenic factors by the osteoblasts such as RANKL, little attention has been given to osteoblast-mediated tumor growth *in vivo*.

Osteoblasts have been reported to express several MMPs but the contribution of osteoblast-derived MMPs to the vicious cycle has not been explored to date [81, 152-157]. MMPs have been implicated as important mediators of cell-cell communication, therefore investigating their contributions to osteoblast functions in a tumor setting is of interest.

In assessing MMP expression in human and murine breast-to-bone metastases, we found that one MMP in particular, MMP-2 was localized to osteoblasts, osteocytes and other cell types throughout the tumor/stroma compartment. This localization of MMP-2 in osteoblast and osteocyte is in keeping with studies examining MMP-2 roles in skeletal development. Thus far, the contribution of MMP-2 in the pathological context of the metastatic tumor-bone microenvironment has not been examined. Given the importance of the osteoblasts in driving the vicious cycle, the current study focused on determining if and how host-derived MMP-2 impacted tumor induced osteolysis.

Results

Expression of MMP-2 in human and mouse samples of breast to bone metastasis

A recent study reported the high expression of several MMPs (MMP-2, -3, -7, -9 and -13) at the tumor-bone interface in a murine model of tumor growth in the bone [116]. In human samples of breast to bone metastasis and tumor bearing limbs of wild type animals, expression of these 5 MMPs was assessed. As shown in Figure 5, MMP-7 and MMP-9 were primarily localized in TRAcP positive multinucleated osteoclasts present at the tumor-bone interface. In contrast, MMP-2 expression appeared more diffuse throughout the tumor/stroma compartment (Figure 16A-B) but osteoblasts and osteocytes were consistently positive for MMP-2 (Figure 16A-B). Interestingly, osteoclasts in the tumor-bone microenvironment were rarely positive for MMP-2 (Figure 16A). These

observations are in agreement with recent studies reporting osteocyte and osteoblast expression of MMP-2 in normal murine bone [68, 69]. Since osteoblasts have a central role in promoting osteoclast activation and subsequent bone resorption in the vicious cycle, we examined the potential impact of the ablation of host MMP-2 in a murine model of an osteolytic tumor-bone microenvironment.

Host MMP-2 significantly impacts mammary tumor growth in the bone

Trabecular bone is a highly metabolically active bone, rich in growth factors, that undergoes continuous remodeling [8]. During tumor-induced osteolysis, trabecular bone is typically the first bone to be resorbed [158]. As MMP-2 deficient mice have a transient bone phenotype, we determined the baseline of trabecular bone volume/tissue volume (BV/TV) ratios in tibias of 6 week old wild type and MMP-2 deficient animals and found not difference between the two groups using high resolution μCT (Figure 16C).

To determine the contribution of host derived MMP-2 in mammary tumor growth in the bone, the PyMT-Luc mammary tumor cell line which expresses MMP-2 *in vitro* (Figure 16D), was injected into the tibia of syngeneic immunocompetent FVB wild type and MMP-2 deficient animals. Quantitation of the bioluminescent signal from the PyMT-Luc tumor cells showed an increased tumor growth rate between the wild type and MMP-2 null mice from day 3 post-injection onwards (Figure 17A and B). These

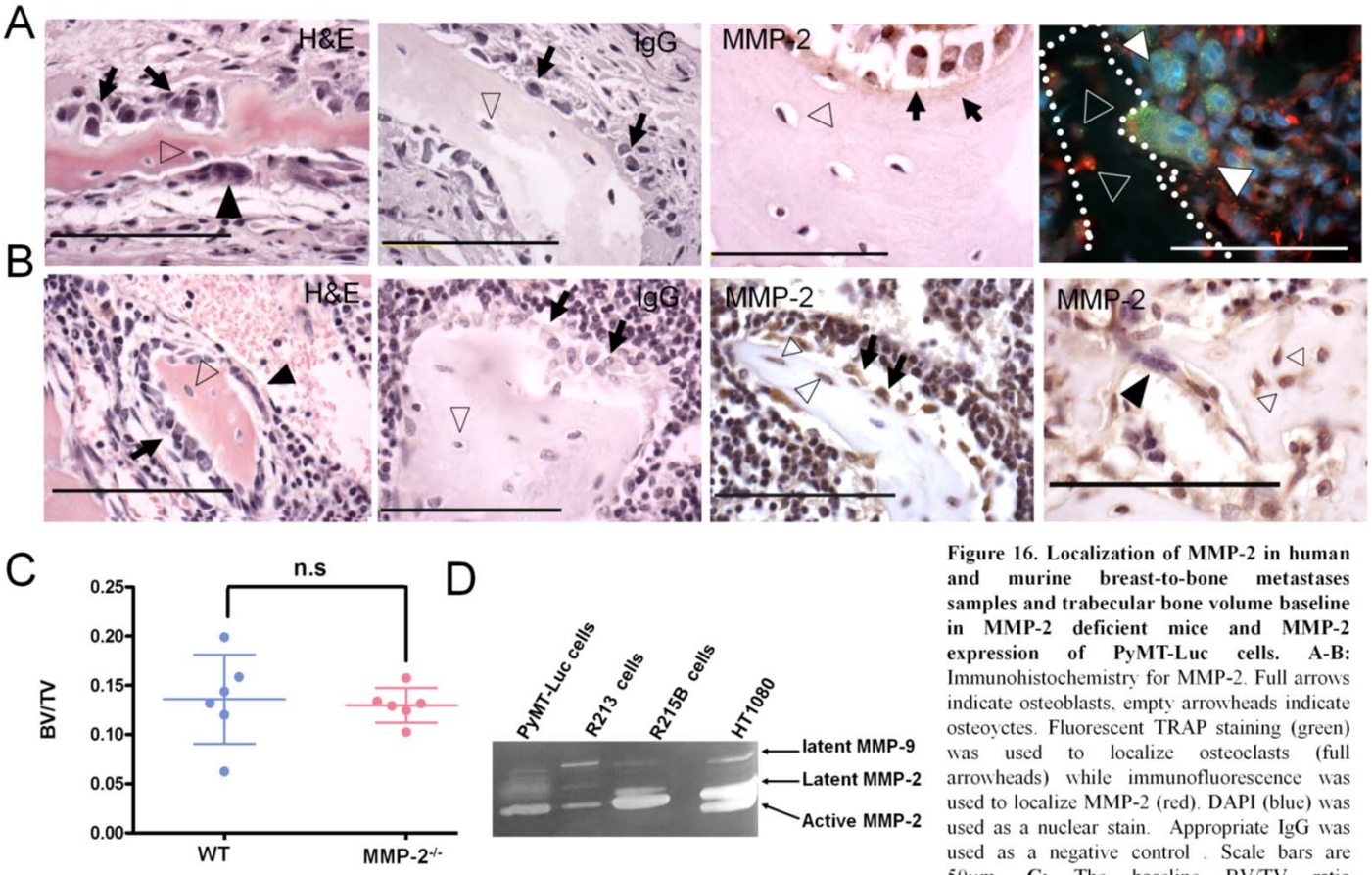


Figure 16. Localization of MMP-2 in human and murine breast-to-bone metastases samples and trabecular bone volume baseline in MMP-2 deficient mice and MMP-2 expression of PyMT-Luc cells. A-B: Immunohistochemistry for MMP-2. Full arrows indicate osteoblasts, empty arrowheads indicate osteoyctes. Fluorescent TRAP staining (green) was used to localize osteoclasts (full arrowheads) while immunofluorescence was used to localize MMP-2 (red). DAPI (blue) was used as a nuclear stain. Appropriate IgG was used as a negative control . Scale bars are 50µm. **C:** The baseline BV/TV ratio immunocompetent 6 week old non-injected FVB WT (n=6) and MMP-2$^{-/-}$ (n=6) mice was assessed by high resolution µCT scan analysis. **D:** MMP-2 expression was assessed by gelatine zymography in 3 mammary tumor cell lines including PyMT-Luc cells.

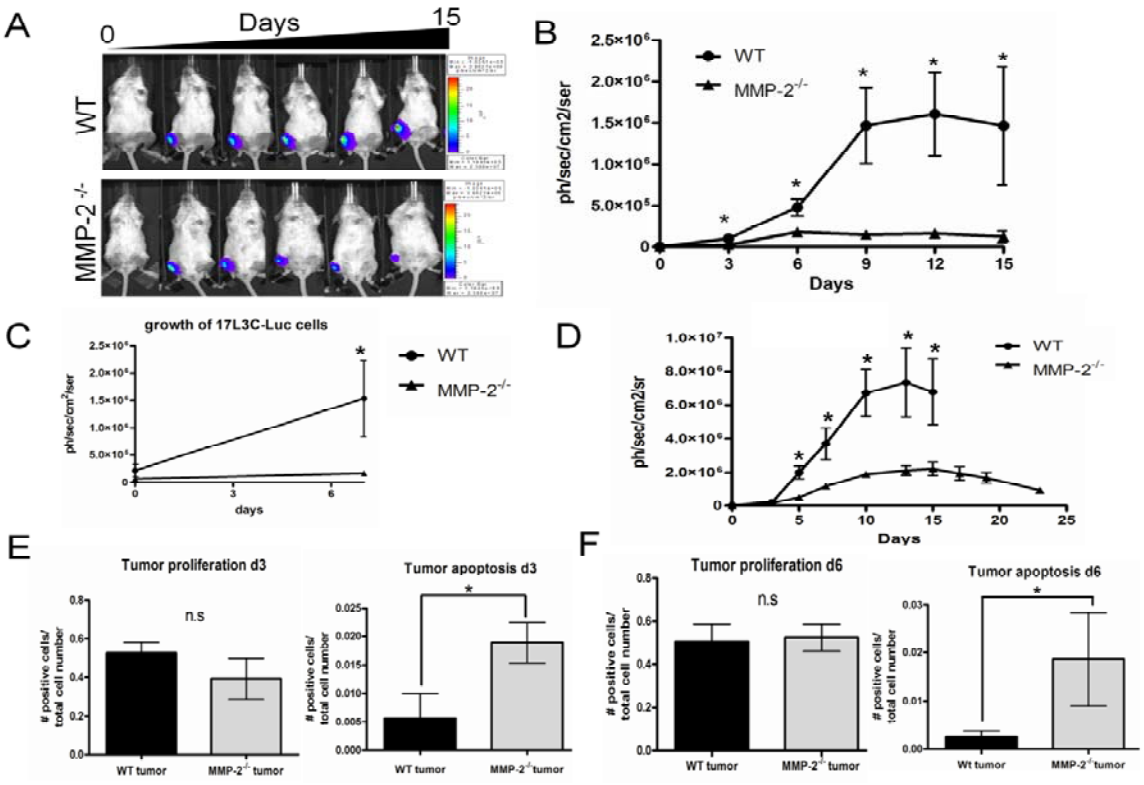


Figure 17. Host-derived MMP-2 impacts mammary tumor growth in the bone microenvironment. A-B: PyMT-Luc cells were intratibially injected into FVB wild type (WT; n=10) or MMP-2 null (MMP-2$^{-/-}$; n=10) mice. The contralateral limb received a sham injection of saline and luciferase activity was assessed as a measure of tumor growth in WT and MMP-2$^{-/-}$ animals. **C:** 17L3C-Luc cells were intratibially injected into FVB wild type (WT; n=10) or MMP-2 null (MMP-2$^{-/-}$; n=10) and tumor growth was assessed by bioluminescent imaging modality. **D:** PyMT-Luc cells were intratibially injected into FVB wild type (WT; n=10) or MMP-2 null (MMP-2$^{-/-}$; n=10) mice and tumor growth was followed for 15 days for WT mice and 25 days for MMP-2 null mice. **E:** Tumor proliferation and apoptosis was assessed by immunohistochemical staining for Mcm2 and cleaved caspase-3 respectively, in tumor bearing tibias of WT and MMP-2$^{-/-}$ deficient mice, at 3 days post-surgery. **F:** At 6 days after injection, tumor proliferation and apoptosis was determined by immunohistochemical staining for respectively, MCM2 and cleaved caspase-3 on tumor bearing limbs of WT and MMP-2$^{-/-}$ deficient mice. Data are mean ± SD; n.s. implies a non-significant p value ($p>0.05$).

experiments with similar numbers of animals per group were conducted independently on several occasions and similar results were observed. The observed result on tumor growth was confirmed using another independent mammary tumor cell line, 17L3C-Luc cells [146] (Figure 17C). To delineate whether reduced tumor growth was due to survival or slow growth rate *in vivo*, tumors in the MMP-2 deficient animals were imaged for at least 25 days. The bioluminescent signal remained significantly less than that obtained in the wild type mice throughout the entire time period (Figure 17D). These data suggested that perhaps host MMP-2 was important for the initial survival and establishment of tumor cells.

To further analyze the impact of host MMP-2 to mammary tumor growth in the bone, we first decided to use a flow cytometry approach to specifically determine tumor versus host proliferation and apoptosis. PyMT cells expressing Dsred were intratibially injected into wild type and MMP-2 deficient mice therefore. Three days after surgery, tumor injected tibias were harvested and proliferation and apoptosis were assessed by flow cytometry using phospho Histone H3 and cleaved caspase-3 specific antibodies coupled with fluorophores (and pacific blue, respectively). Therefore, tumor proliferative cells will be Dsred/ Alexa fluor® 647 positive and tumor apoptotic cells will be Dsred/pacific blue positive. However, we determined that FVB bone marrow cells contains a sub population of autofluorescent cells (less than 2 % of total cells) conferring a high background signal which did not allow for an accurate assessment of tumor proliferation and apoptosis. As a result, immunohistochemical stainings for Mcm2 and cleaved caspase- 3 were performed to assess tumor proliferation and apoptosis, respectively. No difference in tumor proliferation was observed between the two groups

of animals at numerous time points (Figure 17E and F). However, MMP-2 deficient mice showed a significant higher level of apoptotic tumor cells as early as 3 days after intratibial injection compared to wild type mice (Figure 17E). Furthermore, at 6 days post-injection, the apoptotic tumor levels remained significantly higher in the MMP-2 deficient animals compared to controls and tumor proliferation did not differ between the two groups of animals (Figure 17F). These data show for the first time that host-MMP-2 impacts tumor growth in the bone. In addition host MMP-2 mediates its effect on tumor growth by enhancing tumor survival as early as 3 days post-injection and this high rate of apoptosis observed in the MMP-2 deficient mice persisted until day 6.

