THE ROLE OF FOCAL ADHESION KINASE IN BREAST CANCER MEDIATED OSTEOLYSIS

By

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ABSTRACT

Breast cancer most commonly metastasizes to the bone, where it perpetuates the vicious cycle leading to osteolytic lesions. This occurs when secreted factors from breast cancer cells disrupt bone homeostasis by deregulation of osteoblast bone formation, and enhance osteoclast bone degradation thereby releasing bone matrix bound growth factors leading to further tumor growth. Although the use of osteoclast targeting agents, such as bisphosphonates and RANK-L inhibitors, are common practice for the treatment of bone metastasis, they have not been shown to increase patient survival. We therefore sought to investigate the role of focal adhesion kinase (FAK), a potential therapeutic target, in the treatment of breast cancer mediated osteolysis. FAK is a non-receptor tyrosine kinase known to directly regulate tumor progression and metastasis; it is also expressed in all of the cell types involved in breast cancer mediated osteolysis. Thus, we hypothesized that the inhibition of FAK would restore normal bone homeostasis, as well as mediate direct anti-tumor activity. FAK depletion resulted in the decrease of expression of several osteolytic factors secreted by breast cancer cells. However, the use of FAK depleted breast cancer conditioned media did not prevent breast cancer mediated osteoclastogenesis in an osteoblast/osteoclast coculture. In monoculture however, using the FAK inhibitor PF-271, we have shown that FAK inhibition leads to increased apoptosis of mature osteoclasts, and their decreased ability to degrade mineralized bone matrix, perhaps in part due to reduced expression of lytic factors such as tartrate resistant acid phosphatase and cathepsin K. Further, FAK inhibition in osteoblast monoculture led to a decrease in their ability to express the maturation factor alkaline phosphatase, and also inhibited their ability to induce mineralization. This inhibition may be due in part to the specific effects of FAK inhibition using PF-271, which may result in decreased levels of p53 in treated osteoblasts. These results suggests that the pharmacological inhibition of FAK can effect all three cell types involved in the vicious cycle of bone metastasis, and as such could be a beneficial therapeutic for patients with bone metastasis resulting in prevention of bone degradation along with direct inhibition of tumor growth. However, it may require further evaluation in animal models to determine if observed effects on osteoblast activity in vitro also occurs in vivo with possible detrimental effects on restoration of damaged bone.

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ABBREVIATIONS

AAB	Ascorbic acid and β-glycerophosphate
ALP	Alkaline phosphatase
AMEM	Alpha Modified Eagle's Medium
BMP2	Bone morphogenic protein 2
BSP	Bone sialoprotein
CCL5	C-C motif chemokine 5
CSF1R	Receptor colony stimulating factor 1
CXCR4	C-X-C Chemokine receptor type 4
DCIS	Ductal carcinoma in situ
DKK	Dikkopf related proteins
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
ERK	Extracellular Regulated Kinase
FAK	Focal Adhesion Kinase
FAT	Focal adhesion targeting
FBS	Fetal Bovine Serum
FERM	Four-point-one ezrin, radixin, moesin
FGF	Fibroblast growth factor
FRNK	FAK-related non-kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
HER2	Human epidermal growth factor receptor 2
IDC	Invasive ductal carcinoma
IL	Interleukin
ILC	Invasive lobular carcinoma
LCIS	Lobular carcinoma in situ
LOX	Lysyl oxidase
LRP5	Low-density lipoprotein receptor protein 5
M-CSF	Macrophage colony stimulating factor
MaCSC	Mammary cancer stem cells
MAPK	Mitogen activated protein kinase
Mdm-2	Mouse double minute 2 homolog
MITF	Micropthalmia transcription factor
MMP	Matrix Metalloproteinase
MMTV	Mouse mammary tumor virus
MV	Microvesicle
NES	Nuclear export signal
NFATc1	Nuclear factor of activated T-cells
NFKβ	Nuclear factor kappa β
NLS	Nuclear localization signal
OCL	Osteoclast-like cells
OPG	Osteoprotegrin
OPGL	Osteoprotegrin ligand
OPN	Osteopontin
PARP	Poly ADP-Ribose Polymerase

PBS	Phosphate Buffered Saline
PDGF-bb	Platelet-derived growth factor bb
PI3K	Phosphoinositide 3-Kinase
PPi	Inorganic pyrophosphate
PR	Progesterone receptor
PRR	Proline rich regions
PTHrP	Parathyroid hormone related protein
PYK2	Protein tyrosine kinase 2 beta
PyMT	Polyoma middle T oncoprotein
RANK	Receptor activator of nuclear factor kappa β
RANK-L	Receptor activator of nuclear factor kappa β ligand
RPMI	Roswell Park Memorial Medium
RUNX2	Runt-related transcription factor 2
SCC	Squamous carcinoma cells
SCID	Severe combined immune deficient
SH domain	Src Homology domain
Shh	Sonic hedgehog
siRNA	Small Interfering Ribonucleic Acid
SPARC	Secreted protein acidic and cysteine rich
SRE	Skeletal related event
TAF9	TATA-box binding protein (TBP)-associated factor 9
TCL/LEF	T cell factor/lymphoid enhancer factor
TGF-β	Transforming growth factor β
TRAP	Tartrate resistant acid phosphatase
Wnt	Wingless

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1. INTRODUCTION

1.1 BREAST CANCER

Although the overall 5-year survival for breast cancer in Canada is 88%, it is greatly decreased when stage is accounted for, with stage IV breast cancer having a 20% 5-year survival rate (1). This decrease in survival rate is largely attributed to the disease having spread to metastatic site(s) which are largely incurable (1). The main types of breast cancer can be distinguished by their location within the breast. Breast cancer can arise from the milk ducts (ductal) or the lobules (lobular), and can either be in situ or invasive into surrounding tissue (2). These types are ductal carcinoma in situ (DCIS), or invasive ductal carcinoma (IDC), lobular carcinoma in situ (LCIS) or invasive lobular carcinoma (ILC) (2). Breast cancer can also be a mixture of both ductal and lobular.

The site of distant metastasis is largely variable between breast cancer subtypes (3). These subtypes, which include luminal A, luminal B, basal-like, normal breast-like and ErbB2+ (a proto-oncogene) all possess distinct gene expression patterns and survival outcomes (4,5). Luminal, meaning originating from the luminal epithelium has two subtypes, A and B that are associated with low grade disease, while basal-like and ErbB2+ breast cancers are high grade disease and generally have reduced survival (4). These breast cancer subtypes can be generally classified by their expression of the estrogen receptor (ER) and progesterone receptor (PR), which when present make them hormone receptor positive and luminal in origin, or the human epidermal growth factor receptor-2 (HER2), the protein encoded by the ErbB2 gene, which when expressed classifies them as HER2+ breast cancers (4). Breast cancers that lack all three of these important receptors are considered triple negative (basal-like origin) (6). To understand the importance of subtype on predicting distant metastatic site, a tissue microarray analysis was performed from the British Columbia Cancer Agency, and the Estrogen Receptor Tissue bank using 3,726 women (3). Within the subtypes available, they classified the patients by luminal A, luminal B, luminal/HER2 positive, basal-like and triple negative (3). Although liver, lung and brain metastases were frequent, bone

was the most frequent site of metastases (3). Classified by subtype, bone metastases were highest in the more differentiated luminal A, luminal B, hormone positive, HER2 subtypes, and lowest in the less differentiated basal-like and triple negative subtypes (3). With bone metastases being so prevalent within breast cancer patients, there is a need to better understand the process of metastatic tumor growth in the bone by breast cancers in order to increase patient survival.

1.2 NORMAL BONE PHYSIOLOGY

Upon metastasis to and progression in the bone, breast cancers tend to cause osteolysis, or bone degradation. To understand the effects of metastatic breast cancer and its ability to disrupt the bone microenvironment, it is important to understand normal bone physiology. Bone is constantly being remodeled to account for physical changes in its strength, as well as regulation of the homeostatic levels of calcium and phosphate (reviewed in 7). In healthy individuals this is a tightly regulated process involving two major cells types, the osteoblast and the osteoclast. These two cell types work in tandem to keep a healthy bone density through constant remodeling of old bone by osteoclasts, with deposition of new bone by osteoblasts (8). This process has many sequential phases, activation, resorption, reversal, formation and mineralization (9).

Remodeling starts through secretion of factors such as parathyroid hormone (PTH) that cause osteoblasts to secrete factors that activate osteoclast precursors by macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa β ligand (RANK-L) which then leads to the activated cells fusing into large multinucleated osteoclasts (10). These osteoclasts attach to the bone matrix through integrins to form a sealing zone on the bone surface which allows them to create an acidic compartment beneath them (11). This change in pH in the created compartment occurs through secretion of hydrogen ions by an ATPase H Pump (12). The osteoclasts will then secrete lysosomal enzymes such Cathepsin K, and Tartrate resistant acid phosphatase (TRAP) that will work optimally in the acidified environment to degrade the bone matrix causing a resorption lacuna (or resorption pit) (13).

In the reversal phase, these resorption sites attract pre-osteoblasts to these remodeling sites to begin their differentiation (9). One possible mechanism for this induced chemotaxis is through osteoclast secreted platelet-derived growth factor bb (PDGF-bb) that binds the PDGF receptor on osteoblasts (14). Sanchez-Fernandez *et al*. (2008), found that when PDGF-bb was depleted in the pre-osteoclast cell line RAW 264.7, they observed impaired chemotaxis of the pre-osteoblast murine cell line MC3T3 E1 cells (14). Moreover, this impairment was rescued through addition of recombinant PDGF-bb (14). In normal bone turnover, following the recruitment of osteoblasts to the sites of resorption, osteoclasts subsequently undergo apoptosis, and are replaced by differentiating osteoblasts (9). Further, the release of factors from the bone matrix including transforming growth factor β (TGF-β) causes proliferation of pre-osteoblasts and stimulates their differentiation (9,15,16). With differentiation into mature osteoblasts, these cells begin to secrete osteoid matrix such as type I collagen that will begin mineralization of hydroxyapatite (9). Osteoblasts will then either undergo apoptosis or will become entombed by the matrix and become osteocytes (9).

1.3 OSTEOCLASTS

Osteoclasts are derived from hematopoietic stem cells. From the monocyte common progenitor lineage they become mononuclear osteoclasts before fusion into large, multinucleated osteoclasts through stimulation with RANK-L and M-CSF (Figure 1a) (17-19,22). The importance of M-CSF in osteoclast differentiation was established when it was observed that *Op/op* mice which lack functional M-CSF, are osteopetrotic (meaning stone bone, where bones increase in density) due to a lack of osteoclasts (17-19). M-CSF acts to induce pre-osteoclast proliferation, helps induce their differentiation and bone resorption while also promoting their survival (17-19). RANK-L was identified simultaneously by multiple groups as osteoprotegrin ligand (OPGL), and TRANCE (20-22). It is essential for osteoclast differentiation, with RANK-L knockout mice also displaying an osteopetrotic phenotype due to a lack of osteoclasts (23).

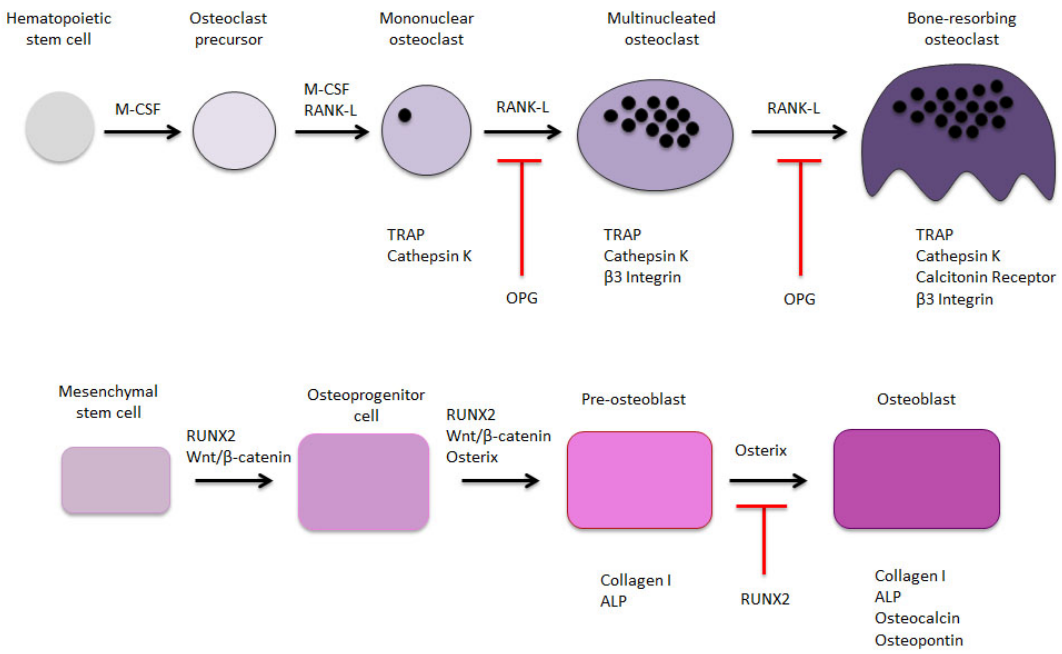

Hematopoietic stem cell
Osteoclast precursor
Mononuclear osteoclast
Multinucleated osteoclast
Bone-resorbing osteoclast
M-CSF
M-CSF
RANK-L
RANK-L
RANK-L
TRAP
Cathepsin K
OPG
TRAP
Cathepsin K
β3 Integrin
OPG
TRAP
Cathepsin K
Calcitonin Receptor
β3 Integrin
Mesenchymal stem cell
RUNX2
Wnt/β-catenin
Osteoprogenitor cell
RUNX2
Wnt/β-catenin
Osterix
Pre-osteoblast
Osterix
Osteoblast
Collagen I
ALP
RUNX2
Collagen I
ALP
Osteocalcin
Osteopontin

Figure 1. Differentiation steps of osteoclasts and osteoblasts. A) Osteoclasts are derived from hematopoietic stem cells. They are expanded into bi-potential osteoclast precursors or monocytes using M-CSF. Through exposure to RANK-L they become TRAP positive mononuclear osteoclasts and with fusion to other mononuclear osteoclasts they become large, multinucleated, TRAP positive cells. B) Osteoblasts are derived from mesenchymal stem cells. They become osteoprogenitor cells with expression of runt-related transcription factor 2 (RUNX2) and Wnt/β catenin. With expression of RUNX2 and Osterix, they become pre-osteoblasts that express Collagen I and alkaline phosphatase (ALP) before differentiating into mature osteoblasts that are high in osteocalcin. This process can take up to 21 days.

In order to differentiate into mature osteoclasts, multiple transcription factors are activated. First, PU.1 is an important mediator in differentiating hematopoietic stem cells into monocyte/myeloid cells that are capable of responding to M-CSF (24). Mice lacking PU.1 are not only osteopetrotic due to a lack of osteoclasts, but have a lack of macrophages within the bone marrow (24). Once M-CSF binds to its receptor colony stimulating factor 1 (CSF1R), it induces survival of the lineage through activation of micropthalmia transcription factor (MITF), with MITF mutant mice also displaying an osteopetrotic phenotype (25,26). RANK-L can then bind to receptor activator of nuclear factor kappa β (RANK) on pre-osteoclasts to commit the cells to an osteoclast fate. RANK-L is responsible for activation of c-Fos, nuclear factor kappa β (NFKβ), and nuclear factor of activated T-cells (NFATc1) (27). NFATc1 transcriptional activities are modulated by calcineurin, a calcium sensitive phosphatase that allows for nuclear translocation of the NFAT proteins (27). It is not surprising that RANK-L also induces calcineurin activity through sustained Ca2+ oscillation, allowing for nuclear localization of NFATc1 (27). NFATc1 can then act with c-Fos to induce transcription of osteoclast genes such as TRAP and Calcitonin receptor (27). There are therefore many transcription factors and signaling pathways to consider when investigating the differentiation of osteoclasts.

Osteoclastogenesis can be inhibited by osteoprotegrin (OPG), a soluble decoy receptor for RANK-L (28). Recent work has provided evidence that OPG can function to regulate RANK-L trafficking within the osteoblast (29). OPG-/- osteoblasts showed accumulation of RANK-L in the Golgi apparatus, which resulted in increased trafficking to the cell surface where it could therefore bind to the RANK receptor on osteoclasts (29). Rescue experiments introducing recombinant OPG caused RANK-L to localize to the lysosome (29). The balance of OPG and RANK-L levels thus controls osteoclastogenesis, with a shift towards either one initiating less or more osteoclastogenesis, respectively.

Mature osteoclasts have a highly polarized structure, with the sealing zone and ruffled border on the basal side of the cell in contact with the bony matrix (Figure 2). The sealing zone is formed from a large F-actin ring rich in integrins, specifically α5β3, which anchors the osteoclast to the bone matrix signaling (11).

The osteoclast is then able to create an acidic pH using carbonic anhydrase II, and H+APTase to remove the mineralized portion of the bone thereby forming a resorption lacuna (12). This low pH allows for optimal enzyme function, such as Cathepsin K activity, which degrades collagen and other organic matrix components (12). These bone degradation products are transported by transcytosis to the apical side of the cell (30).

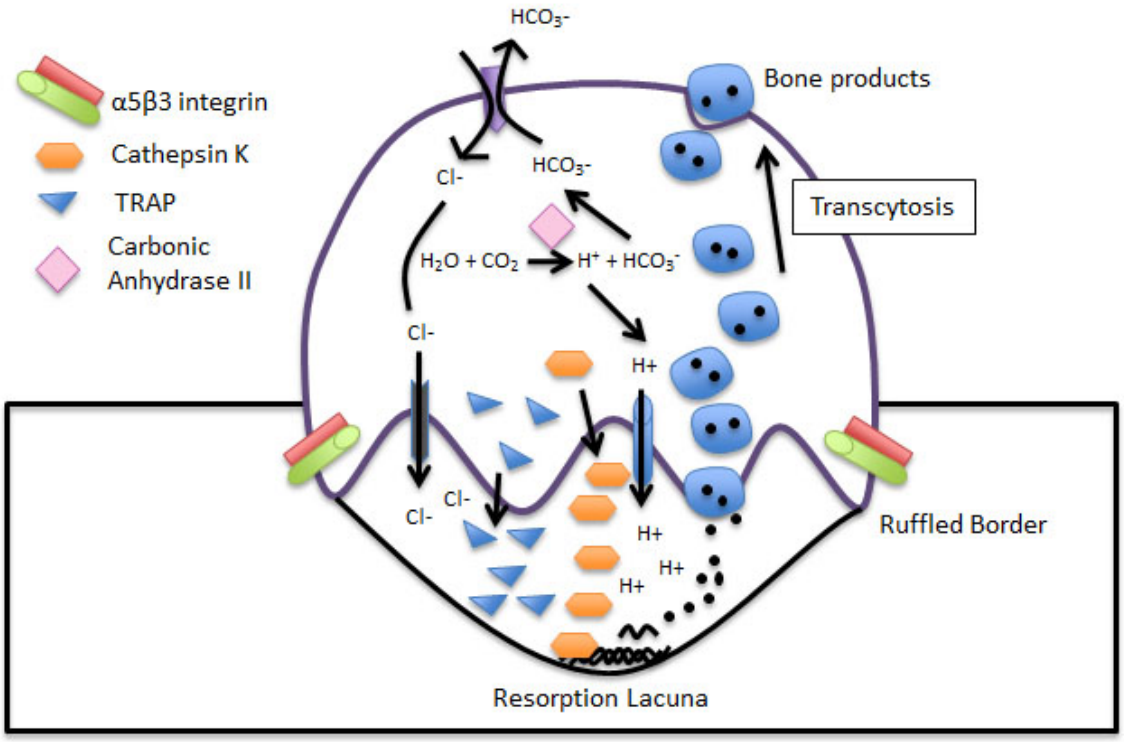

α5β3 integrin
Cathepsin K
TRAP
Carbonic Anhydrase II
HCO3-
Bone products
Cl-
HCO3-
Transcytosis
H2O + CO2
H+ + HCO3-
Cl-
H+
Cl-
Cl-
H+
H+
H+
Ruffled Border
Resorption Lacuna

Figure 2. Osteoclasts induce osteolysis through secretion of proteolytic enzymes. Mature osteoclasts fuse with the bone extracellular matrix through α5β3 integrin to create a sealing zone. The osteoclast is highly polarized, and forms a ruffled border on the basal side of the cell through which it secretes and transports various products. Carbonic anhydrase II works to create H^+ to transport across the ruffled border to induce an acidic pH to allow optimal conditions for proteolytic enzymes. The osteoclast also secretes enzymes such as Cathepsin K that can degrade collagen. This creates a resorption lacuna underneath the osteoclast, where osteolysis takes place. Bone products can be transported to the apical side through transcytosis.

Also released into resorption lacuna is the secreted protein TRAP, which is used as a histochemical marker of osteoclasts and marker for bone turnover (31,32). It was implicated in bone development through the generation of TRAP -/- mice by targeted gene disruption, with mice showing skeletal abnormalities, including increased bone density with age (33). Studies have therefore focused on elucidating the mechanisms by which TRAP affects the bone. It was found that TRAP dephosphorylates osteopontin (OPN), a protein responsible for attaching osteoclasts to the bone matrix, causing osteoclasts to be released from their attachment to the bone surface (34). With osteoblasts differentiating preferentially around resorption lacuna created by the osteoclast, the effects of TRAP on osteoblasts were assessed. TRAP was found to induce expression of the phenotypic markers RUNX2, OPG, and collagen I by osteoblasts, inducing their differentiation (35). Thus, TRAP is an important regulator of both osteoclast and osteoblast function.

1.4 OSTEOBLASTS

Osteoblasts are derived from mesenchymal stem cells, which along with osteoblasts can also differentiate into other cells including adipocytes, chondrocytes and myoblasts. There are three main transcription factors necessary for their differentiation into mature osteoblasts, RUNX2, Osterix and canonical Wnt/β-catenin signaling (Figure 1b) (36-39). Both RUNX2-null and Osterix-null mice show a lack of bone formation, with RUNX2-null mice showing no Osterix expression, indicating that Osterix is downstream of RUNX2 in the osteoblast differentiation lineage (36-38). Mice lacking low-density lipoprotein receptor protein 5 (LRP5), a Wnt co-receptor, show low bone mass and decreased osteoblast proliferation (39), with mutations in LRP5 causing the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (40).

Transcription and activity of RUNX2, also known as Cbfa1, is regulated by multiple factors including bone morphogenic protein 2 (BMP2), parathyroid hormone related protein (PTHrP),

fibroblast growth factor (FGF), and binding of the extracellular matrix to integrins (reviewed in 41). The expression and activation of RUNX2 initiates differentiation into a pre-osteoblast by inducing expression of bone matrix proteins such as type I collagen, osteopontin (OPN) and osteocalcin (41). Osteocalcin, the only true osteoblast specific marker, functions as a non-collagenous protein which binds calcium and is thought to regulate bone metabolism by limiting bone formation (42). Canonical Wnt/β-catenin signaling is then responsible for securing the pre-osteoblast into an osteoblast by inhibiting their differentiation into chondrocytes, with precursor cells lacking β-catenin expression differentiating into chondrocytes rather than osteoblasts (43). Canonical Wnt signaling, as reviewed by Macdonald et al. (2009), is activated when a secreted Wnt ligand such as Wnt3a binds to a Frizzled receptor and one of its co-receptors LRP 5 or 6 (44). This activation results in the accumulation of β-catenin, allowing it to translocate to the nucleus, form a complex with its DNA-bound partner T cell factor/lymphoid enhancer factor (TCL/LEF), and induce transcription of Wnt targets (44). This pathway was shown to be responsible for inducing ALP expression, with Wnt-3a inducing ALP activity in C3H10T1/2 mesenchymal cells (45). Further, inhibition with a BMP inhibitor, Noggin, also decreased the ability of Sonic hedgehog (Shh), another stimulator of osteoblast differentiation, and BMP2, a protein that induces its signal transduction through SMADs, to induce ALP expression (45,46). Osterix expression, which is downstream of RUNX2 activity and Wnt/β-catenin signaling, is also required for mature osteoblast differentiation (47,48). Interestingly p53 inhibits the expression of Osterix, and thus acts as a repressor of osteoblast differentiation (49).

A major initiator of osteoblast differentiation is the extracellular matrix. Once osteoblasts begin differentiating they deposit large quantities of extracellular matrix such as type I collagen (50). Collagen I has been shown to be required for ALP expression, as treatment of ascorbic acid stimulated MC3T3 cells with a collagen I inhibitor 3,4-dihydropyran blocked ALP expression

(50). Moreover pre-osteoblasts plated on type I collagen show an increase in ALP expression (51,52). This induction of osteoblast differentiation by the extracellular matrix was modulated by integrin signaling (52). Blockade of α1 or α2 integrin reduced BMP-2 induction of ALP, with dual blockade of both subunits approaching the effects of collagen I blockade in early differentiating osteoblasts (52). Further it was shown that blockade of α2β1 integrin, a known collagen I receptor, decreased osteoblast mineralization in a dose-dependent and reversible manner (53). It is therefore important to consider multiple pathways when assessing the factors affecting bone disease.

Osteoblasts are responsible for the deposition of new bone through secretion of extracellular matrix proteins, followed by the initiation of mineralization. The organic components include collagen, and non-collagenous proteins such as, OPN, secreted protein acidic and cysteine rich (SPARC) and bone sialoprotein (BSP). These non-collagenous proteins are thought to modulate collagen mineralization through binding of Ca2+, while also being highly phosphorylated and therefore serving as substrates for enzymes such as ALP (54,55). The mineralized component of the bone, hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, is thought to be regulated by osteoblast matrix vesicles (MV). MVs, as reviewed in Golub (2009), arise from budding off of the osteoblast plasma membrane and are rich in annexins, ALP and matrix metalloproteinase proteins (MMPs) (56). Although the mechanism by which MV cause mineralization is not well defined, there are multiple theories (56). In one theory, MVs serve as the initiation site for apatite formation where Annexins allow for internalization of Ca2+, while the type III Na+ pump transports PO_4^{3-} into the MV to initiate apatite formation within the vesicle (56). Other theories suggest that MV solely regulate ion concentrations, where ALP functions to lower the concentration of pyrophosphate (PPi), an inhibitor of mineralization, and increase the concentration of inorganic phosphate (Pi),

an inducer of mineralization (56,57). The apatite is then deposited into collagen fibrils, causing mineralization of the bone.

1.5 BREAST CANCER POTENTIATES A VICIOUS CYCLE IN THE BONE

Breast cancer most often metastasizes to the bone, with 69% of advanced stage patients having bone metastases (58). For bone metastases to occur, cells must detach from the basement membrane and disseminate from the primary tumor; this occurs through production of enzymes such as MMPs or through loss of tight junctions through loss of E-cadherin (59,60). Cells then enter the blood stream and are attracted metastatic sites through various signals. One of these signals is RANK-L, where tumors that express its receptor RANK, may be attracted towards the bone where RANK-L is highly expressed on osteoblasts (61). Breast cancer cells expressing C-X-C Chemokine receptor type 4 (CXCR4) are attracted to their ligand stromal derived factor 1 (SDF-1), which is also expressed on osteoblasts (62). Other signals include matrix proteins such as collagen I and fibronectin that attract the tumor cells to the bone. Interestingly, breast cancer cells have also been shown to induce a pre-metastatic niche through secretion of lysyl oxidase (LOX) (63,64). Inhibition of breast cancer LOX expression significantly reduced osteolytic lesions (64). Once metastatic cells arrive in the bone, they disrupt normal bone homeostasis by secreting many factors. Mundy (1997) first described breast cancer as potentiating a vicious cycle within the bone microenvironment through secretion of factors that act on bone cells, which then induces the bone cells to secrete factors that act on the tumor to induce its proliferation (reviewed in 65). The vicious cycle is depicted in Figure 3. Here, studies suggest that an increase PTHrP secretion from the tumor cells induces increased osteoclast activity (66). The importance of PTHrP in bone metastases was seen when its inhibition using a neutralizing antibody reduced the number of osteolytic bone lesions (66). Other factors including M-CSF and interleukins such as IL-6 and IL-11 have also been implicated in mediating osteolytic disease, with IL-11 sustaining the pre-osteoclast population (67).

These soluble factors can also act directly on pre-osteoclasts to induce their proliferation and differentiation. Mancino *et al.,* (2001) showed that MDA-MB-231 cells produce sufficient M-CSF to induce osteoclastogenesis, as evidenced by the ability of murine hematopoietic cells cultured with MDA-MB-231 cells and RANK-L being able to differentiate into multinucleated osteoclasts (68). Moreover, they showed that conditioned media from MDA-MB-231 cells was able to induce macrophage differentiation, which was inhibited by an M-CSF antibody (68). These factors can also act indirectly on osteoblasts to induce osteolysis. One mechanism by which these factors induce osteolysis is by changing the ratio of RANK-L/OPG resulting in osteoclastogenesis. Osteoblasts cocultured with MDA-MB-231 or MCF7 cells show an increase in RANK-L expression and a decrease in OPG expression (69,70). RANK-L can then bind to the RANK receptor on pre-osteoclast causing them to differentiate into large, multinucleated cells capable of resorbing bone. Breast cancer cells thus produce M-CSF and cause an increase in RANK-L levels that are sufficient to induce osteoclastogenesis.

Bone-derived growth factors play an important role in perpetuating the vicious cycle. Many growth factors that are produced remain embedded within the bone matrix, and become released through osteoclastic resorptive enzymes such as MMPs making them available for binding to tumor cells (71). These factors include TGF-βs, bone morphogenetic proteins, insulin-like growth factors, and platelet-derived growth factors (71). These factors can then act back on the tumor to induce its proliferation and further secretion of factors to induce osteolysis. Yin *et al.,* (1999) showed the importance of TGF-β secretion in this cycle, when MDA-MB-231 cells transfected with a dominant negative mutant TGF-β type II receptor displayed reduced bone destruction and increased survival compared to control (72). They went on to show that the addition of a constitutively active TGF-β type I receptor increased breast cancer secretion of PTHrP, increased osteolytic lesions, and decreased survival (72). This process is depicted in Figure 3.

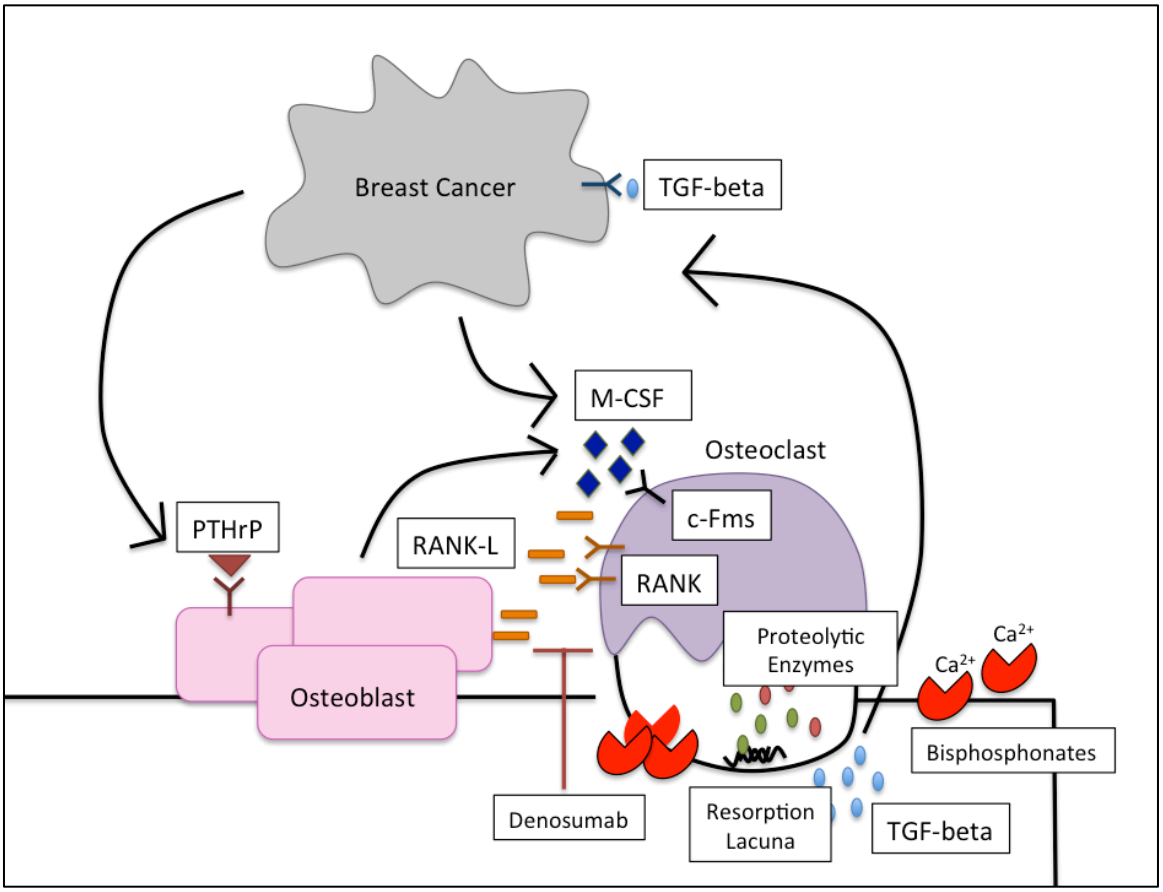

Breast Cancer
TGF-beta
M-CSF
Osteoclast
c-Fms
PTHrP
RANK-L
RANK
Proteolytic Enzymes
Ca2+
Ca2+
Osteoblast
Bisphosphonates
Denosumab
Resorption Lacuna
TGF-beta

Figure 3. Breast cancer potentiates a vicious cycle. When breast cancer cells invade the bone microenvironment they secrete soluble factors such as PTHrP. This can bind to the osteoblast and cause them to secrete RANK-L and M-CSF. These factors can bind to the osteoclast via the RANK and c-FMS receptors respectively, and induce osteoclast activation. This includes the secretion of proteolytic enzymes into the resorption lacuna that directly degrade the bone. A byproduct of osteolysis is the release of factors within the bone such as TGF-β. Free TGF-β can then act back on the tumor to directly induce tumor cell growth and their further secretion of PTHrP, potentiating a vicious cycle of bone metastasis favoring osteolysis and tumor growth.