Host MMP-2 contributes to mammary tumor growth induced osteolysis

In the tumor-bone microenvironment, pathological bone remodeling requires osteoblast-mediated activation of osteoclasts. Osteoblasts are crucial for the induction of osteoclast activation via the expression of osteoclastogenic factors such as RANKL and M-CSF resulting in an extensive bone resorption that is thought to enhance tumor growth [10]. Since a decrease in tumor growth was observed, we next assessed whether there was a concomitant decrease in osteolysis in the MMP-2 null tumor-bone microenvironment. High resolution μ-CT and histomorphometry analyses of the BV/TV ratios of wild type and MMP-2 deficient mice were performed at the end of the study period. Tumor bearing limbs of wild type mice showed a significant decrease of the trabecular bone content compared to tumor injected tibias of MMP-2 deficient animals (Figure 18 A-B). These results demonstrate a decrease in tumor-induced bone resorption in the MMP-2

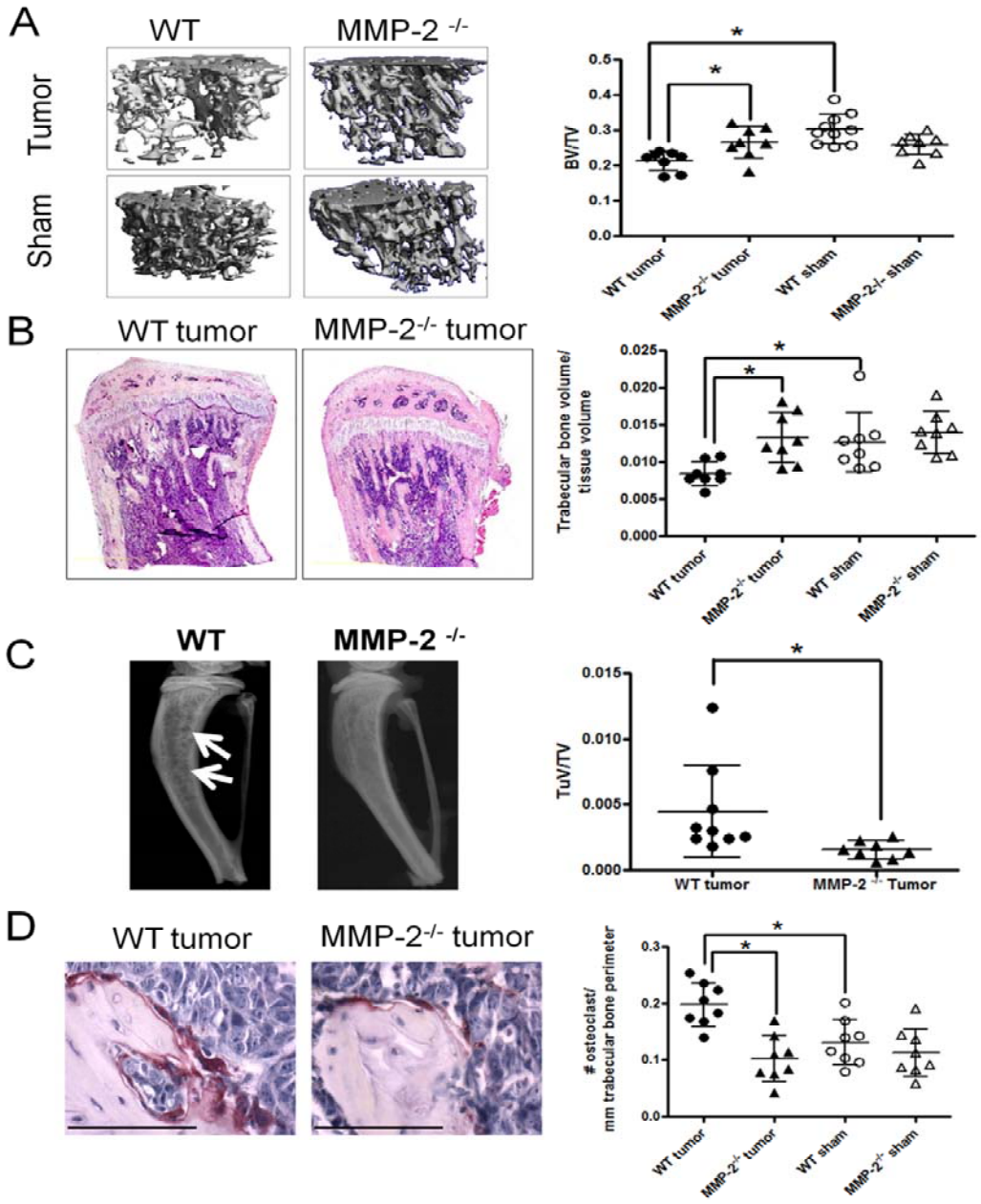


Figure 18. Tumor mediated osteolysis is attenuated in the absence of host derived MMP-7. A: μCT scans of trabecular bone from tumor bearing and sham injected limbs of WT and MMP-2$^{-/-}$ mice allowed for the calculation of the BV/TV ratio. **B:** Representative H&E stained photomicrographs of tumor bearing tibias from WT and MMP-2$^{-/-}$ mice. Scale bars are 1mm. The ratio of trabecular bone volume (BV) to tissue volume (TV) was determined several non-serial sections of tumor injected tibias obtained from WT (n=8) and MMP-2 null animals (n=8). **C:** Representative radiographic images from tumor injected WT and MMP-2$^{-/-}$ animals at day 9. Arrow indicates lytic tumor lesions in the wild type animals. The tumor volume (TuV) over tissue volume (TV) for tumor injected limbs of WT and MMP-2$^{-/-}$ animals was assessed. Data are mean ± SD. Asterisk denotes that $p<0.05$ while n.s. indicates a non-significant p value.

deficient mice compared to the wild type controls. In addition, X-ray analysis revealed that MMP-2 deficient mice have a significantly lower tumor burden compared to the wild type control mice which is in agreement with our tumor growth and histomorphometry data (Figure 18C).

As osteoclasts are the bone cells responsible for osteolysis, the number of mature osteoclasts was assessed in both groups of animals by counting the number of TRAcP positive multinucleated osteoclasts in several non-consecutive sections from multiple animals. A significantly higher number of osteoclast per unit length of bone was observed in tumor injected tibia of wild type mice compared tumor bearing limbs of MMP-2 deficient animals (Figure 18D). This decrease in osteoclast number in the MMP-2 deficient mice is in agreement with the higher content of trabecular bone and the decreased tumor growth rate, i.e. less tumor growth induces less bone resorption therefore, a lower number of mature osteoclasts.

Although we did not observe MMP-2 localization in osteoclasts, we tested whether absence of MMP-2 could affect osteoclast maturation and activation. Osteoclast precursor cells (CD11b positive cells) were isolated from wild type and MMP-2 deficient mice and their ability to migrate towards a chemokine gradient (10 % serum) was assessed in a transwell assay (Figure 19A). No difference between wild type and MMP-2 deficient osteoclast precursor cells was observed in terms of number of migrating cells (Figure 19A). The capacity of wild type and MMP-2 deficient CD11b positive cells to respond to osteoclast differentiating factors such as RANKL and M-CSF was tested (Figure 19B). The same number of TRAcP positive cells was found in MMP-2 deficient

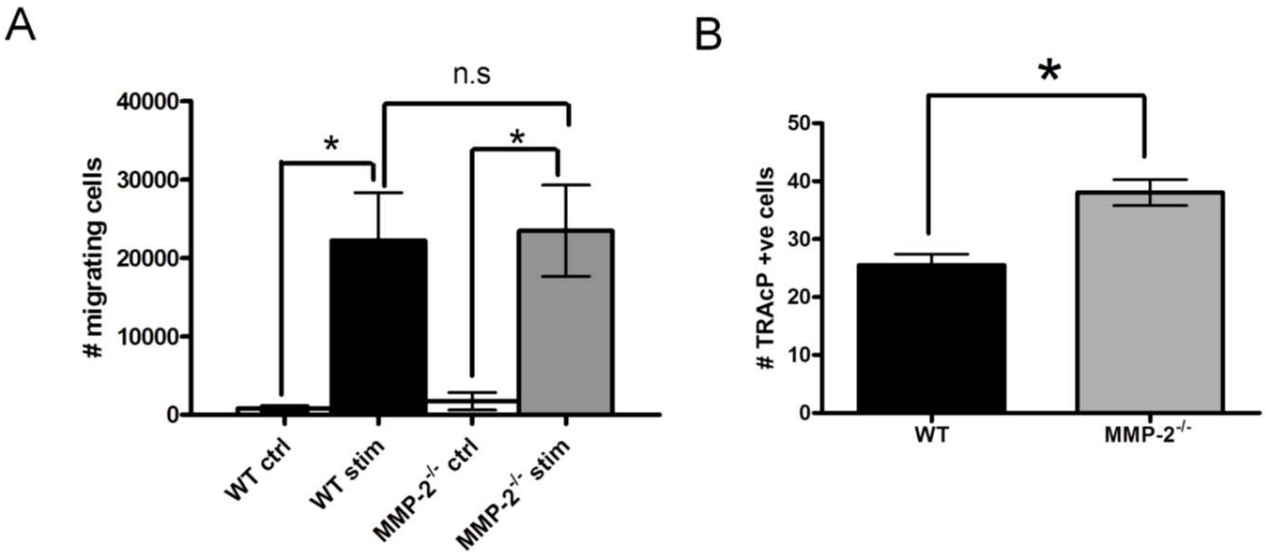


Figure 19. MMP-2 does not affect migration and maturation of osteoclast precursor cells. A: Migration of CD11 positive cells isolated from wild type and MMP-2 deficient mice was assessed in a transwell assay. 10 % serum was used as a chemoattractant. Number of migrating cells after 5 hours were counted in each condition. **B:** CD11 positive cells isolated from wild type and MMP-2 deficient mice were treated with 75 ng/ml RANKL and 30 ng/ml M-CSF for a period of 15 days. Cells were then fixed and stained for TRAcP. Number of multinucleated TRAcP positive cells in multiple fields were counted for each condition. Data are mean ± SD. Asterisk indicates $p<0.05$ and n.s indicates non significance.

osteoclast precursor cells treated with RANKL and M-CSF compared to wild type cells (Figure 19B). Therefore demonstrating that absence of MMP-2 does not impact the ability of osteoclast precursor cells to migrate towards a chemokine gradient and differentiate in presence of osteoclast activating factors.

To address how host MMP-2 impacts tumor survival and tumor-induced osteolysis, we focused our attention on the osteoblasts since 1) they are a rich source of MMP-2 and 2) they are the common link in the vicious cycle between tumor growth and osteoclast recruitment, maturation and activation. We initially decided to examine tumor/osteoblast interactions since the contribution of MMP-2 in this setting has thus far, not been explored.

Osteoblast-derived MMP-2 mediates mammary tumor survival in the bone via the release of active TGF-β

Given that MMP-2 has been shown to impact osteoblast function and that our human and mouse tissues samples showed an osteoblast localization of MMP-2, we determined if osteoblast-derived MMP-2 could mediate tumor growth and survival. In order to assess this, we pursued a more straightforward *in vitro* approach. The effect of conditioned media from wild type and MMP-2 deficient primary osteoblast to modulate PyMT-Luc cells growth and survival was assessed using MTT growth and clonogenic soft agar assays (Figure 20A and B). Clonogenic assay was developed in the mid 1950s by Puck and Marcus to assess the ability of single mammalian cells to form colonies [159]. Since,

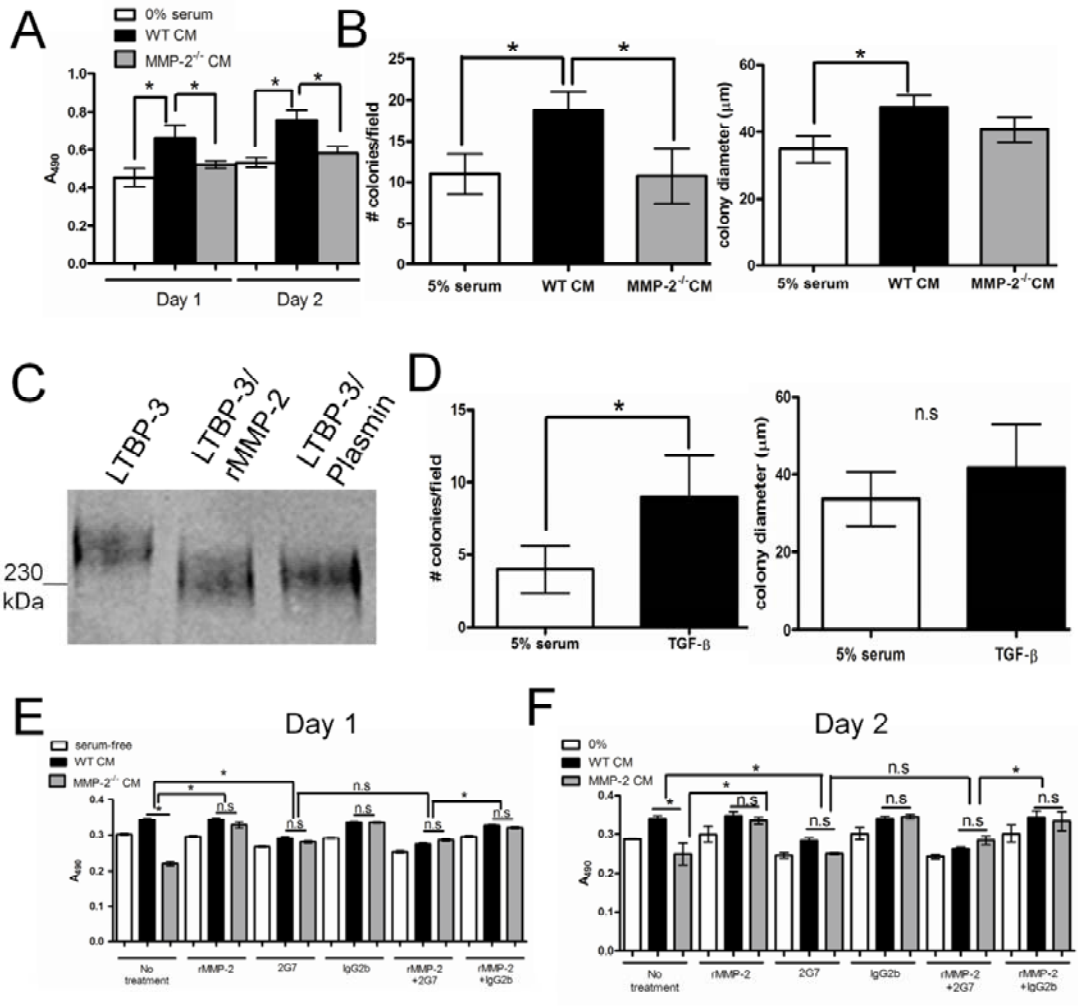


Figure 20. Osteoblast-derived MMP-2 and TGF-β mediate tumor survival. PyMT-Luc cells were treated with conditioned media from wild type (WT CM)or MMP-2 null (MMP-2$^{-/-}$ CM)osteoblasts. Ability of the tumor cells to proliferate and survive was assessed by MTT and soft agar colony formation assays. **A, E and F:** Metabolic activity of PyMT-Luc cells treated with WT CM or MMP-2$^{-/-}$ CM in presence of 2G7 (TGF-β neutralizing antibody), IgG2b (control antibody) or recombinant MMP-2 (rMMP-2) was assessed by MTT assay. **B and D:** Tumor survival and growth in presence of WT CM, MMP-2$^{-/-}$ CM or 1 ng/ml TGF-β was determined by soft agar colony formation assay. Number of colonies and colony diameter were reported for each condition. **C:** Cleavage assay of LTBP-3 by recombinant MMP-2 or plasmin . Data are mean ± SD. Asterisk indicates $p<0.05$ and n.s indicates non significance.

then, this assay, slightly modified had become the 'gold standard assay' in radiology to assay the efficacy of radiation treatment to induce apoptosis in tumor cells [160]. Clonogenic soft agar assay includes all forms of cell death and the ability of single cells to proliferate i.e assesses the ability of cells to escape anoikis (detachment-induced cell death) and therefore survive to proliferate and form colonies [161]. To this end, we utilized clonogenic soft agar assay to assess the ability of PyMT-Luc cells to survive in an anchorage-independent condition as well as proliferate to form colonies under various conditions. Conditioned media derived from wild type primary osteoblasts induced significantly higher metabolic activity of tumor cells and a statistical higher number of tumor colonies compared to tumor cells incubated with conditioned media from MMP-2 deficient osteoblasts (Figure 20A). However, no difference was observed in the average size of the colonies between the two conditions, suggesting that the absence of MMP-2 in osteoblasts affects tumor survival but not tumor growth which is in agreement with our *in vivo* data (Figure 20B). To confirm that osteoblast-derived MMP-2 mediates this phenomenon, recombinant MMP-2 was added to conditioned media from MMP-2 deficient primary osteoblasts prior to treatment of PyMT-Luc cells. Addition of exogenous MMP-2 to conditioned media from MMP-2 deficient osteoblasts rescued metabolic tumor cell activity (Figure 20E and F). These *in vitro* assays demonstrated for the first time that osteoblast-derived MMP-2 contributes to tumor survival.