While breast cancer induces osteolysis, it also inhibits new bone formation through inhibiting osteoblast maturation. When pre-osteoblast MC3T3 E1 cells were treated with MDA-MB-231 conditioned media, it blocked the induction of AAB as shown by a down regulation in expression of osteoblast markers BSP, ALP and osteocalcin while also inhibiting mineralization (73). This process was inhibited with the addition of a neutralizing antibody to TGF-β to the MDA-MB-231 conditioned media (73). In another study, the knockdown of Dkk1, a Wnt/β-catenin antagonist, in MDA-MB-231 cells was able to restore osteoblast differentiation as shown by an increase in ALP and OPG expression (74). Thus breast cancer not only induces osteolysis through induction of osteoclastogenesis, but also inhibits the deposition of new bone by inhibiting osteoblastogenesis. As this process continues over time, the bone continues to stay in an osteolytic state and as a result induces skeletal related events (SRE), which include fractures, hypercalcemia and pain. Although treatments have been developed to aid in the reduction of SRE, these bone metastases remain incurable and patients ultimately succumb to their metastatic disease.

1.6 TREATMENT OF BONE METASTASES

Current breast cancer treatments include endocrine therapy (for hormone positive subtypes), chemotherapies, and radiation with recent efforts having developed bone specific treatments. These bone specific treatments include bisphosphonates such as zoledronic acid and monoclonal antibodies such as denosumab. Bisphosphonates are chemically stable inorganic pyrophosphate (PPi) analogues, which preferentially go to the bone due to their affinity to bind hydroxyapatite (reviewed in 75). Once bound, the osteoclasts ingest them through resorption, which results in the inhibition of the mevalonate pathway (76). This causes inhibition of resorption by osteoclasts while also inhibiting hydroxyapatite calcification (76). More recently, Denosumab, a monoclonal antibody that targets RANK-L, an activator of osteoclastogenesis, has been assessed for the use in

breast cancer bone metastases (77). Recent studies have shown that Denosumab was more effective at reducing skeletal related events than bisphosphonates (78,79). Although both of these drugs have been shown to decrease the time to the first SRE, and the number of SRE, neither have increased patient overall survival (80). Moreover, recent studies have shown that while the bisphosphonate zoledronic acid can reduce TRAP serum levels, it also decreases osteocalcin levels, a marker of new bone formation and decreases osteoblast viability *in vitro* (81). As such, while it may inhibit further bone degradation, it does not result in restoration of bone formation due to the potential inhibition of osteoblasts. Thus, there is a need to assess novel targets for the treatment of breast cancer bone metastasis to improve bone homeostasis and ultimately increase patient survival.

1.7 FOCAL ADHESION KINASE

Focal adhesion kinase (FAK) is a 125 kDa non-receptor tyrosine kinase that is highly conserved among mammals including humans and mice (82,83). Its N-terminus contains a four-point-one ezrin, radixin, moesin (FERM) domain that confers its ability to bind integrins and growth factor receptors including epidermal growth factor receptor (EGFR) (84). The FERM domain also forms an intra-molecular interaction with its kinase domain in its inactive state (85). Recent data has also identified that the FERM domain contains a nuclear localization signal (NLS), and two nuclear export signals (NES) that reside within the FERM domain and the kinase domain (86,87). However it has been suggested that it is the NES within the kinase domain modulates the nuclear trafficking of FAK (87). The C-terminus of FAK contains a focal adhesion targeting (FAT) domain that targets FAK to the cytoskeleton in focal adhesions, which are large assemblies of proteins that mediate binding to the extracellular matrix (88). Moreover, the C-terminus contains two proline rich regions (PRR) that act as docking sites for SH3 domain-containing proteins such as p130cas, and Graf (89,90). Lastly, FAK contains a central kinase domain which once activated induces a conformational change that allows for auto-

phosphorylation at tyrosine 397 (Y397) (91). This phosphorylation confers FAKs activity through binding of SH2 domain-containing proteins such as Src, allowing for activation of many downstream signaling pathways (91). Its structure is depicted in Figure 4a.

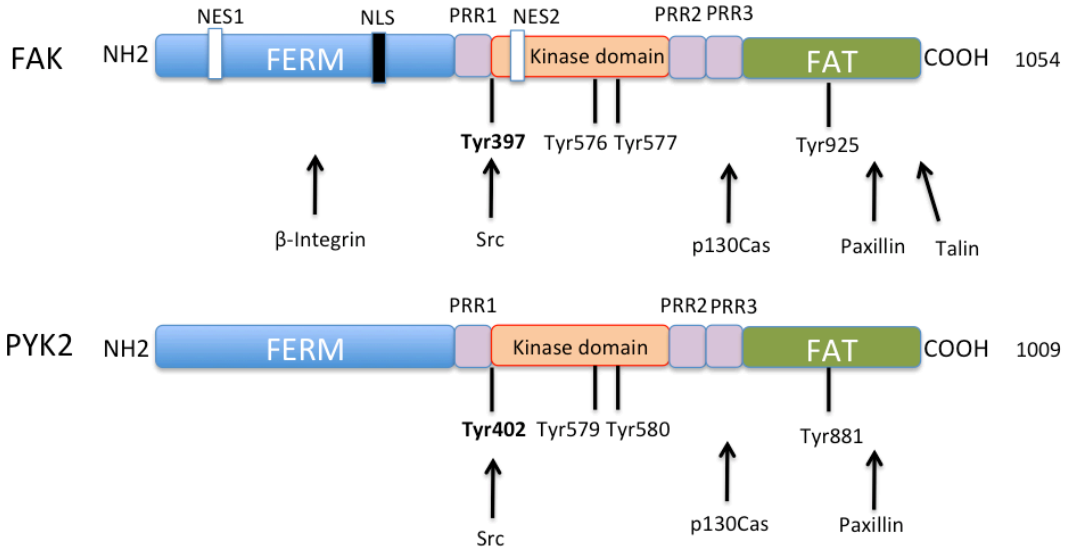

FAK
NH2
NES1
NLS
PRR1
NES2
PRR2 PRR3
FERM
Kinase domain
FAT
COOH
1054
Tyr397
Tyr576 Tyr577
Tyr925
β-Integrin
Src
p130Cas
Paxillin
Talin
PYK2
NH2
PRR1
PRR2 PRR3
FERM
Kinase domain
FAT
COOH
1009
Tyr402
Tyr579 Tyr580
Tyr881
Src
p130Cas
Paxillin

Figure 4. FAK and PYK2 family tyrosine kinases. A) The N terminus of FAK contains a FERM domain that confers its ability to interact with β integrins and some growth factor receptors. Its kinase domain becomes activated through auto-phosphorylation of Y397, with phosphorylation of Y576 and Y577 by Src enhancing its affects. Y397 becomes an SH2 binding domain for Src to bind. There are three proline-rich regions (PRR). The C-terminus of FAK contains a FAT domain where proteins such as paxillin and talin can bind. B) PYK2 also contains a FERM, kinase and FAT domain with approximately 45% homology to FAK. Some differences include the tyrosine residues at which phosphorylation occurs, with activation being induced by auto-phosphorylation of Y402.

FAK null mutation in mice induced embryonic lethality at embryonic day 8.5 with a phenotype similar to that of a fibronectin-deficient embryo, which lacked mesoderm development (92). Therefore, important lessons regarding the function of FAK came through the generation of FAK null (-/-) cells. In FAK null fibroblasts, it was shown that although FAK is not required for focal adhesion formation, it reduced focal adhesion maturation (92). It also hindered focal adhesion turnover, with many focal adhesion proteins including talin and paxillin, showing increased expression in FAK null cells (92). These findings suggest an important role for FAK in mediating focal adhesions, and therefore attachment and signaling from the extracellular matrix.

1.7.1 CYTOPLASMIC FAK

Amongst the most prominent activators of FAK are integrins. Integrins are transmembrane receptors composed of an α and β subunit that bind to extracellular matrix proteins and allow for intracellular signal transduction (93). There are 18 α subunits and 8 β subunits, with different combinations accounting for different ligand specificity (93). For example, α(1) or α(2) subunit paired with a β (1) subunit binds type I collagen, a prominent extracellular matrix protein in the bone (93). When bound to their extracellular matrix subunit, integrins cluster forming focal contacts that are assembled and disassembled in order to propel cell movement (94). These contacts include many other proteins including FAK, talin, and paxillin that aid in focal adhesion turnover, creating intracellular signaling, as well as acting as intracellular scaffolding (95). Once integrins are activated, they can bind to the FERM domain of FAK, which removes FAKs auto-inhibition causing subsequent activation through Y397 auto-phosphorylation (96). This phosphorylated site can then bind to Src, creating the FAK-Src complex capable of initiating many downstream signaling pathways (Figure 5) (97,98).

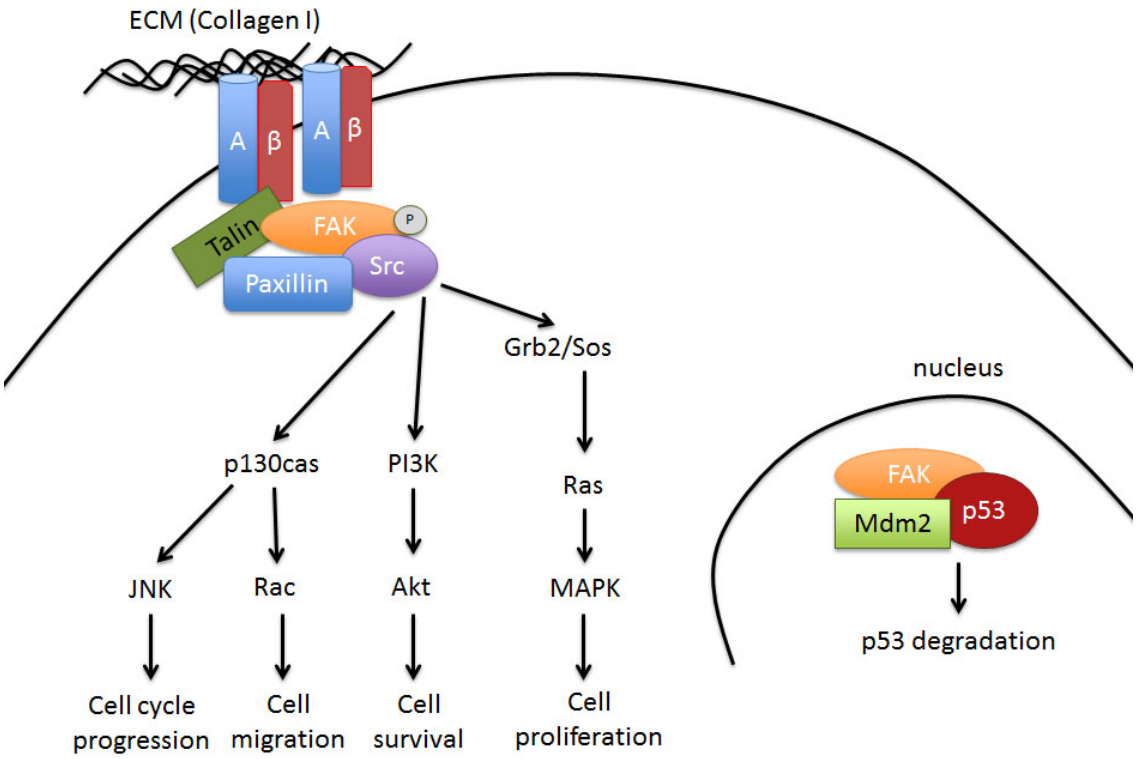

ECM (Collagen I)
A
β
A
β
Talin
FAK
P
Src
Paxillin
Grb2/Sos
p130cas
PI3K
Ras
JNK
Rac
Akt
MAPK
Cell cycle progression
Cell migration
Cell survival
Cell proliferation
nucleus
FAK
Mdm2
p53
p53 degradation

Figure 5. FAK induces many downstream signaling pathways. One of the major components of bone is the extracellular matrix protein collagen I. Collagen I is a potent activator of integrins, which induce the activation of FAK. FAK can then bind with talin and paxillin, or with Src and create the FAK-Src complex. The FAK-Src complex can activate many cytoplasmic pathways that include p130Cas that can induce cell cycle progression through JNK or cell migration through Rac. It can also activate phosphatidylinositol 3-kinase (PI3K) that leads to Akt phosphorylation and cell survival. The FAK-Src complex can also activate Grb2/Sos to induce cell proliferation through MAPK signaling. FAK can also translocate to the nucleus where it can act as a scaffold for Mdm2 and p53 to allow for ubiquitin mediated p53 degradation.

There are many substrates of the FAK-Src complex. p130cas was shown to bind FAK through its SH3 domain in mouse fibroblasts (99). It is phosphorylated in a FAK-dependent manner, but through Src-mediated phosphorylation, where Y397 phosphorylated FAK binds both Src and p130Cas simultaneously resulting in Src phosphorylating Cas (100-102). When the proline rich region of FAK that binds p130cas was mutated P712/715A, p130cas-FAK association was decreased and fibronectin stimulated cell migration was impaired (103). Further, mutation of p130cas SH3 domain in FAK overexpressing cells also resulted in decreased cell migration (103). Downstream, phospho-p130cas leads to activation of Rac1, which causes membrane ruffling and lamellipodium formation (102,104). The FAK-Src complex can also phosphorylate paxillin, a vinculin-binding protein (105). Paxillin phosphorylation can then modulate vinculin, which regulates F-actin binding to the membrane creating focal adhesions and cell adhesion with the extracellular matrix (105). The FAK-Src complex therefore plays important roles in cell migration and attachment.

Further to its role in cell migration, it has been found that FAK can enhance cell invasion through secretion of MMP enzymes responsible for the degradation of ECM proteins such as collagen. In ovarian cancer cells, it was found that FAK was required for fibronectin induced MMP-9 secretion (106). Moreover, a FAK Y397F mutant had reduced MMP-9 expression compared to control wild type FAK expressing OPN stimulated chondrosarcoma cells (107). Sein *et al.,* (2000) also established the role of FAK in stimulation of MMP-2 and MMP-9 secretion induced by concanavalin A, an inducer of MMP secretion (108). In lung cancer, FAK was shown to regulate MMP secretion, where expression of the naturally occurring truncated form of FAK containing the C-terminus, FAK-related non-kinase (FRNK) that localizes to focal contacts and inhibits normal FAK function, eliminated MMP-9 expression (109,110). Taken together, these studies suggest that FAK can regulate secretion of MMP, and thus cellular migration in many cell types. Therefore, it is important to consider the effects of FAK in bone metastases where there is

a prominent accumulation of extracellular matrix proteins and secretion of MMP to regulate extracellular matrix remodeling.

The activation of the FAK-Src complex can also lead to activation of the mitogen activated protein kinase (MAPK) and PI3K signaling pathways. In integrin-engaged fibronectin plated NIH3T3 cells, FAK was found to co-immunoprecipitate with Grb2 and Sos, which resulted in MAPK activation (111). Grb2-FAK binding was abrogated by mutating the FAK phosphorylation site Y925 (111). FAK can also mediate cell survival signaling through PI3K activation. In fibroblasts, β1 integrin binding to collagen I was shown to activate FAK phosphorylation, which in turn phosphorylated PI3K and Akt through Src leading to cell survival (112). Overexpression of a FRNK construct in the same cells decreased PI3K and Akt phosphorylation, and induced apoptosis (112). Taken together, integrin activation of the FAK-Src complex has important ramifications for cell survival.

FAK also plays a role in cell survival through regulating the tumor suppressor p53. FAK was shown to regulate apoptosis in a p53-mediated manner in anchorage dependent serum-starved fibroblasts (113). Further, FAK was found to directly interact with p53 and inhibit its transcriptional activity, with FAK suppressing p53-mediated luciferase activity of p21, mouse double minute 2 homolog (Mdm-2) and BAX (114). Interestingly, assessment of FAK-/- embryo lysates found accumulation of p53, and its responsive genes such as Mdm-2 (86). Introduction of a p53 mutation into FAK-/- embryos did not restore embryonic viability, however p53-/-FAK-/- cells did proliferate more readily as compared to p53+/+FAK-/- cells (86). These findings regarding p53, a largely nuclear protein, led researchers to further evaluate a putative role for nuclear FAK.

1.7.2 NUCLEAR FAK

With both a NLS and NES sequence, FAK is able to traffic in and out of the nucleus. Through further investigation, Lim *et al.,* (2008) found that FAK regulated p53 turnover with FAK residues 1-402 (which contains the NLS sequence) causing a greater than 80% reduction in p53 levels in FAK-/-p21-/- fibroblasts (86). This effect was shown to be mediated by nuclear FAK when K190/191A or R177/178A mutations were introduced into the FERM domain, and the protein was localized to the cytoplasm and did not reduce p53 levels (86). The FERM domain was found to create a scaffold that bound both p53 and Mdm-2 leading to the ubiquitination and degradation of p53, which could be blocked with the proteasome inhibitor MG132 (86). Lastly, they showed that a decrease in total FAK led to accumulation of p53, causing cells to be more susceptible to cisplatin treatment (86). This effect was regulated by the FAK FERM domain, where overexpression of FAK containing a mutated R177/178A FERM could not protect the cells from apoptosis (86).

More recently, a role for nuclear FAK has been established in controlling chemokine transcription and modulating tumor-immunity. In a syngeneic mouse model, FAK-/- squamous carcinoma cells (SCC) regressed within 21 days of implantation, compared to normal tumor growth found with FAK+/+ cells (115). This was attributed to an accumulation of immune-suppressive Tregs, caused by FAK inducing transcription of C-C motif chemokine ligand 5 (CCL5), a chemokine involved in recruitment of Tregs, and TGFβ2, a protein involved in Treg expansion (115). Here, nuclear FAK was able to co-immunoprecipitate with TBP-associated factor 9 (TAF9), part of the transcription factor IID complex that is predicted to regulate CCL5 (115). Moreover, through interaction network analysis, FAK was predicted to bind with AP-1, C/EBP and NFKβ, also supporting its putative role in regulation of transcription factor activity (115). Interestingly, SCC cells overexpressed for a FAK mutant R177/178A and K190/191/216/218A incapable of

translocating to the nucleus showed the same tumor regression as the FAK-/- SCC tumor cells (115). Thus, nuclear FAK plays an important role in regulating tumor immunity.

1.7.3 FAK FAMILY PROTEIN, PYK2

When discussing FAK, it is important to discuss its family member protein tyrosine kinase 2 beta (PYK2) with approximately 45% sequence homology (Figure 4b)(116,117). Unlike FAK null mice that display embryonic lethality, PYK2 null mice are viable but display mild osteopetrosis and impairment of macrophage migration (116,117). PYK2 activation has been linked to many stimuli, including increased intracellular Ca2+, engagement of integrins, and engagement of immune receptors from T cells, B cells and mast cells (117-119). It has both Cas and paxillin binding sites, but unlike FAK, it does not bind talin.

Although they display different tissue distributions, with PYK2 being more localized to hematopoietic and neuronal tissues, PYK2 has been shown to compensate for some functions of FAK in FAK depleted cells. In one study, PYK2 expression was elevated in FAK-/- fibroblasts compared to FAK+/+ control cells (120). Upon fibronectin stimulation of these cells, PYK2 was activated in FAK-/- cells but not in FAK +/+ cells (120). Although it was not sufficient to overcome the FAK-/- decreased migration, PYK2 may become activated in attempt to compensate for FAK (120). In mammary cancer stem cells (MaCSC) lacking FAK, PYK2 was increased in metastatic lung nodules compared to control FAK expressing MaCSC lung nodules (121). The dual FAK/PYK2 small molecule ATP mimetic inhibitor PF-271 but not the selective FAK inhibitor PF-573,228 was able to abolish these lung nodules (121). This suggests a compensatory role for PYK2 in establishing metastatic sites in FAK depleted cells. *In vivo*, both FAK and dual FAK/PYK2 inhibition decreased the metastatic ability of FAK expressing MaCSC when injected into the tail vein, however only the dual FAK/PYK2 inhibitor was able to decrease

metastatic ability of FAK depleted MaCSC (121). These studies provide evidence supporting the use of dual FAK and PYK2 inhibitors in order to block any compensatory effects of PYK2.

1.8 FAK IN OSTEOBLASTS

To assess the role of FAK in osteoblast differentiation, Kim *et al.,* (2009) generated FAK-null osteoblasts by transducing isolated osteoblasts from FAK fl/-;p53-/- and FAK fl/fl;p53-/- double transgenic mice with Cre to remove any remaining FAK (122). They found that 30% of $FAK^{-/-}$ ($p53^{-/-}$) calvarial clones were able to differentiate into osteoblasts. Further, by crossing FAK floxed mice with Cre mice containing the collagen type 1 α1 promoter they generated osteoblast specific FAK mutant embryos (122). Interestingly, there were no skeletal defects throughout their development (122). Adult FAK mutant osteoblasts showed delayed bone formation in response to injury, with a decrease in ALP activity (122). However, the compensatory mechanism of PYK2 for FAK has not been well defined in osteoblasts, with FAK null osteoblasts having increased PYK2 phosphorylation and localization to focal adhesion (122). In MC3T3 cells, ALP activity was shown to be reduced in cells transfected with antisense FAK (asFAK) (123). In another study, expression of asFAK was shown to inhibit BMP-2 induced ALP activity, osteocalcin expression and mineralized nodule formation in MC3T3 cells (124). This inhibition was attributed to decreased Smad1 transcriptional activity, a signal transducer of BMP, as demonstrated by decreased levels of the Smad1 target gene Smad6 in BMP-2 stimulated asFAK cells (124). Thus FAK may play an important role in osteoblast differentiation, and as such it is important to assess osteoblast activity when using FAK inhibition.

1.9 FAK IN OSTEOCLASTS

Both FAK and PYK2 protein levels were increased in differentiating osteoclasts, as confirmed with increased Cathepsin K and TRAP expression (125). To study the effects of FAK in

osteoclastogenesis, Ray *et al.* (2012) established FAK depleted myeloid cells, which includes the osteoclast lineage, using FAK floxed alleles under the myeloid specific lysozyme M promoter (125). While these FAK depleted pre-osteoclasts were able to undergo efficient osteoclast differentiation, they showed a 30% reduction in their ability to resorb bone when plated on bone discs (125). These mice displayed no changes in trabecular bone architecture, however there were low levels of FAK detected within the lineage (125). This suggests that either low FAK levels were sufficient for osteoclast function, or that PYK2 compensates within these cells. To assess the role of both FAK and PYK2, FAK depleted myeloid mice were crossed with PYK2 knockout mice. PYK2-/- mice are osteopetrotic, and PYK2 and FAK crossed mice showed no difference (125). However, in vitro, PYK2-/- FAK-/- cells showed a significant reduction in the spreading area of TRAP-positive multinucleated cells, which was attributed to unorganized podosome structures (125). These studies suggest FAK may have a modest effect in controlling the function of osteoclasts, but not their differentiation.

As PYK2 is the more predominately expressed FAK family kinase in the hematopoietic lineage, studies have focused on exploring the effects of PYK2 in osteoclast differentiation and function. Duong *et al.,* (1998) found that PYK2, but not FAK was highly expressed in osteoclasts (126). Here, PYK2 localized to the sealing zone of the osteoclast, and was activated by the αvβ3 integrin in a c-Src dependent manner (126). Osteoclast-like cells (OCL) infected with adenovirus expressing antisense PYK2 inhibited osteoclast differentiation, and showed decreased spreading and bone resoprtion *in vitro* (127). These cells also displayed decreased paxillin and p130Cas phosphorylation (127). Moreover, pre-osteoclasts expressing the PYK2 mutant Y402F showed decreased osteoclast spreading and bone resorption, implying the importance of the PYK2 kinase domain in regulating osteoclast function (128). These studies suggest that PYK2, and not FAK is more important in osteoclast differentiation and function.

1.10 FAK IN BREAST CANCER

Approximately 78% of invasive breast carcinoma tissues tested showed an increase in FAK expression compared to benign breast epithelium (129). Interestingly FAK amplification rates are low, with only 17.7% of patients testing positive for FAK gene by fluorescence in situ hybridization (FISH) (130). However, patients that tested positive for FAK gene (high polysomy or gene amplification) had significantly reduced overall free survival and relapse-free survival (130). Interestingly, in another study, while the protein expression of FAK was increased in 45 of 55 breast cancer samples compared to normal paired breast tissue, changes in FAK mRNA were not detected (131). Taken together, these results suggest FAK protein overexpression is due to a post-transcriptional or post-translational mechanism (131). Based on the evidence for FAK overexpression, *in vivo* mouse models have been established to assess the role of FAK in breast cancer. In the first study, FAK specific deletion in the mouse mammary epithelium was achieved with floxed FAK and the Cre recombinase under the mouse mammary tumor virus (MMTV) promoter in a polyoma middle T oncoprotein (PyMT) mouse (132). This leads to downstream activation of PI3K and Ras, and development of a highly lung metastatic model (133). Although these mice displayed normal mammary development, tumor initiation was delayed and lung metastasis was impaired compared to FAK expressing PyMT mice (132). This was confirmed by another group, that showed that FAK depleted PyMT mice showed delayed tumor onset, and fewer tumors compared to FAK expressing PyMT tumors (134). Further, when FAK depleted PyMT tumor cells were injected intravenously into severe combined immune deficient (SCID) mice, they showed no lung metastasis while those animals injected with the FAK expressing PyMT tumor cells displayed significant lung metastasis by day 53 (134). It has also been suggested that FAK plays a role in MaCSC, where FAK depleted PyMT cells had a decrease in the Lin-CD24+CD29+CD61+ subpopulation, markers commonly used to identify breast cancer

stem cells (121). Taken together, these studies show an importance for FAK in tumor progression and metastasis and as such FAK may prove to be an important target in breast cancer treatment.

With FAK being important in breast cancer metastasis and with breast cancer predominantly metastasizing to bones in patients, preliminary studies have begun to test the effects of FAK blockade for the treatment of bone metastasis. In an intra-cardiac MDA-MB-231 bone metastasis model, treatment of mice with TAE226, a FAK and IGF-1R inhibitor, significantly reduced osteolytic lesion area, tumor area and number of osteoclasts, while increasing survival (135). In vitro, it was shown that TAE226 inhibited the formation of TRAP-positive multinucleated osteoclasts through decreasing NFATc1 expression (135). Cathepsin K and TRAP levels were also down (135). In assessment of the osteoblasts, it was found that TAE226 inhibited ALP expression and activity in a dose-dependent manner (135). However, because of its ability to simultaneously inhibit IGF-IR, it was unclear whether FAK or IGF-IR was responsible for the observed effects. To more definitively establish the role of FAK, studies have begun investigating the effects of a pharmological FAK inhibitor, PF-562,271 (PF-271) in bone metastasis. A 5mg/kg daily dose of PF-271 in an intra-tibial MDA-MB-231 breast cancer bone metastasis nude rat model showed an increase in osteocalcin suggesting an increase in bone formation, and a reduction of tumor growth (136). However, data was lacking with only one radiograph image published per treatment and the effects on PF-271 on the bone cells were not established (136). In both local subcutaneous and intra-cardiac metastastic prostate cancer bone models, PF-271 treatment showed a significant reduction in tumor growth as detected by bioluminescent imaging (137). These studies provide evidence for the use of FAK inhibitors in the treatment of breast cancer bone metastasis, however more studies need to be done in order to establish the effects of FAK inhibition in all three cell types important to the vicious cycle of bone metastasis.

2. SUMMARY AND HYPOTHESIS:

Breast cancer induces osteolytic bone metastases, and the tumor-induced degradation of the bone results in significant SRE and increased patient morbidity. This osteolytic disease is induced when tumor cells invade the tumor microenvironment and secrete soluble factors that act on the bone resident cells to favour osteoclastogenesis and osteolysis (65). Although drugs such as bisphosphonates and RANK-L have been developed to reduce osteolysis, they have not shown an improvement in patient survival (80). Thus, assessment of novel targeted therapeutics is required to improve patient survival. FAK, is a cytoplasmic tyrosine kinase involved in integrin and growth factor signaling, and regulates cell proliferation, migration and survival. It also has a demonstrated role in breast cancer progression and metastasis. As such it is a promising target for the treatment of bone metastases arising from breast cancer. However, as FAK is also regulating the phenotype of both osteoclasts and osteoblasts, and is thus important for **all three cell types involved in the vicious cycle, inhibition of FAK may decrease breast cancer induced osteolysis, restore bone formation and inhibit further tumor growth in the bone.**

Objectives:

1) Establish the role of FAK in regulating the expression of factors secreted by breast cancer cells to induce osteolysis.
2) Assess the role of FAK in osteoclast differentiation and function
3) Assess the role of FAK in osteoblast differentiation and function

3. APPROACH:

The first objective was to establish the role of FAK in regulating the expression of factors secreted by breast cancer cells that induce osteolysis. It was hypothesized that FAK may play a

role in secretion of osteolytic factors due in part to its implications in regulating expression of MMP, chemokines and initiating survival signals. Moreover, because FAK regulates many pathways that alter gene expression including MAPK, Akt and JNK it is possible that the activation of FAK through collagen bound integrins induces expression of osteolytic genes. Thus through depletion of FAK, a reduction in osteolysis could be achieved. The approach for this objective was to utilize specific siRNA to target FAK to induce a FAK knockdown in common breast cancer cell lines MDA-MB-231, MCF7 and T47D. PCR could then be utilized to assess expression of osteolytic factors in FAK knockdown and compare to a non-targeting (NT) control. Protein expression could then be confirmed for promising targets using ELISA for soluble factors. The ability of conditioned media from breast cancer cells to induce osteoclastogenesis could then be compared between FAK expressing and FAK non-expressing cells. This was performed by adding breast cancer conditioned media to either pre-osteoclasts, or a coculture of osteoclasts and osteoblasts. The number of osteoclasts could then be counted and compared between breast cancer cells expressing or not expressing FAK, with the hopes that decreasing FAK decreases osteoclastogenesis and thus osteolysis.

The second objective was to assess the role of FAK in osteoclast differentiation and function. This was important given that any treatment given for bone metastases would have systemic effects. Thus, inhibition of FAK would affect the tumor cells as well as bone cells. FAK specific siRNA was used to assess the role of FAK in differentiating osteoclasts. Given the role of FAK in mediating cell survival it was hypothesized that FAK inhibition could induce apoptosis in mature osteoclasts. The ability of PF-271 to induce apoptosis, affect pit forming ability and osteolytic factors was assessed.

As previously mentioned, any FAK treatment would be given systemically to patients and thus it is important to test the effects of FAK in all cell types involved in the vicious cycle. Therefore the effects of FAK in osteoblasts, a major mediator of breast cancer induced osteolysis, were

assessed. Moreover, because breast cancer induces a shift towards enhanced osteclastogenesis and reduces new bone formation, it is important to test the effects of FAK inhibition on osteoblast new bone formation. With this in mind, the effects of PF-271 were assessed. This included testing the effects FAK inhibition on osteoblast secretion of osteolytic factors, as well as expression of osteoblastic markers such as ALP. Mineralization assays were also performed to assess the ability of osteoblasts to produce new bone.