Next we examined the molecular mechanism through which osteoblast-derived MMP-2 mediated the tumor survival effect. MMP-2 can process numerous growth and survival factors in particular TGF-β whose role in modulating the vicious cycle, including osteoblast and osteoclast differentiation and function, has been well described [85, 98, 134].

TGF-β is maintained in a latent form via its complex with the latency associated peptide (LAP) and the latent TGF-β binding protein (LTBP-1-4). Successive proteolytic cleavages induce the release of the active growth factor [162, 163]. LTBP-3 deficient mice displayed a distinct cranial phenotype and develop osteopetrosis, therefore suggesting its key role in controlling the bioavailability of TGF-β in the bone [164, 165]. Furthermore, LTBP-3 has been shown to be important for osteogenic differentiation of human mesenchymal stem cells [166]. Therefore, we tested the hypothesis that LTBP-3 is a substrate of MMP-2, and osteoblast-derived MMP-2 induces the release of TGF-β which influences the mammary tumor survival. To assess the susceptibility of LTBP-3 for proteolytic processing, the conditioned medium of COS-7 cells overexpressing the large latent complex of LTBP-3 and LAP-TGF-β was subjected to digestion with recombinant active MMP-2. The molecular weight of the complex was reduced from ~240 kDa to ~230-220 kDa in presence of recombinant active MMP-2, a processing event that has been previously reported to produced a ~ 230 kDa fragment consistent with that of plasmin (Figure 20C) [167]. These data suggest that MMP-2 has the ability to process the latency binding protein that sequesters TGF-β in the bone matrix.

Since our results demonstrate that MMP-2 can process LTBP-3, we then tested the hypothesis that active TGF-β could mediate the survival of PyMT –Luc cells using a clonogenic soft agar assay. Treatment of the tumor cells with TGF-β significantly increased the number of colonies but not the size of the colonies compared to control conditions (Figure 20D). Next, to assess the possibility that TGF-β could influence metabolic tumor cell activity in conditioned media from osteoblasts, neutralizing TGF-β antibody, 2G7, was used to treat cell culture media harvested from wild type and MMP-2

deficient primary osteoblasts. Blocking of TGF-β in conditioned media of wild type osteoblasts significantly reduced tumor metabolic activity in an MTT assay (Figure 20E and F). These data indicate that osteoblast-derived MMP-2 can control the levels of active TGF-β in the tumor-bone microenvironment and thus influence tumor growth.

Osteoblast-derived MMP-2 contributes to the levels of active TGF-β in the bone microenvironment in vivo

Next, we evaluated the levels of latent TGF-β *in vivo* by immunoblotting tissue lysates derived from the tumor bearing limbs of wild type and MMP-2 deficient mice 3 days post-injection for the LAP-TGF-β complex. Samples of MMP-2 deficient tibias injected with tumor showed a significantly higher level of inactive LAP-TGF-β compared to the controls (Figure 21A). To investigate whether higher levels of TGF-β impacted the tumor-bone microenvironment, the status of TGF-β signaling was assessed by immunoblotting for phospho Smad2, the main intracellular effector of TGF-β receptor II/TGF-β signaling pathway [168]. Lysates of tumor bearing tibias from MMP-2 deficient mice displayed a significant lower ratio of phospho smad2 over total smad2 compared to wild type controls (Figure 21B). Taken together, these data show for the first time that an osteoblast-derived MMP, MMP-2, contributes to tumor survival in the tumor-bone microenvironment by controlling the activation of TGF-β.

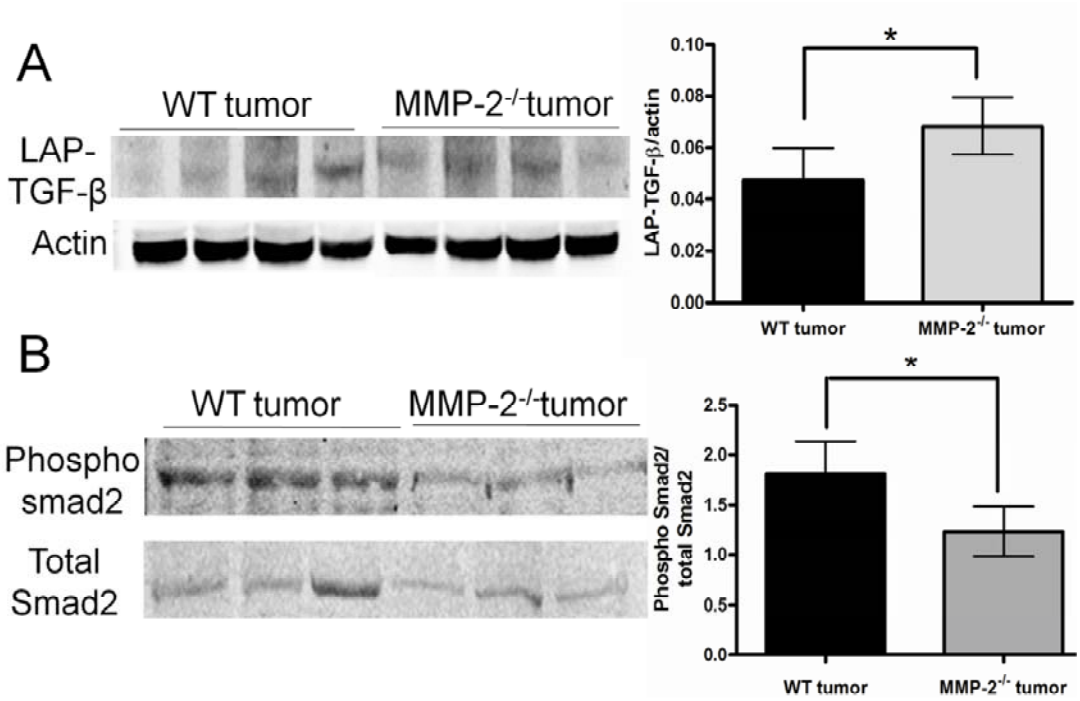


Figure 21. Osteoblast-derived MMP-2 mediates the release of active TGF-β in the tumor-bone microenvironment. A: Immunoblotting for LAP-TGF-β and actin in tumor bearing tibias of wild type (n=4) and MMP-2 deficient mice (n=4) at 3 days after surgery. Densitometry was performed on the levels of LAP-TGF-β and actin and results were expressed as ratio of LAP-TGF-β signal over actin levels for each samples. **B:** Immunoblotting for phospho smad2 and total smad2 in tumor injected limbs of wild type (n=3) and MMP-2 deficient mice (n=3). Densitometry was performed on the levels of phospho smad2 and total smad2 and results were expressed as ratio of phospho smad2 signal over total smad2 levels for each samples. Data are mean ± SD. Asterisk denotes that $p<0.05$. Each experiment has been conducted twice on different samples from independent *in vivo* studies.

Conclusions

Despite the improvement in diagnosis and treatment, human breast-to-bone metastases remain incurable. Therefore, a better understanding of the mechanisms underlying how tumor cells induce the vicious cycle remains a priority in order to develop new therapies that will treat/cure the disease. This study demonstrated for the first time that osteoblast-derived MMP-2 contributes to the survival of two mammary tumor cell lines (PyMT-Luc and 17L3C-Luc) in the bone microenvironment and contributes to tumor-induced osteolysis in immunocompetent animals. Furthermore, our results showed that osteoblast-derived MMP-2 promotes tumor survival but not tumor proliferation via a TGF-β dependent mechanism that potentially induces the processing of a novel MMP-2 substrate, LTBP-3 (Figure 22). Our studies suggest that therapies geared towards selectively inhibiting MMP-2 may be of clinical use for the treatment of breast-to-bone metastasis.

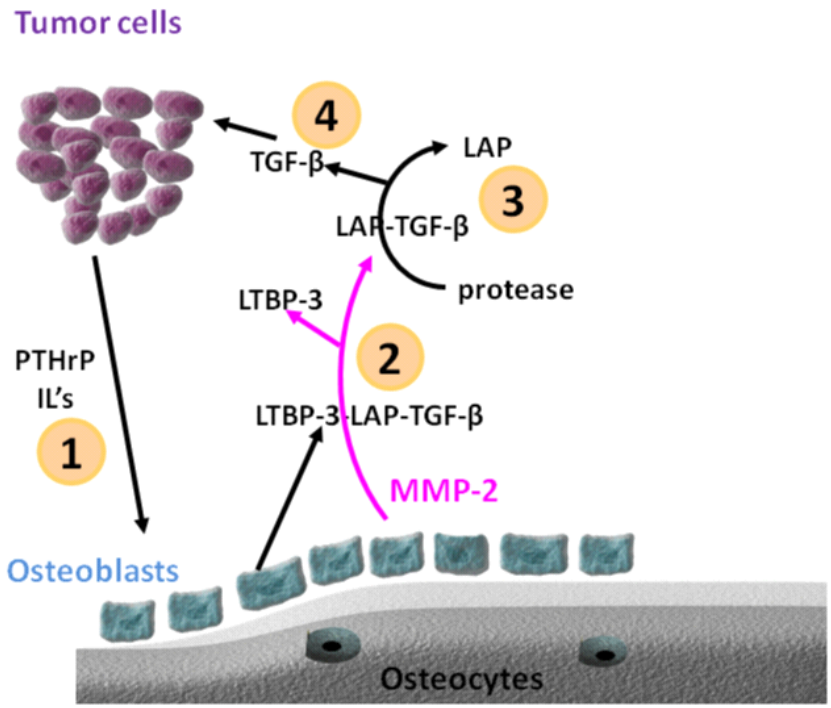


Figure 22. Hypothetical mechanism of osteoblast derived MMP-2 actions in the mammary tumor-bone microenvironment. (1) Metastatic tumor cells stimulate osteoblast expression of factors such as LTBP-3-LAP-TGF-β. **(2)** Osteoblast-derived MMP-2 induce the release of latent TGF-β via the processing of LTBP-3. **(3)** Other proteases, including MMP-2 can then process latent TGF-β to release the active growth factor which in turn will promote tumor survival in the bone microenvironment.

CHAPTER V

DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

Breast to bone metastasis is an incurable disease which affects more than 70 % of patients presenting with advanced breast cancer [5]. Lytic bone lesions cause severe complications that greatly affect the quality of life of the patients [6]. Currently, no cure can be offered to patients suffering with bone metastasis, only palliative treatments such as bisphosphonates, surgery, radiotherapy and chemotherapy are available. Therefore, finding new molecular mechanisms underlying cell-cell communication in the tumor-bone microenvironment is key for the development of better therapies. Although human clinical trials using broad spectrum MMPIs were disappointing in the treatment of cancer, numerous pre-clinical studies demonstrated the potential efficacy of inhibiting MMPs in the context of bone metastasis [135, 137-139, 141]. As a result, bone metastasis appears to be a relevant target for the use of MMPIs; however, the specific role of individual MMPs in the lytic tumor-bone microenvironment must be elucidated so that highly selective MMPIs that lack the side effects noted with broad spectrum inhibitors can be generated [140].

The aims of this dissertation were to understand the contributions of host-derived MMPs to mammary tumor growth and mammary tumor-induced osteolysis in the bone microenvironment. Immunostaining for different MMPs in human breast-to-bone metastasis samples showed MMP-7 and MMP-9 expression primarily within the mature

osteoclasts present at the tumor-bone interface. However, MMP-2 staining was detected in the tumor and host cells such as osteoblasts and osteocytes. Since these three host cell types (osteoclasts, osteoblasts and osteocytes) are the main effectors of the bone remodeling process, we tested the effect of host MMP-2, -7 and -9 ablation in a mouse model of mammary tumor growth and tumor-induced osteolysis using intratibial injection in MMP deficient animals. Our studies established that osteoclast-derived MMP-7 and osteoblast-derived MMP-2 contribute to the vicious cycle through two distinct molecular mechanisms. We demonstrated that osteoclast-derived MMP-7 enhances bone resorption via the solubilization of RANKL and that osteoblast-derived MMP-2 promotes tumor survival in the bone by processing TGF-β latency proteins such as LTBP-3, thereby controlling the levels of active TGF-β. Interestingly, osteoclast-derived MMP-9 does not impact tumor growth and bone resorption in our mouse model. These data demonstrated that although different host MMPs are expressed by mature osteoclasts, they do not all impact bone resorption in a tumor setting. Additionally, our studies showed that MMPs expressed by distinct cell types can contribute differently to tumor growth and bone resorption by multiple molecular mechanisms.

Discussion

Osteoclast-derived MMP-7 contributes to tumor growth-induced bone lesions via the solubilization of RANKL

The results presented in this dissertation show an osteoclast derived protease, MMP-7, can promote osteoclast activation in the tumor-bone microenvironment by generating an active soluble form of the osteoclastogenic factor, RANKL and suggest that selective inhibition of MMP-7 may be of benefit for the treatment of lytic metastases. Our study demonstrate that MMP-7 deficient mice bearing tumor have significantly lower levels of soluble RANKL and statistically reduced bone resorption, therefore suggesting that MMP-7 solubilization of RANKL mediates tumor-induced osteolysis (Figures 11, 12 and 13). However, in tumor bearing MMP-7 deficient mice bone resorption, although significantly attenuated, still occurs, thus MMP-7 may contribute to tumor growth-induced bone lesions via other mechanisms. For example, MMP-7 has been shown to process other members of the TNF family beside RANKL such as Fas ligand (FasL) and TNF-α [72, 169]. Fas/FasL are apoptotic factors and the solublization of FasL by MMP-7 may directly impact tumor survival [148]. Furthermore, Kovacić and coworkers demonstrated the importance of Fas/Fas ligand in osteoblast differentiation by inhibiting progenitor differentiation and therefore, MMP-7 could indirectly affect osteoclastogenesis by impairing osteoblast functions through the processing of FasL [170]. TNF-α has been demonstrated to be important for the final step of osteoclastogenesis i.e. the fusion of tartrate-resistance acid phosphatase-positive mononuclear osteoclasts [171].