4. METHODS

4.1 CELL LINES:

The following murine cell lines were obtained from American Type Culture Collection (ATCC): RAW 264.7 (TIB-71) Abelson leukemia-induced tumor derived macrophages and MC3T3-E1 subclone 4 (CRL-2593) pre-osteoblast cells. The following human breast cancer cell lines were attained from ATCC: MDA-MB-231 (HTB-26), MCF7 (HTB-22), and T47D (HTB-133), which are all mammary epithelial cells derived from pleural effusion. RAW 264.7 and MCF7 cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Logan UT, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan UT, USA). MC3T3-E1 cells were routinely maintained in Alpha Modified Eagle's Medium (AMEM) containing 10% FBS, and 1X sodium pyruvate. T47D cells were routinely maintained in Roswell Park Memorial Medium (RPMI) with 10% FBS (Hyclone, Logan UT, USA). All cells were incubated at 37°C with 5% CO_2.

4.2 REAGENTS AND ANTIBODIES:

Osteoblast differentiation reagents L-ascorbic acid (A4403) and B-glycerophosphate disodium salt hydrate (G9422) were obtained from Sigma (St. Louis, MO, USA). 5X sodium pyruvate (11360-070) was obtained from Gibco (USA). The osteoclast differentiation reagent Recombinant Human TRANCE (390-TN) was obtained from R&D Systems (Minneapolis, MN,

USA). Staining kits: acid phosphatase, leukocyte (TRAP) 387A and leukocyte alkaline phosphatase 86R were obtained from Sigma (St. Louise, MO, USA). Alizarin Red staining solution (TMS-008-C) was obtained from Millipore and Silver nitrate was obtained from Sigma (St. Louis, MO, USA). The FAK inhibitor PF-562,271 (PF-271) was obtained from Shanghai Biochempartner Co. Ltd (Shanghai, China) and was dissolved in DMSO.

The following antibodies were obtained from Cell Signaling (Danvers MA, USA): Phospho-Akt (Ser473), Phospho-FAK (8556S), and Akt (9272S). MMP-2 (ab37150) and MMP-9 (ab38898) antibodies were obtained from Abcam. SPARC and M-CSF antibodies were from R&D Systems (Minneapolis, MN, USA), FAK (610087) from BD Transduction Laboratories (BD Biosciences, San Jose CA, USA) and NFATc1 from Santa Cruz (Santa Cruz, CA). All primary antibodies were used at a 1:1000 dilution. Goat anti-mouse and goat anti-rabbit secondary antibodies were from Calbiochem (San Diego CA, USA) and were used at a dilution of 1:5000.

4.3 SIRNA TRANSFECTIONS:

Oligonucleotides for siRNA transfections were purchased from Dharmacon (Chicago IL, USA). The mouse FAK gene PTK2 ON-TARGET plus FAK6 (J-041099-07) and FAK7 (J-041099-08) had a target sequence of GCGAUUAUAUGUUAGAGAU and GUACAGCUCUUGCAUAU, respectively. A siCONTROL scrambled non-targeting sequence was used as control. The human PTK2 ON-TARGET plus FAK2 (J-003164-14) and FAK4 (J-003164-16) had a target sequence of GGGCAUCAUUCAGAAGAUA and GCUCCAGAGUCAAUCAAUU, respectively. For RNA interference studies, cells were seeded at 1.8x 10^6/10cm dish 24 hours prior to transfection. The siRNA and the oligofectamine were each diluted into Opti-Mem from GIBCO (Grand Island, NY, USA) at an optimized concentration and allowed to sit for 8 minutes. For MDA-MB-231 cells, transfection was performed at a concentration of 80nM of each siRNA. For mouse MC3T3 E1 and

RAW264.7 cells, the transfection was performed at a concentration of 40nM and 20nM respectively. The two mixtures were then combined, and allowed to sit for 25 minutes. The siRNA mixture was then added drop wise to cells in Opti-mem. Following a 4 hour incubation, media containing 30% FBS was added to stop the reaction.

4.4 CONDITIONED MEDIA COLLECTION AND ELISA:

For conditioned media collection, breast cancer cells were seeded at $2x10^6$ cells overnight in 3mL of 10% FBS containing media. The next day the media was changed to 3mL of 2% FBS containing media. Cells were incubated at 37°C with 5% CO_2 for 48 hours prior to collection. Conditioned media was collected, and briefly spun at 1500xg for 5 minutes to remove cell debris. Conditioned media was then aliquoted and stored at -80°C. Quantikine human M-CSF and IL-8 ELISAs were purchased from R&D Systems (Minneapolis, MN, USA). ELISAs were run according to manufacturer's protocol, with standards and each treatment group performed in duplicate. Absorbance was measured at 450nm and corrected at 570nm using a Multiskan Ascent photometer (Thermo Scientific, Rockford IL). Standard curves were created based on the standard protein concentration, and the samples concentrations were determined following interpolation of the standard curve.

4.5 OSTEOCLAST DIFFERENTIATION ASSAY:

RAW 264.7 cells were seeded at 10^4 cells/well in a 24 well dish. The next day, cells were treated with 50ng/ml RANK-L. Cells were treated every 3 days until end point. Treatments were performed in duplicate wells. Cells were then fixed using a mixture of formaldehyde, acetone and citrate for 30 seconds. Cells were then washed and stained using the Sigma TRAP stain according to the manufacturer's directions. Osteoclasts were then visualized under the Nikon Eclipse TE2000-U microscope at 200x and 400x. For quantification, 9 pictures were taken at random

from each well at 200x. The number of TRAP positive cells with 4+ nuclei were counted per well.

4.6 OSTEOBLAST DIFFERENTIATION ASSAY:

MC3T3 E1 cells were seeded at 5 x 10^4 cells/well in a 6-well dish in differentiation media containing 10% FBS, 50μg/mL ascorbic acid and 10mM β-glycerophosphate. Differentiation media was changed every 2-3 days for 7-21 days. For ALP staining, cells were fixed using a mixture of formaldehyde, acetone and citrate for 30 seconds. Cells were washed, and then stained using the Sigma ALP stain kit according to the manufacturer's directions. Cells were counter-stained using hematoxylin. For Von Kossa staining, cells were fixed with 70% ethanol for one hour at room temperature. Cells were then washed with distilled water, and 5% silver nitrate was added until brown spots were visualized. Cells were washed again, and spots were photographed. For Alizarin Red S staining, cells were fixed with 10% formalin for 30 minutes at room temperature. Cells were then washed with distilled water, and Alizarin Red S stain was added for one hour at room temperature. Cells were then washed with distilled water until wash fluid was clear, and red deposits were photographed.

4.7 OSTEOCLAST AND OSTEOBLAST COCULTURE EXPERIMENTS

On day 0, MC3T3 E1 cells were seeded at 1x10^5 cells/well in a 6-well dish in their normal media (10% AMEM containing 1X sodium nitrate). On day 1, RAW 264.7 cells were added at 1x10^4 cells/well in 10% AMEM. Treatments were also initiated on day 1. This included adding 10% breast cancer conditioned media collected as previously described, with or without osteoblast differentiation media containing 50μg/mL ascorbic acid and 10mM β-glycerophosphate for a final 2mL volume/well. Treatments were replenished on day 4. On day 7, cells were fixed using a mixture of formaldehyde, acetone and citrate for 30 seconds. Sigma TRAP stain was then performed as per manufacturer's protocol. Osteoclasts were then visualized under the microscope

at 200x and 400x. For quantification, 9 pictures were taken at random from each well at 200x. The number of TRAP positive cells with 4+ nuclei were counted per well.

4.8 WESTERN BLOTS

Cells were washed twice with ice cold PBS. Cells were then lysed using Radioimmunoprecipitation assay (RIPA) buffer (Tris-HCL pH 7.2, 150mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100), containing 2mM sodium fluoride, 2mM sodium pyrophosphate, 0.2mM phenylmethanesulphonylfluoride, 500uM ammonium vanadate, 2ug/ml aprotinin, and 5ug/ml leupeptin. Lysates were vortexed every 5 minutes for 30 minutes, kept on ice, and then centrifuged at 20,000xg for 20 minutes at 4°C. Protein concentrations were measured using the Bio-Rad protein assay and the Beckman DU 640 spectrophotometer at 595nm. Samples were made with 40μg of protein and 5X sample buffer (250mM Tris-HCL pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 20%(v/v) β-mercaptoethanol, 0.008% (w/v) bromophenol blue). Samples were denatured at 100°C for 5 minutes and then separated on a 10% polyacrylamide gel at 120V. Protein was transferred onto a methanol activated 0.45μM Polyvinylidene fluoride (PVDF) transfer membrane from Immobilon-P (Millipore, Billerca, MA) in Tris-glycine transfer buffer at 100V for 90 minutes. Membranes were then blocked for 1 hour at room temperature in 5% powdered skim milk in TBS-T. Primary antibodies were incubated overnight at 4°C, at a concentration of 1:1000 in 5% powdered skim milk in TBS-T with the exception of phopho-FAK and phospho-Akt which were incubated in 5% bovine serum albumin (BSA). Horseradish peroxidase (HRP)-linked secondary antibodies were incubated for 1 hour at room temperature. HRP-linked chemiluminescence EZ-ECL from Biological Industries (USA) was applied to the membrane surfaces, and images of reactive bands were taken using the GeneGnome Syngene bio imaging system and Genesnap software (Product version 6.05) (Synoptics Ltd, Cambridge UK).

4.9 RNA EXTRACTION, CDNA SYNTHESIS AND PCR

For RNA extraction, media was aspirated and then the extraction was completed using the Qiagen RNeasy kit (Hilden, Germany). Isolated RNA was stored at -80°C. RNA was quantified using a spectrophotometer at 260nm. Complementary DNA (cDNA) was synthesized by combining 1μg of total RNA with 1ul of 10mM dNTP, and 1ul of Oligo (dT) both from Invitrogen (Carlsbad, CA), to prime the reaction. The sample was then heated at 65°C for 5 minutes. A total of 4μL of 5x First Strand Buffer, 1μL of RNAse out, and 2μL of 0.1 M dTT all from Invitrogen were then added to each tube, and the samples were heated at 37°C for 2 minutes. Subsequently, 1uL of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA) was then added to each sample. The samples were then incubated at 37°C for 50 minutes, followed by inactivation at 70ºC for 15 minutes. cDNA was stored at -20°C.

Polymerase chain reaction (PCR) was performed using cDNA, individual primers (forward and reverse) for specific genes of interest, water and RT SYBR Green ROX qPCR Mastermix (Qiagen), using the 7500 Fast Real-Time PCR System (Applied Biosystems by life Technologies, Carlsbad, CA). The reaction was completed with the following steps, denaturation at 95°C for 10 minutes, followed by PCR for 40 cycles, using denaturation of 95°C for 15 seconds and elongation at 60°C for 1 minute. The following primers were used for human and mouse. Primers were purchased from Invitrogen (Carlsbad, CA). Human primer sets were FAK (forward GTCTGCCTTCGCTTCACG, reverse GAATTTGTAACTGGAAGATGCAAG), MCSF (forward GCAAGAACTGCAACAACAGC, reverse ATCAGGCTTGGTCACCACAT) and β-actin (forward CCAACCGCGAGAAGATGA, reverse CCAGAGGCGTACAGGGATAG). Mouse primer sets were Osterix (forward AGCGACCACTTGAGCAAACAT, reverse GCGGCTGATTGGCTTCTTCT), BMP2 (forward GGGACCCGCTGTCTTCTAGT, reverse

TCAACTCAAATTCGCTGAGGAC), Shh (forward AAAGCTGACCCCTTTAGCCTA, reverse TTCGGAGTTTCTTGTGATCTTCC), Wnt3a (forward TGGGTGAGGCCTCGTAGTAG, reverse CAGTGCCTCGGAGATGGTG), MCSF (forward GGTGGAACTGCCAGTATAGAAAG, reverse TCCCATATGTCTCCTTCCATAAA), RUNX2 (forward ATTCAGGGGAACCCAAAAAG, reverse, GCGACTTCATTCGACTTCCT), OPG (forward ACCCAGAAACTGGTCATCAGC, reverse CTGCAATACACACACTCATCACT), NFATc1 (forward TGGAGAAGCAGAGCACAGAC, reverse GCGGAAAGGTGGTATCTCAA), FAK (forward GAACAGCTATTTGTTTCTTCTCAAA, reverse GCTGGAGAAGGCGCTAACTA), TRAP (forward CCAATGCCAAAGAGATCGCC, reverse TCTGTGCAGAGACGTTGCCAAG), Cathepsin K (forward GACGCAGCGATGCTAACTAA, reverse CCAGCACAGAGTCCACAACT), ALP (forward ATCTTTGGTCTGGCTCCCATC, reverse TTTCCCGTTCACCGTCCAC) and β-actin (forward CTAAGGCCAACCGTGAAAAG, reverse ACCAGAGGCATACAGGGACA).

STATISTICAL ANALYSIS:

Statistical analysis was performed using Graph Pad Prism 5 software. Experiments comparing two groups were compared using a student's t-test. Experiments comparing more than two groups were compared using a one-way ANOVA with a Bonferroni post-hoc test. P-values were considered significant at less than 0.05 (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

5. RESULTS

In order to test the hypothesis that FAK, being ubiquitously expressed, plays a key role in mediating breast cancer osteolysis, it was important to assess it's inhibition in all 3 cell types involved in the vicious cycle. In breast cancer cells, the ability of FAK to regulate the production of secreted factors capable of inducing osteolysis was tested. Furthermore, FAK inhibition was tested in osteoclasts in order to assess its ability to regulate differentiation, lytic ability and survival. Lastly, the inhibition of FAK in osteoblasts was assessed in order to test their ability to differentiate and induce mineralization. The results described herein provide evidence regarding the effects that systemic administration of FAK inhibitors would have in the bone microenvironment if used to treat metastatic breast cancer.

5.1 Testing an *in vitro* osteolytic breast cancer bone model

As with any therapeutic target that may be ubiquitously expressed, it is important to determine the effects of its inhibition on the various cell types, which may be affected. As such, we wished to determine the effect of blocking FAK in each major cellular compartment important in the vicious cycle of bone metastases. Initially, however, we wanted to confirm previous findings regarding the effects of breast cancer conditioned media on bone resident cells. To do this, a coculture of RAW 264.7 macrophages (pre-osteoclasts) and MC3T3 E1 (pre-osteoblasts) was tested. MC3T3 E1 cells were plated at $1x10^5$ cells/well and allowed to adhere to surface of tissue culture dishes. The next day, RAW 264.7 cells were then plated on top of adherent MC3T3 E1 cells at a density of $1x10^4$ cells/well. The coculture was then treated with or without ascorbic acid and β-

glycerophosphate (AAB) to initiate differentiation of osteoblasts, 10% final volume breast cancer cell conditioned media, or AAB in combination with addition of breast cancer conditioned media. The breast cancer conditioned media from three commonly used breast cancer cell line models: MCF7, MDA-MB-231 and T47D, was obtained following a 48 hour incubation. The pre-osteoblast/pre-osteoclast coculture was treated as described above every 3 days for 7 days before being TRAP stained to enumerate mature osteoclasts which may have arisen. The numbers of TRAP positive, multinucleated (4+ nuclei) cells (osteoclasts) were then counted in each well and expressed as total number of mature osteoclasts per well (Figure 6a&b). Cell cocultures treated with AAB in combination with breast cancer conditioned media showed higher induction of osteoclastogenesis than breast cancer conditioned media or AAB alone (Figure 6a). All three breast cancer cell lines tested induced osteoclastogenesis with MDA-MB-231 showing the highest and MCF7 showing the lowest induction of osteoclastogenesis (Figure 6a). Thus as expected, breast cancer cells secrete factors that are capable of promoting osteoclastogenesis.

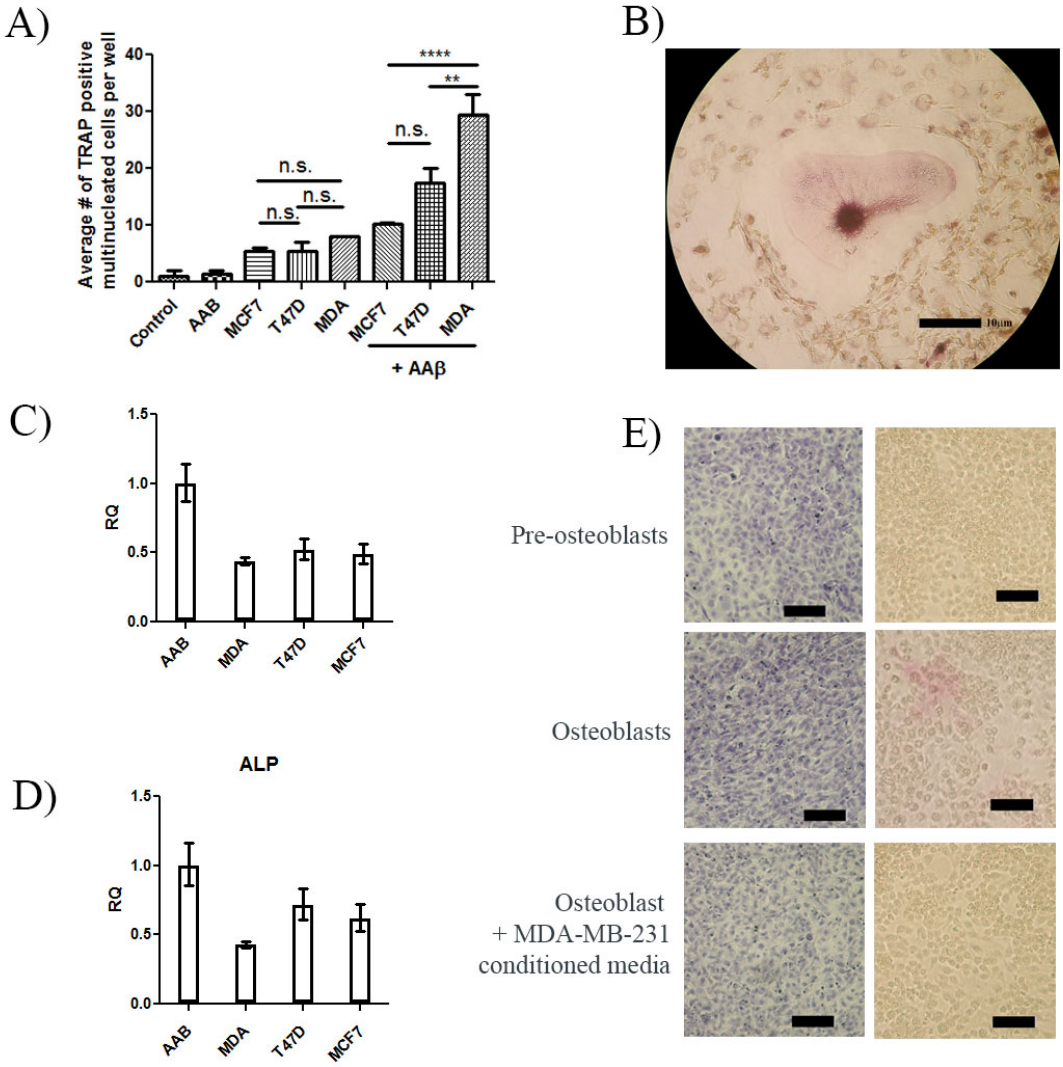

A)
Average # of TRAP positive multinucleated cells per well
40
30
20
10
0
n.s.
n.s.
n.s.
n.s.
****
**
Control
AAB
MCF7
T47D
MDA
MCF7
T47D
MDA
+ AAβ
B)
10μm
C)
RQ
1.5
1.0
0.5
0.0
AAB
MDA
T47D
MCF7
D)
ALP
RQ
1.5
1.0
0.5
0.0
AAB
MDA
T47D
MCF7
E)
Pre-osteoblasts
Osteoblasts
Osteoblast
+ MDA-MB-231
conditioned media

Figure 6. Breast cancer conditioned media induces osteoclastogenesis and inhibits osteoblast differentiation. A) MC3T3 cells were plated at 100,000 cells per well. The next day RAW 264.7 cells were plated on top at 20,000 cells per well. Cells were then treated with 50μg/mL ascorbic acid and 10 mM β-glycerophosphate, 10% breast cancer conditioned media, or combination of the two for 7 days, changing the media every 3 days. On day 7, cells were fixed and stained with TRAP. A) TRAP positive multinucleated cells were then counted in 9 random fields of view, in duplicate wells. Bars represent an average of two biological replicates (n=2, one-way ANOVA was performed with a Bonferroni post hoc test **p<0.01, p****p<0.0001). B) Representative image of a TRAP positive, multinucleated cell at 400X magnification. MC3T3 cells cultured alone were also treated with 10% tumor cell-conditioned media. RNA was collected and RT-PCR was performed to detect the relative expression of C) OPG, and D) ALP, or E) they were fixed and stained with ALP and hematoxylin to detect maturing osteoblasts. Scale bars represent 10μm.

5.2 Assessing the effects of breast cancer conditioned media on osteoblasts

To determine the mechanism by which breast cancer conditioned media induces osteoclastogenesis, the expression of osteoblastic factors were assessed in MC3T3 E1 cells, as it is known that mature osteoblasts can induce osteoclastogenesis. MC3T3 E1 cells were treated with 10% final volume of breast cancer conditioned media for 6 days, or with AAB as a positive control for induction of mature osteoblasts and RNA was subsequently isolated and subjected to RT-PCR to assess expression of mature osteoblast expressed factors OPG and ALP. OPG, an inhibitor of osteoclastogenesis secreted by osteoblasts, was reduced by at least two-fold in all breast cancer conditioned media treated MC3T3 cells (Figure 6c). ALP, a marker for osteoblast differentiation, was also reduced two-fold (Figure 6d). To confirm similar findings at a protein level, ALP staining was also performed on MC3T3 cells treated for 10 days with osteoblast differentiation media AAB alone or in combination with MDA-MB-231 conditioned media. Pink ALP staining was detected in AAB alone treated MC3T3 cells, however ALP was not detected in cells treated with the combination of AAB and MDA-MB-231 conditioned media (Figure 6e), suggesting breast cancer cells secrete factors that inhibit osteoblastogenesis thus confirming previous findings (66,72,74,138), and demonstrating our models behave as expected.

5.3 FAK regulates the secretion of various osteolytic factors by breast cancer cells

As our results from experiments testing the effects of breast cancer conditioned media on osteoclast/osteoblast cocultures suggested an ability to promote osteoclastogenesis and inhibit osteoblastogenesis, we were interested in determining the role of FAK in this process. To test the hypothesis that FAK regulates the expression of osteoclastogenic factors in breast cancer cells, siRNA mediated FAK knockdown was first performed in MDA-MB-231 cells, as they are the primary cell line used in mouse models of breast cancer bone metastasis, and appeared to have the greatest potential to induce osteoclastogenesis in our initial coculture experiments. Initially, we confirmed the efficacy and timing of the siRNA-mediated knock down of FAK at the protein and RNA level. While 80nM of FAK siRNA resulted in a decrease of FAK protein expression at 24 and 48 hours, as detected by Western blot, FAK protein expression returned to normal levels by 96 hours post-transfection (Figure 7a&b). Thus, RNA was collected 48 hours post transfection, given this appeared to be the time of maximal suppression of FAK, and RT-PCR was used to confirm FAK knockdown before further assessment of gene expression of known osteoclastogenic factors (Figure 7c). FAK knockdown resulted in a significant decrease in the expression M-CSF, an inducer of osteoclast differentiation and survival, at the mRNA level (Figure 7d). To assess the expression of M-CSF at a protein level, conditioned media was collected (as most osteoclastogenic factors of interest are secreted proteins) from both control and FAK depleted breast cancer cells and an ELISA was performed. While MDA-MB-231 secreted M-CSF showed a trend to decrease with FAK knockdown, this decrease did not reach statistical significance (Figure 7e). IL-8, a soluble chemokine with known roles in inducing osteoblast RANK-L expression and

osteolysis (139) was also assessed for its regulation by FAK, however it showed no change at the protein level as detected by ELISA with siRNA-mediated FAK knockdown (Figure 7f). Furthermore, other relevant cytokines tested, namely IL-6 and IL-11, also showed no change with FAK knockdown at the mRNA level (Figure 7g and 7h). We also assessed the levels of LOX, a factor recently shown to be involved in the metastatic bone niche (64), however, it also showed no change with FAK knockdown (Figure 7i). Lastly, due to its documented role in inducing breast cancer bone metastases, PTHrP was assessed. Knockdown of FAK in breast cancer cells resulted in a significant decrease in PTHrP at the mRNA level (Figure 7j). Thus, although the depletion of FAK did not decrease all factors known to induce osteolysis, its modest depletion of M-CSF, and depletion of PTHrP, a major modulator of breast cancer osteolysis, may be sufficient to decrease breast cancer mediated osteolysis.

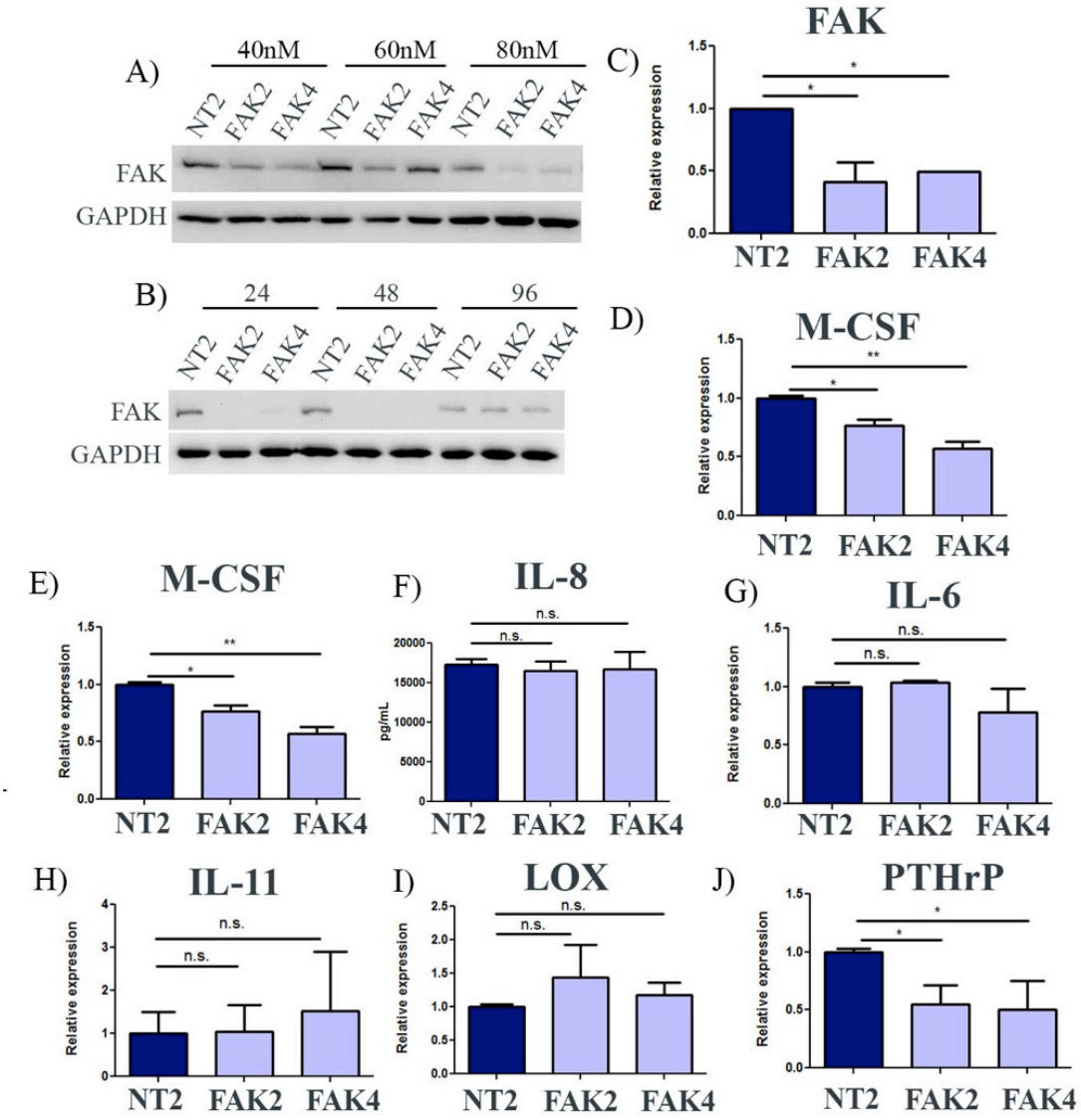
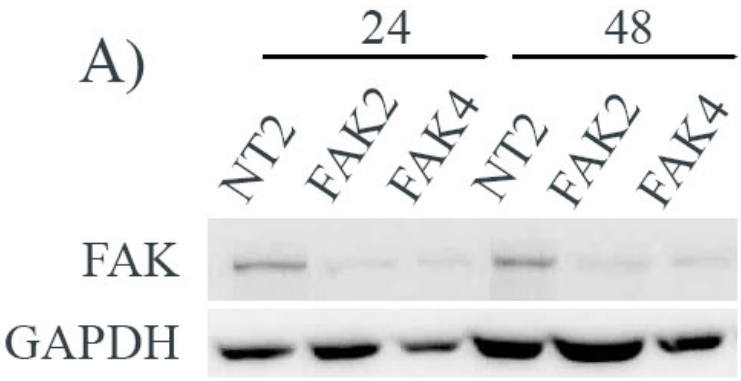

A)
40nM
60nM
80nM
NT2
FAK2
FAK4
FAK
GAPDH
B)
24
48
96
C)
FAK
Relative expression
D)
M-CSF
E)
M-CSF
F)
IL-8
pg/mL
G)
IL-6
H)
IL-11
I)
LOX
J)
PTHrP
n.s.

Figure 7: FAK modulates important mediators of osteolysis. A) MDA-MB-231 cells were plated at equal numbers and transfected with 40nM, 60nM, 80nM of FAK specific siRNA or an equal concentration of non-targeting (NT2) siRNA. B) Using 80nM, rotein was collected at 24, 48 and 96 hours, and a Western blot was performed to assess levels of endogenous FAK. GAPDH was probed as a loading control. C) In parallel experiments, RNA was collected at 48 hours and RT-PCR was performed to confirm the level of FAK mRNA. D) The levels of M-CSF were also assessed by RT-PCR (n=3, T-test performed * $p<0.05$, ** $p<0.01$). Conditioned media was also collected from the cells at 48 hours, and briefly spun down to eliminate cell debris. An ELISA was performed on MDA-MB-231 conditioned media for the known osteoclastogenic factors D) M-CSF and F) IL-8 (n=3, n.s. non-significant). The levels of G) IL-6, H) IL-11 I) LOX and J) PTHrP, were also assessed by RT-PCR and relative levels of expression calculated following normalization to levels of GAPDH as an endogenous control (n=3 biological replicates, with each sample being an average of n=3 wells, t-test, n.s. non-significant, and * $p<0.05$).

Next we wanted to extrapolate these findings to confirm if similar results could be obtained in another breast cancer cell line. siRNA mediated knockdown of FAK was thus performed in MCF7 cells, and protein was collected at 24 and 48 hours to confirm FAK levels by Western blot (Figure 8a). To be consistent with the above MDA-MB-231 RT-PCR, 48 hours post-transfection was chosen for RNA collection from MCF7 cells. While MCF7 cells showed a slight decrease in M-CSF mRNA expression, only one of the two FAK targeting siRNA reduced FAK to statistically significant levels (Figure 8b). However, we could not confirm these changes in M-CSF at the protein level, as secreted protein levels were below the threshold of detection and thus undetectable by ELISA (data not shown). Moreover, when basal levels of PTHrP were assessed by RT-PCR in MCF7 cells, their levels were too low, and the signals arose on too high of a cycle number for accurate detection (data not shown). While MCF7 cells were capable of inducing osteoclastogenesis in our coculture model, they produced significantly fewer osteoclasts per well compared to the number induced by MDA-MB-231 cell conditioned media. Thus, the lower secretion of osteolytic factors of MCF7 cells is consistent with their decreased ability to induce osteoclastogenesis.


A)
24
48
NT2
FAK2
FAK4
NT2
FAK2
FAK4
FAK
GAPDH

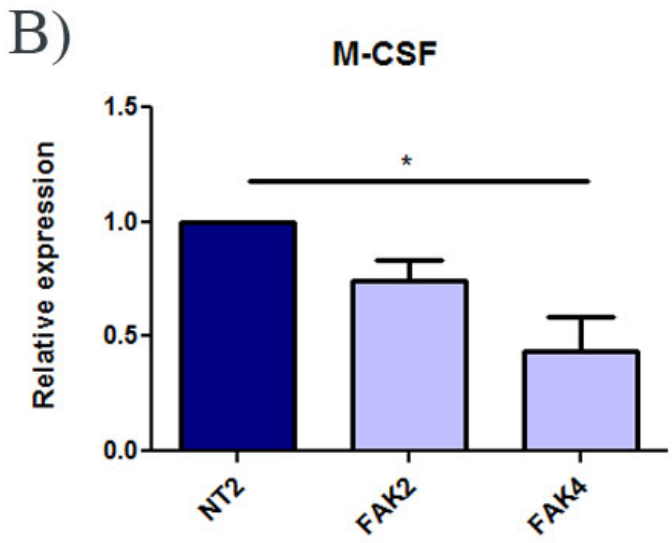

B)
M-CSF
Relative expression
1.5
1.0
0.5
0.0
*
NT2
FAK2
FAK4

Figure 8: FAK depleted MCF7 cells show reduced M-CSF. MCF7 cells were plated at equal numbers and transfected with 40nM of each of two different FAK specific siRNA or non-targeting (NT2) siRNA. A) Protein was collected at 24, 48 hours post-transfection and a Western blot was performed to assess levels of endogenous FAK. GAPDH was probed as a loading control. B) In parallel experiments, RNA was collected at 48 hours post-transfection and RT-PCR was performed to assess the relative levels of M-CSF as normalized to endogenous mRNA levels of GAPDH as a control (n=3 biological replicates, with each sample being an average of n=3 wells, T-test * $p<0.05$).