Release of soluble active TNF-α by MMP-7 may directly promote activation of mature osteoclasts and lead to osteolysis. Therefore, through the processing of different members of the TNF family such as TNF-α, FasL and RANKL, MMP-7 can impact osteoblast and osteoclast biology and ultimately bone resorption.

The direct processing of the bone matrix by MMP-7 may be a possibility. Acidification and cathepsin-K secretion into osteoclast resorption lacunae allows for the demineralization and collagenolysis of the bone matrix respectively [77]. Therefore, the direct resorption of the bone matrix by MMP-7, functional at a more neutral pH, is highly unlikely. However, by a process known as transcytosis, the osteoclast mediates the removal of bone products from the area of bone undergoing resorption [172]. Given the punctuate localization of MMP-7 by immunofluorescent staining it is tempting to speculate that MMP-7 contributes to the further processing of bone matrix components such as osteopontin [66], or the release of growth factors from bone matrix components such as TGF-β and IGFs, within these transcytotic vesicles [57 173]

Since MMP-7 deficient mice used in this study lack the metalloproteinase in the entire stromal compartment, the expression of MMP-7 by other cellular sources may also be a possibility. However, in the tumor-bone microenvironment, we observed that MMP-7 expression was largely confined to osteoclasts (Figures 5 and 8). Nevertheless MMP-7 has also been shown to be expressed by macrophages and given the role of macrophages in tumor induced osteolysis, the contribution of macrophage derived MMP-7 in our model or in humans cannot be discounted [174, 175].

Given the role of MMP-7 in osteoclast function in the pathological setting of tumor induced osteolysis, it is surprising that MMP-7 null animals appear to have a normal skeletal phenotype. Data presented here using μCT scan analysis demonstrate a similar BV/TV ratio between MMP-7 null and wild type control mice at 6 weeks of age (Figure 8). While a role for MMP-7 in bone development has not been explored, a number of reports have revealed that the phenotype of the MMP-7 null animals is often only apparent in response to injury/challenges or disease. For example, in non-pathological conditions such as herniated disc resorption, macrophage derived MMP-7 is critical for the resorption of the herniated disc [72]. In mammary and prostate involution, MMP-7 processing of FasL is important for initiating apoptosis [72, 148, 176]. More often, phenotypes in the MMP-7 null animals have been observed in pathological conditions such as pancreatitis, colon tumorigenesis, mammary gland tumorigenesis and in innate defense wherein MMP-7 null animals show significant delays in disease progression or in response to infection [177-180]. Therefore, although MMP-7 null mice lack an apparent skeletal phenotype, in the context of tumor-bone microenvironment, it is clear based on the results of our study that host MMP-7 plays an important role in the mammary tumor-bone microenvironment. In addition, our observations defining a role for MMP-7 in bone diseases are consistent with previous reports that implicate roles for host MMP-7 in prostate cancer induced osteolysis, osteoarthritis and cartilage/periarticular bone destruction [71, 116, 181].

Finally, our data demonstrate that osteoclast-derived MMP-7 affects mammary tumor growth in the bone by enhancing tumor survival (Figure 9). Our results show that in the MMP-7 deficient animals, a failure to generate active soluble RANKL leads to a

decrease in the number of mature bone resorbing osteoclasts that in turn leads to a decrease in tumor growth by depriving the tumor cells of survival factors such as TGF-β, or IGF. In this regard, the levels of active TGF-β in tissue lysates from wild type or MMP-7 deficient mice bearing tumor were assessed by ELISA and no significant difference were observed between the two groups of animals (Figure 23). Therefore, host MMP-7 impacts tumor survival by an as yet unidentified factor(s) such as IGFs.

Osteoclast-derived MMP-9 does not contribute to mammary tumor growth-induced bone resorption

Although MMP-9 was localized to human and murine osteoclasts, the ablation of host MMP-9 did not appear to impact PyMT-Luc tumor growth and bone resorption compared to the wild-type controls. Analogous results were obtained by Nabha et al., using the same intratibial model but in the context of prostate cancer progression in the bone [147]. Given the importance of MMP-9 in osteoclast migration and recruitment in developing long bones, these results were surprising [75]. It appears that in the tumor-bone microenvironment, MMP-9 is not critical for osteoclast function. The possibility that tumor-derived MMP-9 could overcome the absence of host MMP-9 exists in our model, however, in vivo studies conducted in the Matrisian laboratory have demonstrated that MMP-9 expression by the PyMT-Luc tumor cells is not detectable [146]. Therefore, the ability of tumor derived MMP-9 to circumvent the loss of host derived MMP-9 and impact tumor progression in the bone is unlikely. In comparison, the dramatic effect on tumor growth and bone resorption observed in MMP-2 deficient mice upon tumor

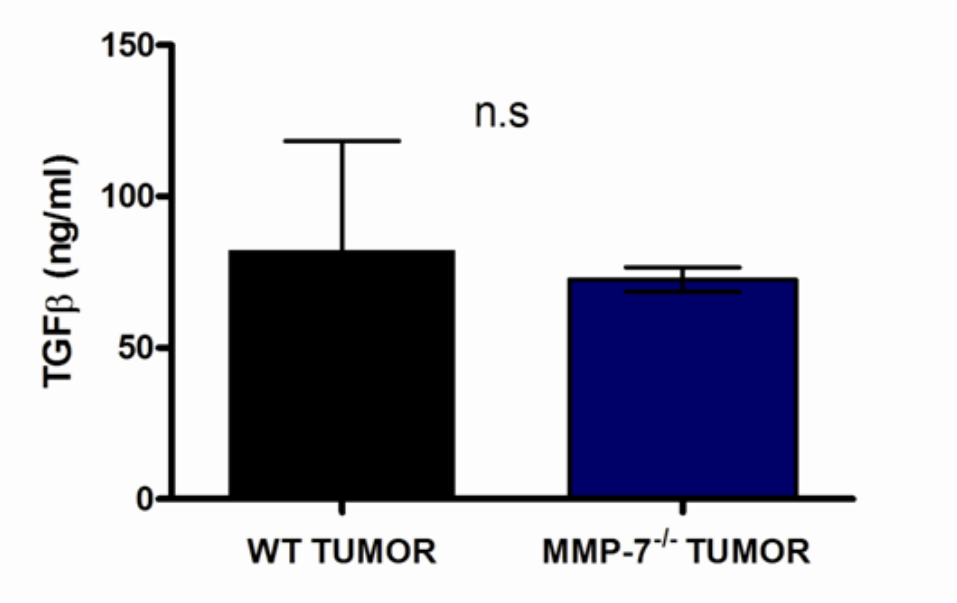


Figure 23. Host-derived MMP-7 does not affect active TGF-β levels in the tumor-bone microenvironment. ELISA analysis of active TGF-β levels in lysates from tumor or sham injected tibias obtained from WT (n=5) or MMP-7$^{-/-}$ (n=5) animals.

injection was surprising since MMP-2 and MMP-9 are closely related in structure. Our studies in the mammary tumor-bone microenvironment suggest that despite their similarities in terms of structure and substrates, MMP-2 and MMP-9 are 1) expressed by different cell types and 2) have very distinct functions with respect to the progression of the osteolytic vicious cycle. In addition, these data reinforce the importance of assessing the contributions of individual MMPs to tumor progression in the bone in a bid to design selective MMPIs that lack the deleterious side effects noticed with broad spectrum inhibitors during human clinical trials.

While our data points towards MMP-9 as not being critical for mammary tumor growth or induced osteolysis, it is important to note that MMP-9 could contribute to other steps of metastasis that are not taken into account with the intratibial model. These include extravasation from the sinusoidal vasculature in the bone and initial survival, the latter of which has been shown to be an important role for host derived MMP-9 in early lung metastasis [182]. Furthermore, MMP-9 has been implicated in tumor angiogenesis by mediating the release of matrix sequestered vascular endothelial growth factor (VEGF) [76]. In the context of the prostate tumor-bone microenvironment, Nabha and colleagues demonstrated a decrease in angiogenesis in MMP-9 null animals compared to wild type controls [147]. Therefore, the selective inhibition of MMP-9 may still prove useful in preventing the establishment and angiogenesis of bone metastases. Studies have demonstrated the importance of MMP-9 in the formation of 'pre-metastatic niches' in a lung and skin metastasis murine models [76, 183-185]. Hiratsuka and coworkers showed that MMP-9 expression by endothelial cells and macrophages was induced in the lungs by distant tumors prior to metastasis [185]. Given the richness in immune cells and the highly

vascularized state of bone, it would be interesting to investigate the potential contribution of host MMP-9 to the establishment of bone metastastic niches in breast cancer progression.

Osteoblast-derived MMP-2 impacts mammary tumor growth-induced bone lesions

Our results demonstrate that osteoblast-derived MMP-2 affects mammary tumor survival in the bone. It is of interest to note that mammary tumor virus (MMTV) long terminal repeat-polyoma middle-T antigen (MMTV-PyMT) mice deficient for MMP-2 do not show any difference in terms of mammary tumor incidence, onset, growth rate and tumor volume compared to wild type control mice, demonstrating that the effect of host MMP-2 are specific to the bone microenvironment (Fingleton and Matrisian, personal communication).

Our study suggests that osteoblast-derived MMP-2 can modulate the release of TGF-β via the processing of LTBP-3 (Figure 20). TGF-β is sequestered in a latency complex comprised of LTBP-1-4 and LAP and that these complexes must be sequentially processed in order to generate active TGF-β [120-124]. The binding of LAP-TGF-β to LTBP is thought to be important for the binding of latent TGF-β to the ECM. The processing of LTBP releases LAP-TGF-β from the ECM which can be activated by further proteolytic processing [186, 187]. Other members of the LTBP family such as LTBP-1 have been shown to be substrates for MMPs, in particular MMP-2 [124, 188, 189]. In addition, a recent study using a multiplex proteomics approach, identified LTBP-4 as a potential novel substrate for MMP-2 [190]. However, of the four members of the LTBP family, only LTBP-3

deficient mice are reported as having skeletal defects including osteoarthritis and osteopetrosis [164, 165]. MMP-2 has also been shown to process LAP-TGF-β leading to the release of the active form of the growth factor [188]. Our study identifies for the first time that osteoblast-derived MMP-2 is responsible for the cleavage of LTBP-3 and we posit that based on other studies that MMP-2 subsequently cleaves LAP-TGF-β to release active TGF-β [122]. This conclusion is supported by our observations (Figures 20 and 21). While we suggest that MMP-2 is critical for TGF-β activation, the role of other proteases that can process TGF-β latency complexes such as plasmin and MMP-9 may also contribute and explain why residual levels of active TGF-β could be identified in the conditioned media of MMP-2 deficient osteoblasts (Figures 20 and 21) [122, 191].

Our results suggest that MMP-2-mediated activation of TGF-β is the primary mechanism responsible for tumor survival in the bone (Figure 20). However, MMP-2 could impact tumor survival through the processing of other bone factors [98]. IGFs are sequestered in a latent complex through their interactions with IGF binding proteins (IGFBP-1 to -4) [125] and several MMPs, including MMP-2 have been shown to process different members of the IGFBP family resulting in the activation of IGFs [131, 192, 193]. However, we determined active IGF signaling levels in tissue lysates of tumor bearing limbs from wild type and MMP-2 deficient mice by immunoblotting for phospho IGF-receptor 1 (IGF-1R). No difference between MMP-2 deficient and wild type control animals was observed in terms of the phosphorylation levels of IGF-1R (data not shown). These data further support our conclusion that MMP-2 activation of TGF-β is a major mechanism mediating tumor survival in the tumor-bone microenvironment. However,

we acknowledge that other reported substrates of MMP-2 may also contribute to tumor survival (see table 2 in chapter I).

A potential molecular mechanism through which TGF-β can mediate its anti-apoptotic effect is the activation of phosphatidylinositol-3 kinase (PI3K)/Akt pathway. By regulating Smad3 activation, Akt has been shown to protect liver cancer cells from apoptotsis [194, 195]. Furthermore, Muraoka-Cook and colleagues demonstrated that TGF-β can signal to PI3K/Akt signaling pathway to enhance mammary tumor survival [196]. In addition, TGF-β has been shown to induce NF-κB activity and promote cell survival. Treatment of activated hepatic stellate cells with TGF-β induced the activation of NF-κB and suppressed apoptosis [197]. Therefore activation of PI3K/Akt or NF-κB pathways in mammary tumor cells by osteoblast-derived TGF-β could potentially mediate tumor survival in our mouse model.

The majority of the studies examining the osteolytic vicious cycle conducted so far have focused almost exclusively on the 'forward' communication i.e. tumor cell control of osteoblast behavior that in turn impacts osteoclastic bone resorption (Figure 24). For the first time, our study demonstrates the *in vivo* 'reverse' communication i.e. osteoblast control of tumor cells behavior in the vicious cycle. For instance, in the 'forward setting', tumor-derived factors such as parathyroid hormone-related peptide (PTHrP) have been extensively studied as its expression by metastatic breast tumor cells induces osteoclastic bone resorption via osteoblast-dependent activation of osteoclast precursors [95, 112, 198]. In addition, osteoclast-mediated bone resorption releases TGF-β that in turn affects PTHrP expression by tumor cells [87, 112, 199]. However, the 'reverse setting'

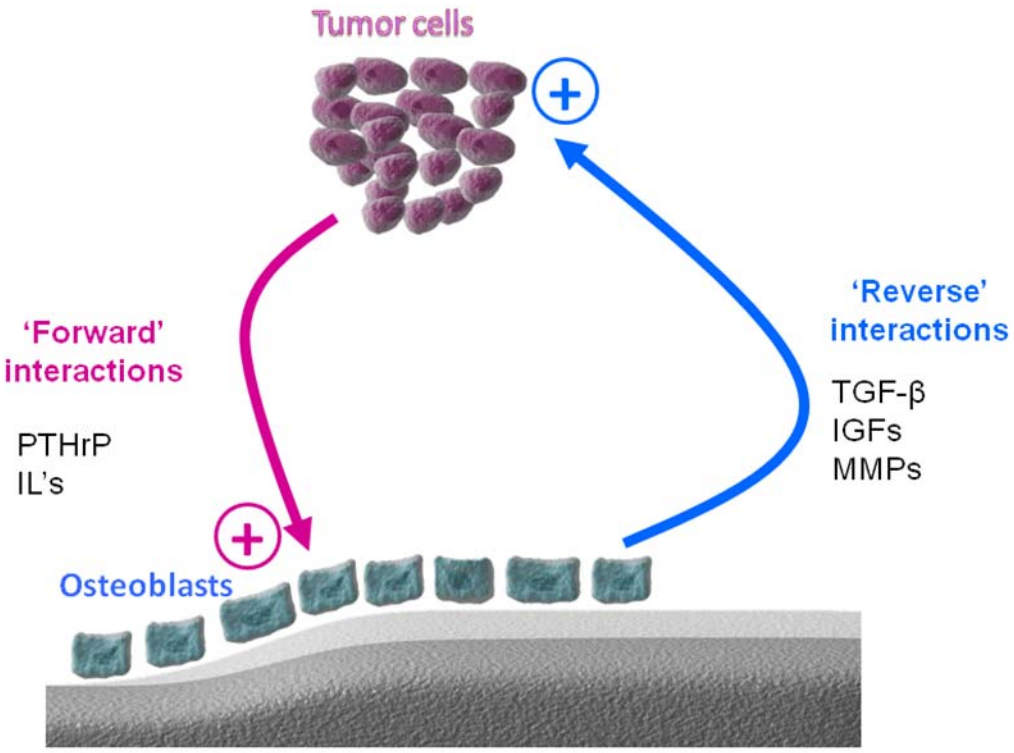


Figure 23. Interactions between tumor cells and osteoblast in the 'vicious cycle'. During tumor growth in the bone, tumor cells secrete factors such as PTHrP and ILs that stimulate RANKL expression by osteoblasts (Forward interactions) which promote osteoclast maturation and subsequent osteolysis. In turn, osteoblasts can influence tumor cell behaviors by secreting molecules such as TGF-β, IGFs and MMPs (Reverse interactions) leading to increased tumor growth in the bone.

i.e. the direct modulation of the tumor cell behavior by osteoblasts and the molecular mechanisms therein, have thus far not been assessed. Our results are the first to demonstrate the importance of osteoblast-derived signals in controlling tumor survival (Figures 17 and 20).

Besides its effects on tumor growth, TGF-β has been shown to modulate both osteoblast and osteoclast functions [200-204]. Therefore, MMP-2-mediated release of active TGF-β may also directly affect osteoblast-osteoclast interactions. Our studies revealed a decrease in tumor-induced osteolysis (Figure 18). While our observations ruled out a direct role for MMP-2 in osteoclast maturation and activation, we speculate that osteoblast-derived MMP-2 impacts osteoclastogenesis via the processing of LTBP-3 and activation of TGF-β (Figures 19 and 20). TGF-β has been shown be sufficient to induce osteoclastogenesis but also to support and/or increase osteoclast activation [205 206, 207]. Therefore, it is possible that the release of TGF-β by osteoblast-derived MMP-2 induces an increase in osteoclastogenesis in addition to its effects on tumor survival. Furthermore, a recent study demonstrated that treatment of mature mice with TGF-β type I receptor kinase inhibitor, SD-208, induces increase bone mineral density, bone mass and mineral concentrations indicating an effect on osteoblast functions [208]. Mice treated with SD-208 had significantly higher number of osteoblasts and significantly reduced number of osteoclasts [208]. Although, we observed a significant decreased in trabecular bone volume in tumor bearing limbs, we acknowledge that an autocrine effect of osteoblast-derived TGF-β could occur in our mouse model.

Our study demonstrates that MMP-2 secreted by osteoblasts impacts mammary tumor growth and mammary tumor-induced osteolysis. However, since MMP-2 is entirely eliminated in the MMP-2 deficient animals, we cannot rule out that MMP-2 derived from other cell types is of importance in mediating tumor survival and bone resorption. For example, immune cells have been shown to express MMPs, including MMP-2 and recent findings demonstrated that T cells (a rich source of MMP-2) modulate tumor-induced bone resorption [209, 210]. Thus, a contribution of MMP-2 secreted by immune cells such as T cells that are present in our syngeneic immunocompetent mice cannot be disregarded. Inoue and coworkers reported the importance of MMP-2 in maintaining proper osteocytic canicular network caused by a defect in osteocyte functions and increased apoptosis [68]. Osteocytes are important in controlling bone mass through the modulation of osteoblast/osteoclast function via the secretion of factors such as sclerostin [15, 16]. As a consequence, we acknowledge that reduced osteolysis in a tumor setting could be caused by osteocyte function to regulate bone remodeling is a possibility in our model but that has not been explored thus far.

Although, MMP-2 does not directly affect osteoclast maturation and activation, its contribution to direct bone resorption may exist. MMP-2 has collagenase activity and hence osteoblast-derived MMP-2 could directly contribute to bone resorption after the exit of the osteoclasts [61]. This degradation of the bone can generate ICTP fragments of type I collagen that in turn can promote further osteoclast precursor cell recruitment [60, 62-64]. While host MMP-2 can potentially work through these suggested mechanisms in our model, our data clearly identifies a role for osteoblast-derived MMP-2 given our results

with treatment of tumor cells with conditioned media derived from osteoblasts *in vitro* and our observation *in vivo* (Figures 20 and 21).

Is there a place for selective MMP-2 and MMP-7 inhibitors in treatment of breast-to-bone metastases?

Breast-to-bone metastases are incurable and with the exception of surgery and radiation, bisphosphonates are the most common approach to treat patients. Despite the success of bisphosphonates, their use is often palliative [211]. Bisphosphonates are classified as non-nitrogen-containing bisphosphonates or nitrogen-containing bisphosphonates (NBPs), according to their chemical structure [212]. NBPs such as pamidronate, ibandronate, risedronate and zoledronic acid have a better anti-resorptive effect than the non-nitrogen-containing bisphosphonates. NBPs prevent bone resorption through the inhibition of osteoclast activity and the induction of osteoclast apoptosis [213, 214]. Despite their proven efficacy in treating breast cancer-associated bone lesions, bisphosphonates are not an ideal long-term therapy since they present minimal effect on survival and they have minimal oral bioavailability and a long half-life which increases the risk to develop osteonecrosis of the jaw [215, 216]. Therefore, there is still a growing interest in developing new therapeutic agents targeting key components of the 'vicious cycle'.

Pre-clinical studies in animal models of tumor bone metastasis have demonstrated that the use of MMPIs remains a relevant approach to treat lytic bone lesions [137-139]. Treatment of tumor bearing animals with broad spectrum MMPIs such as BB-94 have been shown to prevent bone resorption induced by prostate and breast tumor cells [137, 138].

Weber and co-workers showed that the use of Neovastat, a reagent with reported metalloproteinase inhibition ability, can prevent the development of lytic lesions during tumor growth in the bone [217]. Furthermore, the combined effects of a bisphosphonate (ibandronate) and TIMP-2 in a mouse model of mammary tumor-induced osteolysis showed a decrease in bone resorption and an increased survival rate [218]. Taken together, these data demonstrate the potential efficacy of using MMP inhibitors in treating bone lesions during cancer progression. However, a better understanding of the specific roles of individual MMPs to the bone metastasis process is required to avoid musculoskeletal side effects observed during the first clinical trials with broad spectrum inhibitors [135]. In this regard, a recent study reported that SB-3CT, an MMP-2 and MMP-9 selective inhibitor was effective in controlling prostate tumor growth and osteolysis [141]. However, prinomastat, a selective MMP-2 and MMP-9 inhibitor, has been shown to cause musculoskeletal side effects [219]. Based on our results, a generation of reagents with further specificity against MMP-2 but not MMP-9 may provide efficacy without side effects. Our studies also suggest that the selective inhibition of MMP-7 would be effective in halting the vicious cycle. Regardless of the efficacy of the MMP inhibitors, identifying the molecular mechanisms through which individual MMPs mediate their effect can also result in the generation of new therapies, for example LTBP-3 and RANKL that ultimately will be of benefit to patients with lytic bone metastases.

Future directions

Our studies show mature osteoclasts as a rich source of MMP-7. However, to rule out any effects of MMP-7 on mammary tumor growth and bone resorption from other cellular sources, the generation of a mouse specifically deficient for MMP-7 in osteoclasts would be important. Therefore, a specific osteoclast MMP-7 deficient mouse using a Cre/LoxP flanked MMP-7 construct approach could be engineered. These specific MMP-7 deficient mice will allow us to investigate the precise contribution of osteoclast-derived MMP-7 in tumor growth and tumor-induced osteolysis.

We demonstrate in this dissertation that osteoclast-derived MMP-7 affects tumor-induced osteolysis via the solublization of RANKL. The use of a specific transgenic mouse expressing membrane-bound RANKL resistant to proteolytic processing would allow for the assessment of the importance of soluble RANKL versus full length RANKL to mammary tumor growth-induced osteolysis. Since the sequence of the RANKL cleavage site by MMP-7 has previously been reported, using site directed mutagenesis, an MMP-7-resistant RANKL could be engineered [116]. First, an MMP-7-resistant RANKL osteoblast cell line could be established and *in vitro* the functionality of this osteoblastic cell line in terms of ability to induce osteoclast activation could be assessed. Finally, using a systemic knock in MMP-7-reistant RANKL mouse would allow for the assessment of the effect of this protein on the development of the skeleton and in the context of mammary tumor growth in the bone.

Our study showed that osteoclast-derived MMP-9 does not impact tumor growth in the bone. Therefore, the use of intracardiac injection as a model of breast to bone

metastasis would allow us to assess the potential contributions of host-derived MMP-9 in early steps to metastasis. Since MMP-9 has been shown to be important for the establishment of a 'pre-metastatic niche' in experimental lung and skin cancer metastasis models, it would be of interest to investigate the potential contribution of host MMP-9 in the formation of a 'bone metastatic niche' [76, 183-185]. Orthotopic injection of mammary tumor cells and examination of the bone for 'pre-metastatic' markers such as VEGFR1 or fibronectin would assess this interesting question.

Our work in MMP-2 deficient mice demonstrates that MMP-2 secreted by osteoblasts impacts tumor survival in the bone. However, we cannot rule out that MMP-2 derived from other sources is of importance, therefore future studies could directly assess the importance of osteoblast-derived MMP-2 without disturbing other host cell type functions. Using inducible MMP-2 deficient animals such as an MMP-2/Tet transgenic mice where the Tet transactivator is under the control of a collagen type I α1 promoter to allow for specific osteoblast expression. Furthermore, in this dissertation, we posit that MMP-2 controls the release of active TGF-β via LTBP-3 processing. Future studies could identify the cleavage site for MMP-2 on LTBP-3 to generate an MMP-2 resistance LTBP-3 by site directed mutagenesis and establish an MMP-2 resistance LTBP-3 osteoblast cell line. First, the *in vitro* functionality of the MMP-2-resistant LTBP-3 osteoblast cell line in terms of proliferation, differentiation and induction of osteoclast activation and expression levels could be assessed. The importance of LTBP-3 cleavage by MMP-2 to mammary tumor survival in the bone *in vivo* could be analyzed using a knock in MMP-2 resistant mouse.

Finally, with respect to the importance of osteoblast-derived MMP-2 in tumor growth in the bone, it would be interesting to investigate the effect of host MMP-2 ablation in a model of prostate to bone metastasis. Once in the bone, prostate tumor cells induce mixed lesions containing excess of osteoblastic and osteolytic response [220]. To this end, a model of murine prostate cancer in the bone developed by Lynch and colleagues where prostate adenocarcinoma samples are transplanted into the cranial region of immunocompromised MMP-2 deficient mice (RAG-2 deficient mice) would permit the assessment of MMP-2 function in a tumor-induced osteoblastic setting [116].

Conclusions

The results of my thesis demonstrated:

- Mature osteoclasts at the tumor-bone interface are a rich source of MMP-7 and MMP-9.
- Osteoclast-derived MMP-7 impacts mammary tumor growth and tumor induced osteolysis. (First time observation)
    - Molecular mechanism: solublization of membrane-bound RANKL by MMP-7 that mediates maturation/activation of osteoclast precursor cells.
- Osteoclast-derived MMP-9 does not contribute to either mammary tumor growth or bone resorption.
- Osteoblasts are a rich source of MMP-2.

- Osteoblast-derived MMP-2 impacts mammary tumor growth by enhancing tumor survival. (First report to show this)
- Host-MMP-2 impacts mammary tumor-induced osteolysis. (First report)
- MMP-2 can modulate the release of active TGF-β via the processing of its latency protein, LTBP-3. (First report)
- Osteoblast-derived MMP-2 and osteoblast-derived TGF-β mediate mammary tumor survival. (First report)

In conclusion, this dissertation demonstrates that osteoblast-derived MMP-2 controls the levels of active TGF-β via the processing of LTBP-3 and thereby mediates tumor survival in the tumor-bone microenvironment. In addition, our results showed that osteoclast-derived MMP-7 but not MMP-9 mediates mammary tumor-induced osteolysis through RANKL solublization (Figure 25). Therefore, novel therapeutic agents focusing on major components of the 'vicious cycle' will improve the current treatment options offered to breast cancer patients with lytic bone metastases. Development of specific MMP-2 and MMP-7 inhibitors or therapies that target their substrates would benefit patients whose response to bisphosphonates and other traditional treatment strategies are unsatisfactory. Alternatively, these new therapeutic agents may also be useful as adjuvants in combination with bisphosphonates, denosumab (specific RANKL antibody), hormonal therapies or other treatments offered, to hopefully lead to the eradication of breast-to-bone metastasis.

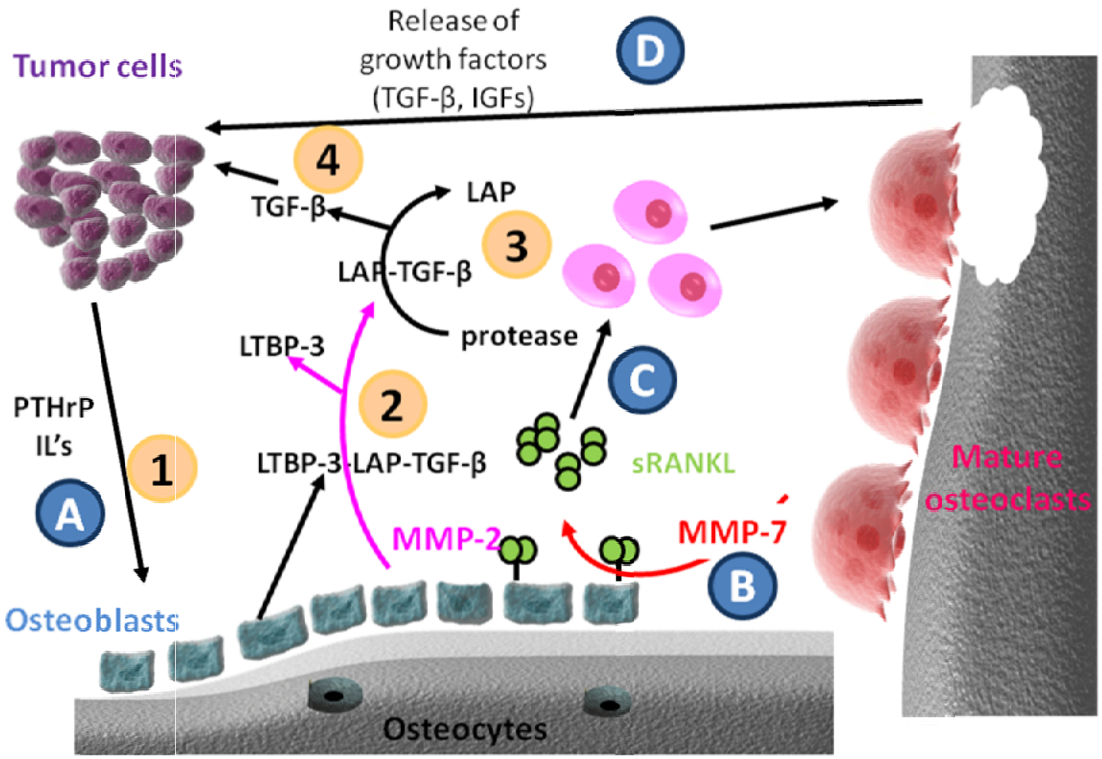


Figure 25. Hypothetical mechanisms of osteoclast derived MMP-7 and osteoblast derived MMP-2 actions in the mammary tumor-bone microenvironment. A: Metastatic tumor cells through the secretion of factors such as PTHrP, stimulate osteoblasts to express full length membrane bound RANKL. B: Osteoclasts express MMP-7 which can process membrane bound RANKL to a soluble active form. C: Soluble RANKL has been shown to be chemotactic for osteoclast precursors (25). In addition to acting as a potential chemotactic molecule, soluble RANKL stimulates can stimulate the maturation and activation of osteoclast precursors. D: Activated osteoclasts in turn execute bone resorption leading to the release of growth factors such as TGF-β and IGFs that promote tumor growth in the bone microenvironment. **(1)** Tumor cells stimulate osteoblast expression of factors such as LTBP-3-LAP-TGF-β. **(2)** Osteoblast-derived MMP-2 induce the release of latent TGF-β via the processing of LTBP-3. **(3)** Other proteases, including MMP-2 can then process latent TGF-β to release the active growth factor which in turn will promote tumor survival in the bone microenvironment.