Because of the significant changes in expression of the osteoclastogenic factor PTHrP in MDA-MB-231 cells upon FAK depletion, the ability of breast cancer conditioned media to induce osteoclastogenesis was assessed using RAW 264.7 cells. RAW 264.7 cells, a pre-osteoclast cell line capable of differentiating into osteoclasts with RANK-L stimulation, were thus supplemented with RANK-L and MDA-MB-231 conditioned media for 7 days, at which time TRAP staining was performed to detect mature osteoclasts. Conditioned media collected from FAK-depleted MDA-MB-231 cells showed a similar ability to induce osteoclastogenesis in a monoculture as compared to control siRNA treated cells (Figure 9a). However, because of the known role of osteoblasts in breast cancer mediated osteolysis as depicted in Figure 6a, a coculture of MC3T3 E1 pre-osteoblast and RAW 264.7 pre-osteoclast cells was also performed. The coculture, as described above, was used to compare the effects of conditioned media from FAK expressing versus FAK-depleted MDA-MB-231 cells on osteoclastogenesis in this system. Unlike when conditioned media was used to directly stimulate the RAW264.7 cells alone, stimulation of the coculture with conditioned media from FAK-depleted tumor cells showed a general decrease in mature osteoclasts when combined with osteoblast differentiation media AAB, however this did not quite reach statistical significance (Figure 9b). To assess the mechanism by which conditioned media from FAK depleted tumor cells resulted in reduced osteoclastogenesis, osteoblast factors OPG and ALP were assessed by RT-PCR using RNA isolated from treated osteoblasts. While increases in the average relative expression of OPG and ALP were observed in MC3T3 E1 cells treated with conditioned media from FAK-depleted breast cancer cells, this was not statistically significant (Figure 9c&d). Although the effects of FAK depleted breast

cancer conditioned media did not show statistically significant effects on osteoclast differentiation in both a mono, and coculture setting, trends were observed that suggest FAK inhibition could provide a beneficial treatment for breast cancer mediated osteolysis. However, because osteolysis is a complex process formed through interactions between 3 cell types, it remains possible that FAK blockade could play a role in inhibiting undesired effects in each cell type individually, resulting in a significant cumulative decrease in osteolysis.

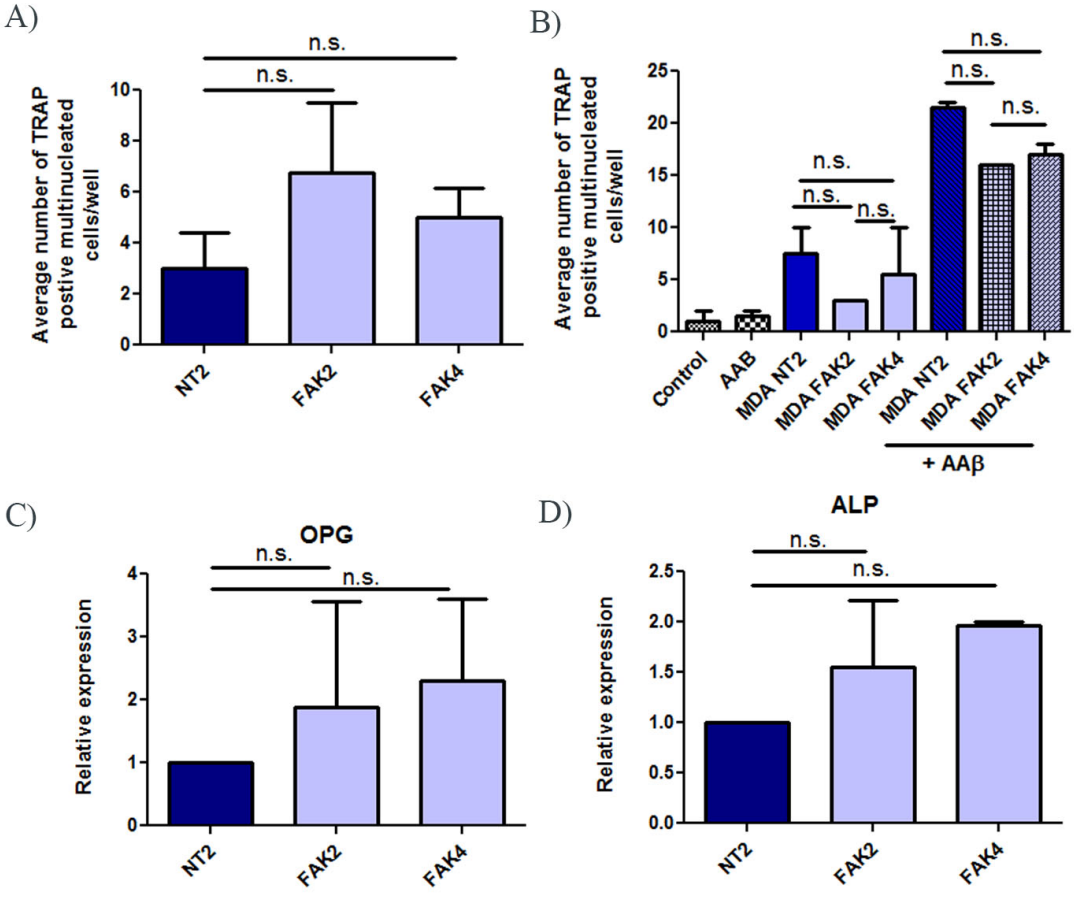

A)
Average number of TRAP postive multinucleated cells/well
n.s.
n.s.
NT2
FAK2
FAK4
B)
Average number of TRAP positive multinucleated cells/well
n.s.
Control
AAB
MDA NT2
MDA FAK2
MDA FAK4
MDA NT2
MDA FAK2
MDA FAK4
+ AAβ
C)
OPG
Relative expression
D)
ALP
Relative expression

Figure 9. Depletion of FAK in breast cancer cells does not inhibit breast cancer induced osteoclastogenesis. MDA-MB-231 cells were plated in equal numbers and transfected with FAK specific siRNA or non-targeting (NT2) siRNA. The next day equal amounts of 2% FBS supplemented media were plated, and conditioned for 48 hours. Conditioned media was collected from the cells, and briefly spun down to eliminate cell debris. A) RAW 264.7 cells were plated in monoculture in a 24-well tissue culture plate. Wells were treated in duplicate with 20ng/ml RANK-L and 10% MDA-MB-231 conditioned media every 3 days for 7 days. Cells were then fixed and TRAP stained. The average number of TRAP positive multinucleated cells was counted per 9 fields of view in duplicate (each bar represents an average of 2 wells done in n=2 biological replicates, one-way ANOVA was performed with a Bonferroni post hoc test, n.s. non significant). B) MC3T3 and RAW264.6 cells were plated in a 24 well dish coculture. Cells were treated every 3 days for 7 days with 10% MDA-MB-231 conditioned media, 50μg/ml ascorbic acid and 10mM β-glycerophosphate (AAB). Cells were then fixed and TRAP stained. The average number of TRAP positive multinucleated cells was counted per well in duplicate. Each bar represents an average of 9 fields per view, wells done in n=2 biological replicates, n.s. non significant). RNA was collected from MC3T3 cells treated in mono-culture with 10% MDA-MB-231 conditioned media for 6 days, replacing media every 3 days. An RT-PCR was performed to detect relative expression of C) OPG and D) ALP (n=2 biological replicates, with each sample being an average of n=3 wells, n.s. non significant).

5.4 FAK inhibition induces apoptosis and decreases the lytic ability of osteoclasts

Our initial studies using osteoblast/osteoclast cocultures, suggested that FAK depletion in tumor cells may have a small effect on osteoclastogenesis in the presence of osteoblasts, but we were unable to determine the exact mechanism by which this happens in our subsequent experiments. In addition, given the complexity of trying to model bone microenvironment cell interactions in vitro, it remains possible that the necessity of using potent stimulators in combination with tumor conditioned media could mask FAK-mediated effects. As such, we decided to investigate the effects of FAK inhibition in bone cells directly given that these cells would also potentially be directly exposed to pharmacological FAK inhibition in a therapeutic setting. It is possible that inhibition of FAK in mature osteoclasts could affect their survival or their lytic activity. In the context of pre-osteoclasts, FAK inhibition could affect their survival and differentiation. As such we set out to investigate each of these possibilities.

To determine the role of FAK in regulating osteoclast differentiation, we initially evaluated the expression of known osteolytic factors in osteoclasts as markers of differentiation, following siRNA mediated FAK knockdown in RAW 264.7 cells. Multiple concentrations of siRNA were used to assess the effectiveness of mouse specific FAK siRNA within RAW 264.7 cells. A concentration of 20nM of siRNA was found to be the most effective in mediating depletion of FAK in pre-osteoclasts, with the siRNA sequences FAK6 and FAK7 functioning better than FAK5 (Figure 10a). Thus, further experiments utilized sequences FAK6 and FAK7 at a concentration of 20nM. To assess the role for FAK in osteoclast differentiation, FAK depleted RAW 264.7 cells were treated with RANK-L 24 hours post transfection, for 7 days, changing the media every 3

days, and TRAP staining was performed thereafter. FAK depletion using either FAK-targeting siRNA sequence showed no effect on the ability of RAW 264.7 cells to undergo osteoclastogenesis as detected following enumeration of multinucleated TRAP+ cells in this assay (figure 10b). Although FAK depletion did not appear to play a role in osteoclast differentiation, at least in this model system, it may play a role in affecting their lytic ability. As such, RNA was collected from siRNA mediated FAK depleted RAW 264.7 cells stimulated with RANK-L for 48 hours. RT-PCR was then performed to determine the expression of factors known to play a role in the lytic ability of osteoclasts. TRAP and Cathepsin K are two of the most important bone degrading enzymes mediating the osteolytic ability of osteoclasts. Forty-eight hours post siRNA mediated FAK depletion in RAW264.7 cells, TRAP mRNA expression was decreased two-fold with FAK knockdown (figure 10c). To confirm this result at a protein level, FAK depleted RAW264.7 cells were fixed and stained for TRAP levels 48 hours post RANK-L stimulation. TRAP staining was decreased in FAK depleted cells stimulated with RANK-L as assessed using two different FAK-specific siRNAs (figure 10f). We also examined the level of mRNA expression of Cathepsin K, a protease responsible for collagen degradation. In this context, Cathepsin K expression levels were variable between FAK siRNA sequences (figure 10d), and as such we cannot conclude any potential direct role of FAK mediating its expression. While assessment of TRAP at early time points post-transfection suggested a putative effect of FAK depletion in the lytic ability of osteoclasts, it is known to take many days for osteoclasts to fully mature. As such, while FAK may play a role in TRAP expression early in osteoclast differentiation, this may not translate into phenotypes that are more dependent on fully

mature osteoclasts such as cathepsin K, which was found to have a 7-fold increase in expression between day 3 and day 6 of RANK-L stimulation (125). In addition to restoration of FAK expression by 96 hours post-transfection which could affect our observations, PYK2, the FAK family member which is up-regulated during osteoclastogenesis, may compensate for FAK loss in these cells as osteoclasts mature confounding the interpretation of our results.

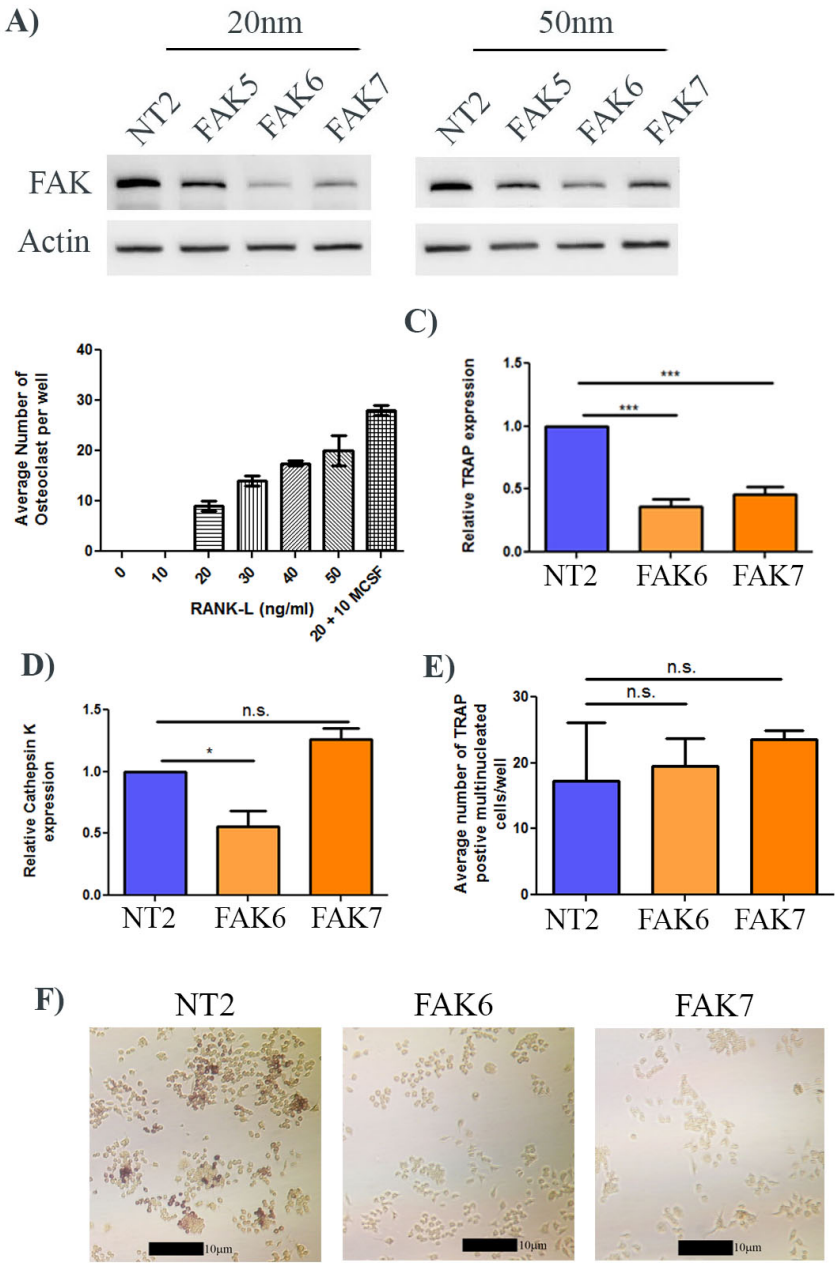
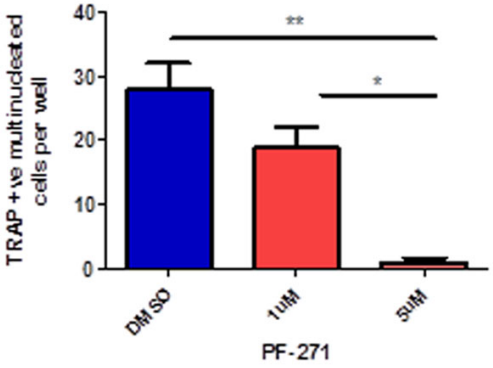

A)
20nm
50nm
NT2
FAK5
FAK6
FAK7
FAK
Actin
C)
Average Number of Osteoclast per well
RANK-L (ng/ml)
0
10
20
30
40
50
20 + 10 MCSF
Relative TRAP expression
***
***
NT2
FAK6
FAK7
D)
Relative Cathepsin K expression
n.s.
*
E)
Average number of TRAP postive multinucleated cells/well
n.s.
n.s.
F)
10µm

Figure 10. FAK regulates the expression of TRAP in RAW264.7 cells but does not affect osteoclastogenesis. RAW264.7 cells were transfected with non-targeting or mouse FAK specific siRNA (FAK5, FAK6, and FAK7). A) Protein was collected from RAW 264.7 cells treated with doses of 20nM and 50nM siRNA, and a Western blot was performed to assess their ability to efficiently knockdown FAK at 48 hours post transfection. Actin was used as a loading control for total protein. B) RAW 264.7 cells were stimulated with different concentrations of RANK-L every 3 days for 7 days. The number of TRAP positive multinucleated cells were then counted. Twenty-four hours post FAK depletion, RAW 264.7 cells were stimulated with 50ng/mL RANK-L for 48 hours. RNA was collected and RT-PCR was performed to assess the level of mRNA expression of C) TRAP and D) Cathepsin K (n=3 biologial replicates, * $p<0.05$, ***$p<0.001$, n.s. non-significant, one-way ANOVA was performed with a Bonferroni post hoc test.) E) siRNA-transfected RAW264.7 cells were treated with RANK-L for 7 days, changing the media every 3 days. On Day 7, cells were fixed, stained for TRAP and TRAP positive multinucleated cells were counted per well, in duplicate (bars are an average of two wells done in n=2 biological replicates, n.s. non significant, one-way ANOVA was performed with a Bonferroni post hoc test). F) siRNA-transfected RAW264.7 cells were fixed and TRAP stained 48 hours post RANK-L treatment. Images were taken at 100x. Scale bar represents 10μm.