REFERENCES

1. American Cancer S: Cancer Facts and Figures 2008 www.acs.org 2008,

2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ: Cancer statistics, 2008, CA Cancer J Clin 2008, 58:71-96

3. Chambers AF, Groom AC, MacDonald IC: Dissemination and growth of cancer cells in metastatic sites, Nature Reviews Cancer 2002, 2:563-572

4. Mundy GR: Metastasis to bone: causes, consequences and therapeutic opportunities, Nature Reviews Cancer 2002, 2:584-593

5. Coleman RE, Rubens RD: The clinical course of bone metastases from breast cancer, British Journal of Cancer 1987, 55:61-66

6. Mundy GR: Mechanisms of bone metastasis, Cancer 1997, 80:1546-1556

7. Paget S: The distribution of secondary growths in cancer of the breast, Lancet 1889, 1:571-573

8. Hadjidakis DJ, Androulakis, II: Bone remodeling, Ann N Y Acad Sci 2006, 1092:385-396

9. Robey PG: Vertebrate mineralized matrix proteins: structure and function, Connect Tissue Res 1996, 35:131-136

10. Sims NA, Gooi JH: Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption, Semin Cell Dev Biol 2008, 19:444-451

11. Canalis E, McCarthy TL, Centrella M: Growth factors and cytokines in bone cell metabolism, Annu Rev Med 1991, 42:17-24

12. Lanyon LE: Osteocytes, strain detection, bone modeling and remodeling, Calcif Tissue Int 1993, 53 Suppl 1:S102-106; discussion S106-107

13. Bonewald LF: Osteocytes as dynamic multifunctional cells, Ann N Y Acad Sci 2007, 1116:281-290

14. Marotti G: The osteocyte as a wiring transmission system, J Musculoskelet Neuronal Interact 2000, 1:133-136

15. Verborgt O, Gibson GJ, Schaffler MB: Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo, J Bone Miner Res 2000, 15:60-67

16. van Bezooijen RL, Roelen BA, Visser A, van der Wee-Pals L, de Wilt E, Karperien M, Hamersma H, Papapoulos SE, ten Dijke P, Lowik CW: Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist, J Exp Med 2004, 199:805-814

17. Teitelbaum SL: Osteoclasts; culprits in inflammatory osteolysis, Arthritis Res Ther 2006, 8:201

18. Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Koga T, Martin TJ, Suda T: Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells, Proc Natl Acad Sci U S A 1990, 87:7260-7264

19. Teitelbaum SL, Ross FP: Genetic regulation of osteoclast development and function, Nat Rev Genet 2003, 4:638-649

20. Allan EH, Hausler KD, Wei T, Gooi JH, Quinn JM, Crimeen-Irwin B, Pompolo S, Sims NA, Gillespie MT, Onyia JE, Martin TJ: EphrinB2 Regulation by Parathyroid Hormone (PTH) and PTHrP Revealed by Molecular Profiling in Differentiating Osteoblasts, J Bone Miner Res 2008,

21. Allan EH, Hausler KD, Wei T, Gooi JH, Quinn JM, Crimeen-Irwin B, Pompolo S, Sims NA, Gillespie MT, Onyia JE, Martin TJ: EphrinB2 regulation by PTH and PTHrP revealed by molecular profiling in differentiating osteoblasts, J Bone Miner Res 2008, 23:1170-1181

22. Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, Suda T, Matsuo K: Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis, Cell Metab 2006, 4:111-121

23. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation, Cell 1998, 93:165-176

24. Aubin JE, Bonnelye E: Osteoprotegerin and its ligand: A new paradigm for regulation of osteoclastogenesis and bone resorption, Medscape Womens Health 2000, 5:5

25. Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S: The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene, Nature 1990, 345:442-444

26. Teitelbaum SL: Bone resorption by osteoclasts, Science 2000, 289:1504-1508

27. Delaisse JM, Engsig MT, Everts V, del Carmen O, Ferreras M, Lund, Vu TH, Werb Z, Winding B, Lochter A, Karsdal MA, Troen T, Kirkegaard T, Lenhard T, Heegaard AM, Neff L, Baron R, Foged NT: Proteinases in bone resorption: obvious and less obvious roles, Clinica Chimica Acta 2000, 291:223-234

28. Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Moritz JD, Schu P, von Figura K: Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice, Proc Natl Acad Sci U S A 1998, 95:13453-13458

29. Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, Foged NT, Delmas PD, Delaisse JM: The collagenolytic activity of cathepsin K is unique among mammalian proteinases, J Biol Chem 1998, 273:32347-32352

30. Everts V, Beertsen W, Schroder R: Effects of the proteinase inhibitors leupeptin and E-64 on osteoclastic bone resorption, Calcif Tissue Int 1988, 43:172-178

31. Everts V, Beertsen W: Phagocytosis of collagen fibrils by periosteal fibroblasts in long bone explants Effect of concanavalin A, Tissue and Cell 1992, 24:935-941

32. Everts V, Delaisse JM, Korper W, Beertsen W: Cysteine proteinases and matrix metalloproteinases play distinct roles in the subosteoclastic resorption zone, Journal of Bone and Mineral Research 1998, 13:1420-1430

33. Cauwe B, Steen PE, Opdenakker G: The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases, Crit Rev Biochem Mol Biol 2007, 42:113-185

34. Nagase H, Visse R, Murphy G: Structure and function of matrix metalloproteinases and TIMPs, Cardiovasc Res 2006, 69:562-573

35. Nagase H, Woessner JF, Jr.: Matrix metalloproteinases, Journal Biological Chemistry 1999, 274:21491-21494

36. Van Wart HE, Birkedal-Hansen H: The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family, Proceedings of the National Academy of Sciences of the United States of America 1990, 87:5578-5582

37. Sternlicht MD, Werb Z: How matrix metalloproteinases regulate cell behavior, Annual Review of Cell and Developmental Biology 2001, 17:463-516

38. Murphy G, Nguyen Q, Cockett MI, Atkinson SJ, Allan JA, Knight CG, Willenbrock F, Docherty AJP: Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant, Journal Biological Chemistry 1994, 269:6632-6636

39. Shipley JM, Doyle GAR, Fliszar CJ, Ye QZ, Johnson LL, Shapiro SD, Welgus HG, Senior RM: The structural basis for the elastolytic activity of the 92-kDa and 72-kDa gelatinases, Journal Biological Chemistry 1996, 271:4335-4341

40. Strongin AY, Collier IE, Bannikov G, Marmer BL, Grant GA, Goldberg GI: Mechanism of cell surface activation of 72-kDa type IV collagenase Isolation of the activated form of the membrane metalloprotease, Journal Biological Chemistry 1995, 270:5331-5338

41. Fini ME, Cook JR, Mohan R, Brinckerhoff CE, Parks WC, Mecham RP: Regulation of MMP gene expression. Edited by Mecham RP. San Diego, Academic Press, 1998, p. pp. 299-356

42. Pei D, Weiss SJ: Furin-dependent intracellular activation of the human stromelysin-3 zymogen, Nature 1995, 375:244-247

43. Woessner JF, Nagase H: Matrix Metalloproteinases and TIMPs. Edited by New York, Oxford University Press,Inc., 2000, p

44. Overall CM, Tam E, McQuibban GA, Morrison C, Wallon UM, Bigg HF, King AE, Roberts CR: Domain interactions in the gelatinase A.TIMP-2.MT1-MMP activation complex. The ectodomain of the 44-kDa form of membrane type-1 matrix metalloproteinase does not modulate gelatinase A activation, J Biol Chem 2000, 275:39497-39506

45. Brew K, Dinakarpandian D, Nagase H: Tissue inhibitors of metalloproteinases: evolution, structure and function, Biochimica et Biophysica Acta 2000, 1477:267-283

46. Baker AH, Edwards DR, Murphy G: Metalloproteinase inhibitors: biological actions and therapeutic opportunities, J Cell Sci 2002, 115:3719-3727

47. Fridman R, Fuerst TR, Bird RE, Hoyhtya M, Oelkuct M, Kraus S, Komarek D, Liotta LA, Berman ML, Stetler-Stevenson WG: Domain structure of human 72-kDa gelatinase/type IV collagenase Characterization of proteolytic activity and identification of the tissue inhibitor of metalloproteinase-2 (TIMP-2) binding regions, Journal Biological Chemistry 1992, 267:15398-15405

48. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP: Tissue inhibitors of metalloproteinases: structure, regulation and biological function, European Journal of Cell Biology 1997, 74:111-122

49. Guedez L, McMarlin AJ, Kingma DW, Bennett TA, Stetler-Stevenson M, Stetler-Stevenson WG: Tissue inhibitor of metalloproteinase-1 alters the tumorigenicity of Burkitt's lymphoma via divergent effects on tumor growth and angiogenesis, American Journal of Pathology 2001, 158:1207-1215

50. Seo DW, Li H, Guedez L, Wingfield PT, Diaz T, Salloum R, Wei BY, Stetler-Stevenson WG: TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism, Cell 2003, 114:171-180

51. Delaisse JM, Eeckhout Y, Vaes G: Bone-resorbing agents affect the production and distribution of procollagenase as well as the activity of collagenase in bone tissue, Endocrinology 1988, 123:264-276

52. Otsuka K, Sodek J, Limeback H: Synthesis of collagenase and collagenase inhibitors by osteoblast-like cells in culture, Eur J Biochem 1984, 145:123-129

53. Meikle MC, Bord S, Hembry RM, Compston J, Croucher PI, Reynolds JJ: Human osteoblasts in culture synthesize collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines, J Cell Sci 1992, 103 (Pt 4):1093-1099

54. Gack S, Vallon R, Schmidt J, Grigoriadis A, Tuckermann J, Schenkel J, Weiher H, Wagner EF, Angel P: Expression of interstitial collagenase during skeletal development of the mouse is restricted to osteoblast-like cells and hypertrophic chondrocytes, Cell Growth and Differentiation 1995, 6:759-767

55. Fuller K, Chambers TJ: Localisation of mRNA for collagenase in osteocytic, bone surface and chondrocytic cells but not osteoclasts, Journal of Cell Science 1995, 108:2221-2230

56. Chambers TJ, Fuller K: Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact, J Cell Sci 1985, 76:155-165

57. Imai K, Hiramatsu A, Fukushima D, Pierschbacher MD, Okada Y: Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta 1 release, Biochemical Journal 1997, 322:809-814

58. Holliday LS, Welgus HG, Fliszar CJ, Veith GM, Jeffrey JJ, Gluck SL: Initiation of osteoclast bone resorption by interstitial collagenase, J Biol Chem 1997, 272:22053-22058

59. Zhao W, Byrne MH, Boyce BF, Krane SM: Bone resorption induced by parathyroid hormone is strikingly diminished in collagenase-resistant mutant mice, J Clin Invest 1999, 103:517-524

60. Garnero P, Ferreras M, Karsdal MA, NicAmhlaoibh R, Risteli J, Borel O, Qvist P, Delmas PD, Foged NT, Delaisse JM: The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation, Journal of Bone and Mineral Research 2003, 18:859-867

61. Krane SM, Inada M: Matrix metalloproteinases and bone, Bone 2008, 43:7-18

62. Berruti A, Dogliotti L, Gorzegno G, Torta M, Tampellini M, Tucci M, Cerutti S, Frezet MM, Stivanello M, Sacchetto G, Angeli A: Differential patterns of bone turnover in relation to bone pain and disease extent in bone in cancer patients with skeletal metastases, Clin Chem 1999, 45:1240-1247

63. Blomqvist C, Risteli L, Risteli J, Virkkunen P, Sarna S, Elomaa I: Markers of type I collagen degradation and synthesis in the monitoring of treatment response in bone metastases from breast carcinoma, British Journal of Cancer 1996, 73:1074-1079

64. Malone JD, Teitelbaum SL, Griffin GL, Senior RM, Kahn AJ: Recruitment of osteoclast precursors by purified bone matrix constituents, J Cell Biol 1982, 92:227-230

65. Davis GE: Affinity of integrins for damaged extracellular matrix: alpha v beta 3 binds to denatured collagen type I through RGD sites, Biochem Biophys Res Commun 1992, 182:1025-1031

66. Agnihotri R, Crawford HC, Haro H, Matrisian LM, Havrda MC, Liaw L: Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin), Journal Biological Chemistry 2001, 276:28261-28267

67. Itoh T, Ikeda T, Gomi H, Nakao S, Suzuki T, Itohara S: Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice, Journal Biological Chemistry 1997, 272:22389-22392

68. Inoue K, Mikuni-Takagaki Y, Oikawa K, Itoh T, Inada M, Noguchi T, Park JS, Onodera T, Krane SM, Noda M, Itohara S: A crucial role for matrix metalloproteinase 2 in osteocytic canalicular formation and bone metabolism, J Biol Chem 2006, 281:33814-33824

69. Mosig RA, Dowling O, DiFeo A, Ramirez MC, Parker IC, Abe E, Diouri J, Aqeel AA, Wylie JD, Oblander SA, Madri J, Bianco P, Apte SS, Zaidi M, Doty SB, Majeska RJ, Schaffler MB, Martignetti JA: Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth, Hum Mol Genet 2007, 16:1113-1123

70. Wilson CL, Heppner KJ, Labosky PA, Hogan BL, Matrisian LM: Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin, Proc Natl Acad Sci U S A 1997, 94:1402-1407

71. Gjertsson I, Innocenti M, Matrisian LM, Tarkowski A: Metalloproteinase-7 contributes to joint destruction in Staphylococcus aureus induced arthritis, Microb Pathog 2005, 38:97-105

72. Haro H, Crawford HC, Fingleton B, Shinomiya K, Spengler DM, Matrisian LM: Matrix metalloproteinase-7-dependent release of tumor necrosis factor-alpha in a model of herniated disc resorption, Journal of Clinical Investigation 2000, 105:143-150

73. Battaglino R, Kim D, Fu J, Vaage B, Fu XY, Stashenko P: c-myc is required for osteoclast differentiation, J Bone Miner Res 2002, 17:763-773

74. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z: MMP-9/Gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes, Cell 1998, 93:411-422

75. Engsig MT, Chen QJ, Vu TH, Pedersen AC, Therkidsen B, Lund LR, Henriksen K, Lenhard T, Foged NT, Werb Z, Delaisse JM: Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones, Journal of Cell Biology 2000, 151:879-890

76. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D: Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis, Nature Cell Biology 2000, 2:737-744

77. Delaisse JM, Andersen TL, Engsig MT, Henriksen K, Troen T, Blavier L: Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities, Microscopy Research and Technique 2003, 61:504-513

78. Nakashima A, Tamura M: Regulation of matrix metalloproteinase-13 and tissue inhibitor of matrix metalloproteinase-1 gene expression by WNT3A and bone morphogenetic protein-2 in osteoblastic differentiation, Front Biosci 2006, 11:1667-1678

79. Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C, Krane SM: Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification, Proceedings of the National Academy of Sciences of the United States of America 2004, 101:17192-17197

80. Irie K, Tsuruga E, Sakakura Y, Muto T, Yajima T: Immunohistochemical localization of membrane type 1-matrix metalloproteinase (MT1-MMP) in osteoclasts in vivo, Tissue Cell 2001, 33:478-482

81. Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H: MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover, Cell 1999, 99:81-92

82. Holmbeck K, Bianco P, Pidoux I, Inoue S, Billinghurst RC, Wu W, Chrysovergis K, Yamada S, Birkedal-Hansen H, Poole AR: The metalloproteinase MT1-MMP is required for normal development and maintenance of osteocyte processes in bone, J Cell Sci 2005, 118:147-156

83. Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K: Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I, Proceedings of the National Academy of Sciences of the United States of America 2000, 97:4052-4057

84. Holmbeck K, Bianco P, Chrysovergis K, Yamada S, Birkedal-Hansen H: MT1-MMP-dependent, apoptotic remodeling of unmineralized cartilage: a critical process in skeletal growth, J Cell Biol 2003, 163:661-671

85. Guise TA, Mundy GR: Cancer and bone, Endocr Rev 1998, 19:18-54

86. Chambers AF, Matrisian LM: Changing views of the role of matrix metalloproteinases in metastasis, Journal National Cancer Institute 1997, 89:1260-1270

87. Kakonen SM, Mundy GR: Mechanisms of osteolytic bone metastases in breast carcinoma, Cancer 2003, 97:834-839

88. Suda T, Udagawa N, Nakamura I, Miyaura C, Takahashi N: Modulation of osteoclast differentiation by local factors, Bone 1995, 17:87S-91S

89. Dobnig H, Turner RT: Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells, Endocrinology 1995, 136:3632-3638

90. Amizuka N, Henderson JE, Hoshi K, Warshawsky H, Ozawa H, Goltzman D, Karaplis AC: Programmed cell death of chondrocytes and aberrant chondrogenesis in mice homozygous for parathyroid hormone-related peptide gene deletion, Endocrinology 1996, 137:5055-5067

91. Miao D, He B, Jiang Y, Kobayashi T, Soroceanu MA, Zhao J, Su H, Tong X, Amizuka N, Gupta A, Genant HK, Kronenberg HM, Goltzman D, Karaplis AC: Osteoblast-derived PTHrP is a potent endogenous bone anabolic agent that modifies the therapeutic efficacy of administered PTH 1-34, J Clin Invest 2005, 115:2402-2411

92. Liu BY, Guo J, Lanske B, Divieti P, Kronenberg HM, Bringhurst FR: Conditionally immortalized murine bone marrow stromal cells mediate parathyroid hormone-dependent osteoclastogenesis in vitro, Endocrinology 1998, 139:1952-1964

93. Southby J, O'Keeffe LM, Martin TJ, Gillespie MT: Alternative promoter usage and mRNA splicing pathways for parathyroid hormone-related protein in normal tissues and tumours, Br J Cancer 1995, 72:702-707

94. Cramer SD, Chen Z, Peehl DM: Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts, J Urol 1996, 156:526-531

95. Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, Bennett RC, Martin TJ: Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites, Cancer Research 1991, 51:3059-3061

96. Southby J, Kissin MW, Danks JA, Hayman JA, Moseley JM, Henderson MA, Bennett RC, Martin TJ: Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer, Cancer Res 1990, 50:7710-7716

97. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR: Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis, J Clin.Invest 1996, 98:1544-1549

98. Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M: Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose 2, J Biol.Chem. 1986, 261:12665-12674

99. Boyce BF, Yoneda T, Guise TA: Factors regulating the growth of metastatic cancer in bone, Endocr Relat Cancer 1999, 6:333-347

100. Lucas PA: Chemotactic response of osteoblast-like cells to transforming growth factor beta, Bone 1989, 10:459-463

101. Pfeilschifter J, Oechsner M, Naumann A, Gronwald RGK, Minne HW, Ziegler R: Stimulation of bone matrix apposition in vitro by local growth factors: a comparison between insulin-like growth factor I, platelet-derived growth factor, and transforming growth factor beta, Endocrinology 1990, 127:69-75

102. Hughes FJ, Aubin JE, Heersche JN: Differential chemotactic responses of different populations of fetal rat calvaria cells to platelet-derived growth factor and transforming growth factor beta, Bone Miner 1992, 19:63-74

103. Noda M, Rodan GA: Type beta transforming growth factor (TGF beta) regulation of alkaline phosphatase expression and other phenotype-related mRNAs in osteoblastic rat osteosarcoma cells, J Cell Physiol 1987, 133:426-437

104. Centrella M, McCarthy TL, Canalis E: Transforming growth factor beta is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone, J Biol Chem 1987, 262:2869-2874

105. Hattersley G, Chambers TJ: Effects of transforming growth factor beta 1 on the regulation of osteoclastic development and function, J Bone Miner Res 1991, 6:165-172

106. Takai H, Kanematsu M, Yano K, Tsuda E, Higashio K, Ikeda K, Watanabe K, Yamada Y: Transforming growth factor-beta stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells, J Biol Chem 1998, 273:27091-27096

107. Kale VP: Differential activation of MAPK signaling pathways by TGF-beta1 forms the molecular mechanism behind its dose-dependent bidirectional effects on hematopoiesis, Stem Cells Dev 2004, 13:27-38

108. Dieudonne SC, Foo P, van Zoelen EJ, Burger EH: Inhibiting and stimulating effects of TGF-beta 1 on osteoclastic bone resorption in fetal mouse bone organ cultures, J Bone Miner Res 1991, 6:479-487

109. Pfeilschifter J, Mundy GR: Modulation of type beta transforming growth factor activity in bone cultures by osteotropic hormones, Proc Natl Acad Sci U S A 1987, 84:2024-2028

110. Pulukkody KP, Norman TJ, Parker D, Royle L, Broan CJ: Journal of the Chemical Society Perkin Transactions 1993, 2:605-620

111. Norgaard P, Damstrup L, Spang-Thomsen M, Poulsen HS: [Transforming growth factor beta. A potent multifunctional growth factor for normal and malignant cells], Ugeskr Laeger 1992, 154:3494-3498

112. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA: TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development, J Clin Invest 1999, 103:197-206

113. Ruchon AF, Marcinkiewicz M, Ellefsen K, Basak A, Aubin J, Crine P, Boileau G: Cellular localization of neprilysin in mouse bone tissue and putative role in hydrolysis of osteogenic peptides, J Bone Miner Res 2000, 15:1266-1274

114. Dall'Era MA, True LD, Siegel AF, Porter MP, Sherertz TM, Liu AY: Differential expression of CD10 in prostate cancer and its clinical implication, BMC Urol 2007, 7:3

115. Smollich M, Gotte M, Yip GW, Yong ES, Kersting C, Fischgrabe J, Radke I, Kiesel L, Wulfing P: On the role of endothelin-converting enzyme-1 (ECE-1) and neprilysin in human breast cancer, Breast Cancer Res Treat 2007,

116. Lynch CC, Hikosaka A, Acuff HB, Martin MD, Kawai N, Singh RK, Vargo-Gogola TC, Begtrup JL, Peterson TE, Fingleton B, Shirai T, Matrisian LM, Futakuchi M: MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL, Cancer Cell 2005, 7:485-496

117. Lum L, Wong BR, Josien R, Becherer JD, Erdjument-Bromage H, Schlondorff J, Tempst P, Choi Y, Blobel CP: Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival, Journal Biological Chemistry 1999, 274:13613-13618

118. Schlondorff J, Lum L, Blobel CP: Biochemical and pharmacological criteria define two shedding activities for TRANCE/OPGL that are distinct from the tumor necrosis factor alpha convertase, Journal Biological Chemistry 2001, 276:14665-14674

119. Chesneau V, Becherer JD, Zheng Y, Erdjument-Bromage H, Tempst P, Blobel CP: Catalytic properties of ADAM19, Journal Biological Chemistry 2003, 278:22331-22340

120. Janssens K, Ten Dijke P, Janssens S, Van Hul W: Transforming growth factor-beta1 to the bone 2, Endocr.Rev. 2005, 26:743-774

121. Dallas SL, Miyazono K, Skerry TM, Mundy GR, Bonewald LF: Dual role for the latent transforming growth factor-beta binding protein in storage of latent TGF-beta in the extracellular matrix and as a structural matrix protein 6, J Cell Biol. 1995, 131:539-549

122. Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis, Genes and Development 2000, 14:163-176

123. Maeda S, Dean DD, Gay I, Schwartz Z, Boyan BD: Activation of latent transforming growth factor beta1 by stromelysin 1 in extracts of growth plate chondrocyte-derived matrix vesicles, Journal of Bone and Mineral Research 2001, 16:1281-1290

124. Maeda S, Dean DD, Gomez R, Schwartz Z, Boyan BD: The first stage of transforming growth factor beta1 activation is release of the large latent complex from the extracellular matrix of growth plate chondrocytes by matrix vesicle stromelysin-1 (MMP-3), Calcified Tissue International 2002, 70:54-65

125. Mohan S, Baylink DJ: Characterization of the IGF regulatory system in bone, Adv Exp Med Biol 1993, 343:397-406

126. Nickerson T, Chang F, Lorimer D, Smeekens SP, Sawyers CL, Pollak M: In vivo progression of LAPC-9 and LNCaP prostate cancer models to androgen independence is associated with increased expression of insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR), Cancer Res 2001, 61:6276-6280

127. Ngo TH, Barnard RJ, Leung PS, Cohen P, Aronson WJ: Insulin-like growth factor I (IGF-I) and IGF binding protein-1 modulate prostate cancer cell growth and apoptosis: possible mediators for the effects of diet and exercise on cancer cell survival, Endocrinology 2003, 144:2319-2324

128. Giles ED, Singh G: Role of insulin-like growth factor binding proteins (IGFBPs) in breast cancer proliferation and metastasis, Clin Exp Metastasis 2003, 20:481-487

129. Wang Y, Nishida S, Elalieh HZ, Long RK, Halloran BP, Bikle DD: Role of IGF-I signaling in regulating osteoclastogenesis, J Bone Miner Res 2006, 21:1350-1358

130. Rajah R, Bhala A, Nunn SE, Peehl DM, Cohen P: 7S nerve growth factor is an insulin-like growth factor-binding protein protease, Endocrinology 1996, 137:2676-2682

131. Fowlkes JL, Enghild JJ, Suzuki K, Nagase H: Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures, Journal Biological Chemistry 1994, 269:25742-25746

132. Manes S, Mira E, Barbacid MM, Cipres A, Fernandez-Resa P, Buesa JM, Merida I, Aracil M, Marquez G, Martinez A: Identification of insulin-like growth factor-binding protein-1 as a potential physiological substrate for human stromelysin-3, Journal Biological Chemistry 1997, 272:25706-25712

133. Nakamura M, Miyamoto S, Maeda H, Ishii G, Hasebe T, Chiba T, Asaka M, Ochiai A: Matrix metalloproteinase-7 degrades all insulin-like growth factor binding proteins and facilitates insulin-like growth factor bioavailability, Biochem Biophys Res Commun 2005, 333:1011-1016

134. Lynch CC, Matrisian LM: Matrix metalloproteinases in tumor-host cell communication, Differentiation 2002, 70:561-573

135. Coussens LM, Fingleton B, Matrisian LM: Matrix metalloproteinase inhibitors and cancer: trials and tribulations, Science 2002, 295:2387-2392

136. Brown PD: Ongoing trials with matrix metalloproteinase inhibitors, Expert Opinion on Investigational Drugs 2000, 9:2167-2177

137. Lee J, Weber M, Mejia S, Bone E, Watson P, Orr W: A matrix metalloproteinase inhibitor, batimastat, retards the development of osteolytic bone metastases by MDA-MB-231 human breast cancer cells in Balb C nu/nu mice, European Journal of Cancer 2001, 37:106-113

138. Winding B, NicAmhlaoibh R, Misander H, Hoegh-Andersen P, Andersen TL, Holst-Hansen C, Heegaard AM, Foged NT, Brunner N, Delaisse JM: Synthetic matrix metalloproteinase inhibitors inhibit growth of established breast cancer osteolytic lesions and prolong survival in mice, Clinical Cancer Research 2002, 8:1932-1939

139. Nemeth JA, Yousif R, Herzog M, Che M, Upadhyay J, Shekarriz B, Bhagat S, Mullins C, Fridman R, Cher ML: Matrix metalloproteinase activity, bone matrix turnover, and tumor cell proliferation in prostate cancer bone metastasis, Journal National Cancer Institute 2002, 94:17-25

140. Matrisian LM, Sledge GW, Jr., Mohla S: Extracellular Proteolysis and Cancer: Meeting Summary and Future Directions, Cancer Research 2003, 63:6105-6109

141. Bonfil RD, Sabbota A, Nabha S, Bernardo MM, Dong Z, Meng H, Yamamoto H, Chinni SR, Lim IT, Chang M, Filetti LC, Mobashery S, Cher ML, Fridman R: Inhibition of human prostate cancer growth, osteolysis and angiogenesis in a bone metastasis model by a novel mechanism-based selective gelatinase inhibitor 1, International Journal of Cancer 2006, 118:2721-2726

142. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, Pollard JW: Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases, American Journal of Pathology 2003, 163:2113-2126

143. Horton RM, Karachunski PI, Conti-Fine BM: PCR screening of transgenic RAG-2 "knockout" immunodeficient mice, Biotechniques 1995, 19:690-691

144. Fingleton B, Powell WC, Crawford HC, Couchman JR, Matrisian LM: A rat monoclonal antibody that recognizes pro- and active MMP-7 indicates polarized expression in vivo, Hybridoma (Larchmt) 2007, 26:22-27

145. Filgueira L: Fluorescence-based staining for tartrate-resistant acidic phosphatase (TRAP) in osteoclasts combined with other fluorescent dyes and protocols, J Histochem Cytochem 2004, 52:411-414

146. Martin M, Carter K, Thiolloy S, Lynch CC, Matrisian L, Fingleton B: Effect of ablation or inhibition of stromal matrix metalloproteinase-9 on lung metastasis in a breast cancer model is dependent on genetic background Cancer Res 2008, 68:E-Pub ahead of print

147. Nabha SM, Bonfil RD, Yamamoto HA, Belizi A, Wiesner C, Dong Z, Cher ML: Host matrix metalloproteinase-9 contributes to tumor vascularization without affecting tumor growth in a model of prostate cancer bone metastasis, Clin Exp Metastasis 2006, 23:335-344

148. Powell WC, Fingleton B, Wilson CL, Boothby M, Matrisian LM: The metalloproteinase matrilysin (MMP-7) proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis, Current Biology 1999, 9:1441-1447

149. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM: OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis, Nature 1999, 397:315-323

150. Breuil V, Schmid-Antomarchi H, Schmid-Alliana A, Rezzonico R, Euller-Ziegler L, Rossi B: The receptor activator of nuclear factor (NF)-kappaB ligand (RANKL) is a new chemotactic factor for human monocytes, Faseb J 2003, 17:1751-1753

151. Wilson TJ, Nannuru KC, Futakuchi M, Sadanandam A, Singh RK: Cathepsin G enhances mammary tumor-induced osteolysis by generating soluble receptor activator of nuclear factor-kappaB ligand, Cancer Res 2008, 68:5803-5811

152. Bord S, Horner A, Hembry RM, Reynolds JJ, Compston JE: Production of collagenase by human osteoblasts and osteoclasts in vivo, Bone 1996, 19:35-40

153. Bord S, Horner A, Hembry RM, Compston JE: Stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) expression in developing human bone: potential roles in skeletal development, Bone 1998, 23:7-12

154. Kusano K, Miyaura C, Inada M, Tamura T, Ito A, Nagase H, Kamoi K, Suda T: Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption, Endocrinology 1998, 139:1338-1345

155. Breckon JJ, Papaioannou S, Kon LW, Tumber A, Hembry RM, Murphy G, Reynolds JJ, Meikle MC: Stromelysin (MMP-3) synthesis is up-regulated in estrogen-deficient mouse osteoblasts in vivo and in vitro, Journal of Bone and Mineral Research 1999, 14:1880-1890

156. Dew G, Murphy G, Stanton H, Vallon R, Angel P, Reynolds JJ, Hembry RM: Localisation of matrix metalloproteinases and TIMP-2 in resorbing mouse bone, Cell and Tissue Research 2000, 299:385-394

157. Parikka V, Vaananen A, Risteli J, Salo T, Sorsa T, Vaananen HK, Lehenkari P: Human mesenchymal stem cell derived osteoblasts degrade organic bone matrix in vitro by matrix metalloproteinases, Matrix Biol 2005, 24:438-447

158. Mastro AM, Gay CV, Welch DR: The skeleton as a unique environment for breast cancer cells, Clinical and Experimetnal Metastasis 2003, 20:275-284

159. Puck TT, Marcus PI, Cieciura SJ: Clonal growth of mammalian cells in vitro; growth characteristics of colonies from single HeLa cells with and without a feeder layer, J Exp Med 1956, 103:273-283

160. Hoffman RM: In vitro sensitivity assays in cancer: a review, analysis, and prognosis, J Clin Lab Anal 1991, 5:133-143

161. Brown JM, Attardi LD: The role of apoptosis in cancer development and treatment response, Nat Rev Cancer 2005, 5:231-237

162. Dubois CM, Laprise MH, Blanchette F, Gentry LE, Leduc R: Processing of transforming growth factor beta 1 precursor by human furin convertase, J Biol Chem 1995, 270:10618-10624

163. Saharinen J, Hyytiainen M, Taipale J, Keski-Oja J: Latent transforming growth factor-beta binding proteins (LTBPs)--structural extracellular matrix proteins for targeting TGF-beta action 2, Cytokine Growth Factor Rev. 1999, 10:99-117

164. Dabovic B, Chen Y, Colarossi C, Obata H, Zambuto L, Perle MA, Rifkin DB: Bone abnormalities in latent TGF-[beta] binding protein (Ltbp)-3-null mice indicate a role for Ltbp-3 in modulating TGF-[beta] bioavailability, J Cell Biol 2002, 156:227-232

165. Dabovic B, Levasseur R, Zambuto L, Chen Y, Karsenty G, Rifkin DB: Osteopetrosis-like phenotype in latent TGF-beta binding protein 3 deficient mice, Bone 2005, 37:25-31

166. Koli K, Ryynanen MJ, Keski-Oja J: Latent TGF-beta binding proteins (LTBPs)-1 and -3 coordinate proliferation and osteogenic differentiation of human mesenchymal stem cells, Bone 2008, 43:679-688

167. Penttinen C, Saharinen J, Weikkolainen K, Hyytiainen M, Keski-Oja J: Secretion of human latent TGF-beta-binding protein-3 (LTBP-3) is dependent on co-expression of TGF-beta, J Cell Sci 2002, 115:3457-3468

168. de Caestecker MP, Parks WT, Frank CJ, Castagnino P, Bottaro DP, Roberts AB, Lechleider RJ: Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases, Genes Dev 1998, 12:1587-1592

169. Strand S, Vollmer P, van den AL, Gottfried D, Alla V, Heid H, Kuball J, Theobald M, Galle PR, Strand D: Cleavage of CD95 by matrix metalloproteinase-7 induces apoptosis resistance in tumour cells, Oncogene 2004, 23:3732-3736

170. Kovacic N, Lukic IK, Grcevic D, Katavic V, Croucher P, Marusic A: The Fas/Fas ligand system inhibits differentiation of murine osteoblasts but has a limited role in osteoblast and osteoclast apoptosis, J Immunol 2007, 178:3379-3389

171. Hotokezaka H, Sakai E, Ohara N, Hotokezaka Y, Gonzales C, Matsuo K, Fujimura Y, Yoshida N, Nakayama K: Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF-alpha, lipopolysaccharide, or peptidoglycan, J Cell Biochem 2007, 101:122-134

172. Blair HC: How the osteoclast degrades bone, Bioessays 1998, 20:837-846

173. Miyamoto S, Yano K, Sugimoto S, Ishii G, Hasebe T, Endoh Y, Kodama K, Goya M, Chiba T, Ochiai A: Matrix metalloproteinase-7 facilitates insulin-like growth factor bioavailability through its proteinase activity on insulin-like growth factor binding protein 3, Cancer Research 2004, 64:665-671

174. Burke B, Giannoudis A, Corke KP, Gill D, Wells M, Ziegler-Heitbrock L, Lewis CE: Hypoxia-induced gene expression in human macrophages: implications for ischemic tissues and hypoxia-regulated gene therapy, American Journal of Pathology 2003, 163:1233-1243

175. Athanasou NA, Sabokbar A: Human osteoclast ontogeny and pathological bone resorption, Histol Histopathol 1999, 14:635-647

176. Fingleton B, Vargo-Gogola T, Crawford HC, Matrisian LM: Matrilysin [MMP-7] Expression Selects for Cells with Reduced Sensitivity to Apoptosis, Neoplasia 2001, 3:459-468

177. Sawey ET, Johnson JA, Crawford HC: Matrix metalloproteinase 7 controls pancreatic acinar cell transdifferentiation by activating the Notch signaling pathway, Proc Natl Acad Sci U S A 2007, 104:19327-19332

178. Wilson CL, Heppner KJ, Labosky PA, Hogan BLM, Matrisian LM: Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin, Proceedings of the National Academy of Sciences of the United States of America 1997, 94:1402-1407

179. Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC: Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense, Science 1999, 286:113-117

180. Rudolph-Owen LA, Chan R, Muller WJ, Matrisian LM: The matrix metalloproteinase matrilysin influences early-stage mammary tumorigenesis, Cancer Research 1998, 58:5500-5506

181. Ohta S, Imai K, Yamashita K, Matsumoto T, Azumano I, Okada Y: Expression of matrix metalloproteinase 7 (matrilysin) in human osteoarthritic cartilage, Laboratory Investigation 1998, 78:79-87

182. Acuff HB, Carter KJ, Fingleton B, Gorden DL, Matrisian LM: Matrix metalloproteinase-9 from bone marrow-derived cells contributes to survival but not growth of tumor cells in the lung microenvironment 1, Cancer Research 2006, 66:259-266

183. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D: VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche 1, Nature 2005, 438:820-827

184. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S: Recruitment of stem and progenitor cells from the bone marrow niche requires mmp-9 mediated release of kit-ligand, Cell 2002, 109:625-637

185. Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, Shipley JM, Senior RM, Shibuya M: MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis, Cancer Cell 2002, 2:289-300

186. Taipale J, Miyazono K, Heldin CH, Keski-Oja J: Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein, J Cell Biol 1994, 124:171-181

187. Saharinen J, Taipale J, Monni O, Keski-Oja J: Identification and characterization of a new latent transforming growth factor-beta-binding protein, LTBP-4, J Biol Chem 1998, 273:18459-18469

188. Dallas SL, Rosser JL, Mundy GR, Bonewald LF: Proteolysis of latent transforming growth factor-beta (TGF-beta)-binding protein-1 by osteoclasts. A cellular mechanism for release of TGF-beta from bone matrix, J Biol Chem 2002, 277:21352-21360

189. Tatti O, Vehvilainen P, Lehti K, Keski-Oja J: MT1-MMP releases latent TGF-beta1 from endothelial cell extracellular matrix via proteolytic processing of LTBP-1, Exp Cell Res 2008, 314:2501-2514

190. Dean RA, Overall CM: Proteomics discovery of metalloproteinase substrates in the cellular context by iTRAQ labeling reveals a diverse MMP-2 substrate degradome, Mol Cell Proteomics 2007, 6:611-623

191. Sato Y, Rifkin DB: Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture, J Cell Biol 1989, 109:309-315

192. Georgii-Hemming P, Wiklund HJ, Ljunggren O, Nilsson K: Insulin-like growth factor I is a growth and survival factor in human multiple myeloma cell lines, Blood 1996, 88:2250-2258

193. Dunn SE, Hardman RA, Kari FW, Barrett JC: Insulin-like growth factor 1 (IGF-1) alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs, Cancer Res 1997, 57:2687-2693

194. Conery AR, Cao Y, Thompson EA, Townsend CM, Jr., Ko TC, Luo K: Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis, Nat Cell Biol 2004, 6:366-372

195. Remy I, Montmarquette A, Michnick SW: PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3, Nat Cell Biol 2004, 6:358-365

196. Muraoka-Cook RS, Shin I, Yi JY, Easterly E, Barcellos-Hoff MH, Yingling JM, Zent R, Arteaga CL: Activated type I TGFbeta receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression, Oncogene 2006, 25:3408-3423

197. Saile B, Matthes N, El Armouche H, Neubauer K, Ramadori G: The bcl, NFkappaB and p53/p21WAF1 systems are involved in spontaneous apoptosis and in the anti-apoptotic effect of TGF-beta or TNF-alpha on activated hepatic stellate cells, Eur J Cell Biol 2001, 80:554-561

198. Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT: Breast cancer cells interact with osteoblasts to support osteoclast formation, Endocrinology 1999, 140:4451-4458

199. Kakonen SM, Selander KS, Chirgwin JM, Yin JJ, Burns S, Rankin WA, Grubbs BG, Dallas M, Cui Y, Guise TA: Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways, J Biol Chem 2002, 277:24571-24578

200. Ignotz RA, Massague J: Type beta transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts, Proc Natl Acad Sci U S A 1985, 82:8530-8534

201. Ignotz RA, Endo T, Massague J: Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta, J Biol Chem 1987, 262:6443-6446

202. Noda M, Camilliere JJ: In vivo stimulation of bone formation by transforming growth factor-beta, Endocrinology 1989, 124:2991-2994

203. Marcelli C, Yates AJ, Mundy GR: In vivo effects of human recombinant transforming growth factor beta on bone turnover in normal mice, J Bone Miner Res 1990, 5:1087-1096

204. Erlebacher A, Derynck R: Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype, J Cell Biol 1996, 132:195-210

205. Karsdal MA, Hjorth P, Henriksen K, Kirkegaard T, Nielsen KL, Lou H, Delaisse JM, Foged NT: Transforming growth factor-beta controls human osteoclastogenesis through the p38 MAPK and regulation of RANK expression, J Biol Chem 2003, 278:44975-44987

206. Lovibond AC, Haque SJ, Chambers TJ, Fox SW: TGF-beta-induced SOCS3 expression augments TNF-alpha-induced osteoclast formation, Biochem Biophys Res Commun 2003, 309:762-767

207. Takuma A, Kaneda T, Sato T, Ninomiya S, Kumegawa M, Hakeda Y: Dexamethasone enhances osteoclast formation synergistically with transforming growth factor-beta by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts, J Biol Chem 2003, 278:44667-44674

208. Mohammad KS, Chen CG, Balooch G, Stebbins E, McKenna CR, Davis H, Niewolna M, Peng XH, Nguyen DH, Ionova-Martin SS, Bracey JW, Hogue WR, Wong DH, Ritchie RO, Suva LJ, Derynck R, Guise TA, Alliston T: Pharmacologic inhibition of the TGF-beta type I receptor kinase has anabolic and anti-catabolic effects on bone, PLoS One 2009, 4:e5275

209. Goetzl EJ, Bonda MJ, Leppert D: Matrix metalloproteinoses in immunity, Journal of Immunology 1996, 156:1-4

210. Fournier PG, Chirgwin JM, Guise TA: New insights into the role of T cells in the vicious cycle of bone metastases, Curr.Opin.Rheumatol. 2006, 18:396-404

211. Stresing V, Daubine F, Benzaid I, Monkkonen H, Clezardin P: Bisphosphonates in cancer therapy, Cancer Lett 2007, 257:16-35

212. Lyseng-Williamson KA: Zoledronic acid: a review of its use in breast cancer, Drugs 2008, 68:2661-2682

213. Russell RG: Bisphosphonates: mode of action and pharmacology, Pediatrics 2007, 119 Suppl 2:S150-162

214. Dunford JE, Rogers MJ, Ebetino FH, Phipps RJ, Coxon FP: Inhibition of protein prenylation by bisphosphonates causes sustained activation of Rac, Cdc42, and Rho GTPases, J Bone Miner Res 2006, 21:684-694

215. Hillner BE, Ingle JN, Chlebowski RT, Gralow J, Yee GC, Janjan NA, Cauley JA, Blumenstein BA, Albain KS, Lipton A, Brown S: American Society of Clinical Oncology 2003 update on the role of bisphosphonates and bone health issues in women with breast cancer, J Clin Oncol 2003, 21:4042-4057

216. Bamias A, Kastritis E, Bamia C, Moulopoulos LA, Melakopoulos I, Bozas G, Koutsoukou V, Gika D, Anagnostopoulos A, Papadimitriou C, Terpos E, Dimopoulos MA: Osteonecrosis of the jaw in cancer after treatment with bisphosphonates: incidence and risk factors, J Clin Oncol 2005, 23:8580-8587

217. Weber MH, Lee J, Orr FW: The effect of Neovastat (AE-941) on an experimental metastatic bone tumor model, Int J Oncol 2002, 20:299-303

218. Yoneda T, Sasaki A, Dunstan C, Williams PJ, Bauss F, DeClerck YA, Mundy GR: Inhibition of osteolytic bone metastasis of breast cancer by combined treatment with the bisphosphonate ibandronate and tissue inhibitor of the matrix metalloproteinase-2, Journal of Clinical Investigation 1997, 99:2509-2517

219. Whittaker M, Floyd CD, Brown P, Gearing AJH: Design and therapeutic application of matrix metalloproteinase inhibitors, Chemical Reviews 1999, 99:2735-2776

220. Charhon SA, Chapuy MC, Delvin EE, Valentin-Opran A, Edouard CM, Meunier PJ: Histomorphometric analysis of sclerotic bone metastases from prostatic carcinoma special reference to osteomalacia, Cancer 1983, 51:918-924