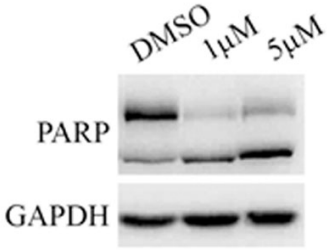
Although our data suggested that FAK was not necessary to induce osteoclast differentiation in RAW264.7 cells in response to RANK-L, the use of siRNA did suggest a role for FAK in the lytic ability of osteoclasts. Moreoever, siRNA is limited by the transient nature of its ability to suppress its endogenous target, and hence given the length of these differentiation assays, it remained possible that effects of FAK suppression were lost in maturing osteoclasts. Thus we moved to a pharmacological method of FAK inhibition in order to be able to assess the effects of its blockade directly in mature osteoclasts and their activity and allow for testing of its inhibition over longer periods of time. To test the hypothesis that FAK plays a role in osteoclast survival, mature osteoclasts were treated with the dual FAK/PYK2 inhibitor, PF-271. A total of 10,000 RAW 264.7 cells were plated per well and differentiated with RANK-L for 6 days, changing the media on day 3. Each well was assessed to ensure an equal number of multinucleated osteoclasts had formed per well prior to treatment of the osteoclasts with PF-271 for 48 hours. Post treatment with FAK inhibitor, cells were fixed and TRAP staining was performed. PF-271 treatment greatly reduced the number of mature osteoclasts per well in a dose-dependent manner (figure 11a). Given we had previously checked for similar numbers of mature osteoclasts in each condition prior to treatment with FAK inhibitors, we speculated that PF-271 may have induced apoptosis of mature osteoclasts. To test this, protein was also collected from the PF-271 treated osteoclasts, and a Western blot was performed. Cleaved Poly ADP-ribose polymerase (PARP) was assessed as a marker of apoptosis, and was found to increase following treatment with both 1μM and 5μM of PF-271 (figure 11b). In parallel, given our results suggesting that FAK depletion could reduce TRAP mRNA expression, at least at early time points, we

also determined whether pharmacological blockade of mature osteoclasts resulted in their impaired activity by assessing their ability to degrade mineralized matrix using Corning Osteo Assay plates. RAW 264.7 cells were plated on the osteo assay plates and then differentiated with RANK-L for 6 days, followed by treatment with PF-271 for 48 hours. Cells were then bleached off, and osteolytic pits were visualized and counted under the microscope. Treatment of mature osteoclasts with PF-271 at 1μM and 5μM inhibited osteolysis compared to DMSO control (figure 11c). The inhibition of osteolysis caused by PF-271 is unlikely to be solely due to apoptosis given that equal numbers of large multinucleated cells (consistent with mature osteoclasts) were visualized in each well prior to PF-271 treatment. Moreover, the 5μM treated wells had 28 osteoclasts and 6 pits, compared to the 1μM dose of PF-271 which had approximately 18 osteoclasts, and no pits (figure 11a&c). These data thus suggest that FAK inhibition has a direct effect on lytic ability. As such, FAK inhibition may prove to be a promising therapeutic in breast cancer mediated osteolysis as it reduces lytic ability and induces apoptosis in mature osteoclasts.

A)



B)



C)

DMSO	PF-271 1μM	PF-271 5μM
6	0	0

D)

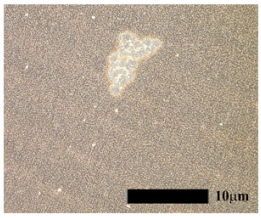


Figure 11. The FAK inhibitor PF-271 induces cell death and decreases osteolytic ability in TRAP+ multinucleated cells. RAW264.7 cells were treated with 50ng/ml of RANK-L every 3 days for 6 days. The differentiated cells were then treated with 1uM or 5uM PF-271 or equal amounts of DMSO as a vehicle control for 48 hours. A) Cells were fixed and TRAP stained, then the number of TRAP + multinucleated cells was counted in each well. Each bar represents the average of two wells done in triplicate biological replicates (n=3, one-way ANOVA was performed with a Bonferroni post hoc test,* $p<0.05$, **$p<0.01$). B) Protein was also isolated from 6-day differentiated osteoclasts treated with PF-271 drug for 48 hours and a Western blot was performed to assess relative protein expression of the marker of apoptosis, cleaved PARP. GAPDH was used as a loading control for equal levels of total protein. C) RAW 264.7 cells were plated on a Corning Osteo Assay mineralized matrix coated plates, and allowed to differentiate in the presence of 50ng/ml RANKL for for 6 days. The differentiated cells were then treated with PF-271 or DMSO for an additional 48 hours. Cells were then lysed with bleach, and 'pits' created as a result of osteoclast degradation of the mineralized matrix were then visualized and counted using the Nikon Eclipse TE2000-U at 100x magnification. D) Scale bar represents 10μm.

5.5 FAK inhibition causes inhibition of osteoblast differentiation and mineralization

In addition to osteoclasts, it is also possible that pharmacological blockade of FAK could directly affect osteoblasts, a major mediator of bone homeostasis. As such, we performed similar analyses to those performed directly in osteoclast precursor cells, to determine whether blockade of FAK activity in pre- or mature osteoblasts has effects on their maturation or activity. To assess the effects of FAK inhibition on osteoblast activity, namely mineralization, mature osteoblasts were treated with PF-271 and multiple staining techniques were performed. MC3T3 E1 cells (pre-osteoblasts) were differentiated for 21 days with AAB, with media being refreshed every 3 days. On day 21, differentiated osteoblasts were treated with PF-271 for 96 hours, with drug being refreshed after 48 hours. Cells were then fixed, and stained for ALP. ALP as detected by red staining, a marker of mature osteoblasts, showed a dose-dependent decrease with increasing concentrations of PF-271 treatment suggesting a reduction in numbers of mature osteoblasts, or minimally their ability to express ALP (figure 12a). To determine the ability of PF-271 treated osteoblasts to lay down mineralized matrix, Von Kossa and Alizarin Red stain were utilized. The non-specific mineralization stain Von Kossa showed a decrease in mineralization with both PF-271 doses (figure 12b). To confirm this finding, we also stained using Alizarin Red stain, a specific red stain that binds calcium. Alizarin Red staining showed a substantial decrease in calcium deposits even at a dose of 1μM PF-271 (figure 12c). Calcium deposits were completely inhibited at the 5μM dose of PF-271 (figure 12c). To confirm that the PF-271 drug inhibited FAK kinase activity in the mouse cells, a Western blot was performed (figure 12d). Both the 1μM and 5μM PF-271 doses inhibited Y397 phosphorylation of FAK, an autophosphorylated

site and hence a marker of active FAK (figure 12d). Moreover, at the higher dose of PF-271, SPARC, a protein that aids in mineralization, was also decreased (figure 12d). In order to extrapolate on the findings that FAK inhibition resulted in a decrease in ALP and mineralization, a Western blot was performed and PARP levels were assessed as a marker of apoptosis. While 1uM and 5uM doses of PF-271 may slightly decrease the levels of total PARP, no cleaved PARP was detected (figure 12e). Taken together, these findings suggest that treatment with the FAK inhibitor PF-271 results in reduced mineralization capacity of osteoblasts, without inducing apoptosis.

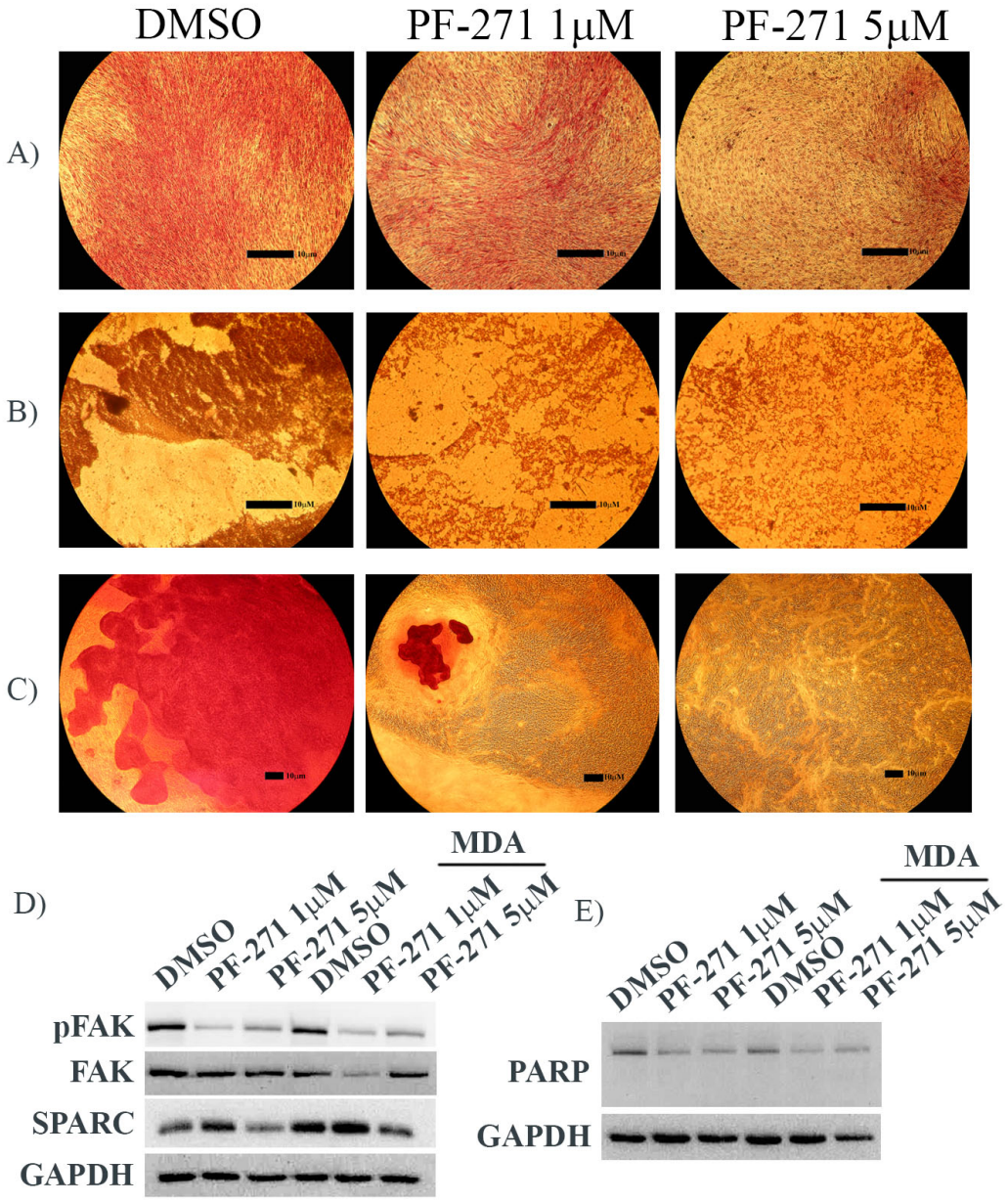

DMSO
PF-271 1μM
PF-271 5μM
A)
B)
C)
D)
MDA
DMSO
PF-271 1μM
PF-271 5μM
DMSO
PF-271 1μM
PF-271 5μM
pFAK
FAK
SPARC
GAPDH
E)
MDA
DMSO
PF-271 1μM
PF-271 5μM
DMSO
PF-271 1μM
PF-271 5μM
PARP
GAPDH

Figure 12. PF-271 treatment results in the inhibition of osteoblast differentiation and mineralization. MC3T3 E1 cells were differentiated with ascorbic acid and β-glycerophosphate for 21 days, changing the media every 2-3 days. Cells were then treated for 96 hours with DMSO or PF-271, changing the media every 2 days. Scale bars represent 10μm A) ALP stain. Cells were fixed with a mixture of acetone, formaldehyde and citrate. Cells were then stained for ALP for 30 minutes at room temperature. Pictures are representative images of n=3 biological replicates. B) Von Kossa stain. Cells were fixed with 70% ethanol for 1 hour at room temperature and then stained with silver nitrate in direct sunlight until brown spots were formed. Pictures are representative images of n=3 biological replicates. C) Alizarin Red Stain. Cells were fixed with 10% formalin for 30 minutes. Cells were then stained with Alizarin Red stain for 45 minutes at room temperature. Pictures are representative images of n=3 biological replicates. D) Protein was also isolated from these cells 96 hours post PF-271 treatment. A Western blot was performed to assess the levels of FAK activity through probing with Y397 FAK, and total FAK. SPARC was also detected by western blot analysis. GAPDH was used as a loading control. E) Using the same samples, a Western blot was performed to assess PARP and cleaved PARP levels. GAPDH was used as a loading control.

5.6 FAK inhibition alters key signaling pathways involved in osteoblast differentiation

While decreasing osteolysis is the main goal of treatment of bone metastatic breast cancers, it may also be important not to inhibit new bone formation. With higher doses of PF-271 significantly inhibiting bone mineralization *in vitro*, it was important to assess the mechanisms by which FAK inhibition may cause this result. FAK inhibition could lead to an inability of mature osteoblasts to lay down mineralized matrix, or it could somehow lead to their de-differentiation and loss of the ability to perform this function. To assess major pathways important for osteoblast maturation and mineralization affected by PF-271 treatment, RNA was collected from 21 day differentiated mature osteoblasts subsequently treated with PF-271 for 96 hours. RT-PCR was then performed to assess expression of the genes known to be important for osteoblast differentiation. Osterix and RUNX2, two transcription factors important for osteoblast differentiation, (36-38) were unaffected by PF-271 treatment (figure 13a&b). With PF-271 treatment resulting in a significant decrease of ALP expression, it was important to assess mediators of ALP expression in osteoblasts. Wnt3a, a canonical Wnt agonist, BMP2, part of the TGF-β superfamily, and Shh, all have documented roles in inducing ALP expression (45,36,52,140). Interestingly, expression of Wnt3a, BMP2 and Shh were increased by 4, 6 and 5 fold, respectively, in mature osteoblast cells treated with the 5μM dose of PF-271 (figure 13c, d & e). To assess signaling pathways downstream of FAK, which may be affected by PF-271 treatment, a Western blot was performed (figure 13g). Interestingly, while the phosphorylation of Akt, known to regulate cell survival, was decreased with the 5μM dose of PF-271, Erk phosphorylation was increased. Erk phosphorylation has been

documented to induce apoptosis (141), and as such it is possible that high doses of PF-271 may begin to induce apoptosis in osteoblasts, however as shown above, no cleaved PARP was detected and as such this finding requires further investigation to determine the relevance of elevated phospho-ERK levels in response to PF-271 (141). MMP2 levels were unaffected by PF-271 treatment (figure 13g). Taken together, these findings suggest that treatment of mature osteoblasts with PF-271 may induce dedifferentiation, and begin to affect their survival. In assessing mechanisms through which FAK inhibition may regulate osteoblast differentiation and mineralization, p53 was examined, as it has recently been linked to FAK where nuclear FAK plays a role in mediating Mdm-2 mediated p53 degradation (86). P53, a tumor suppressor, has well documented roles in negatively regulating osteoblast differentiation, as well as negatively regulating transcription of the significantly upregulated targets Wnt3a and BMP2 (49). Thus, p53 levels were assessed by Western blot. Interestingly p53 levels decreased with increasing doses of PF-271 treatment (figure 13h). Thus, FAK inhibition may result in impaired osteoblast differentiation through inhibiting key signals required for their maturation.

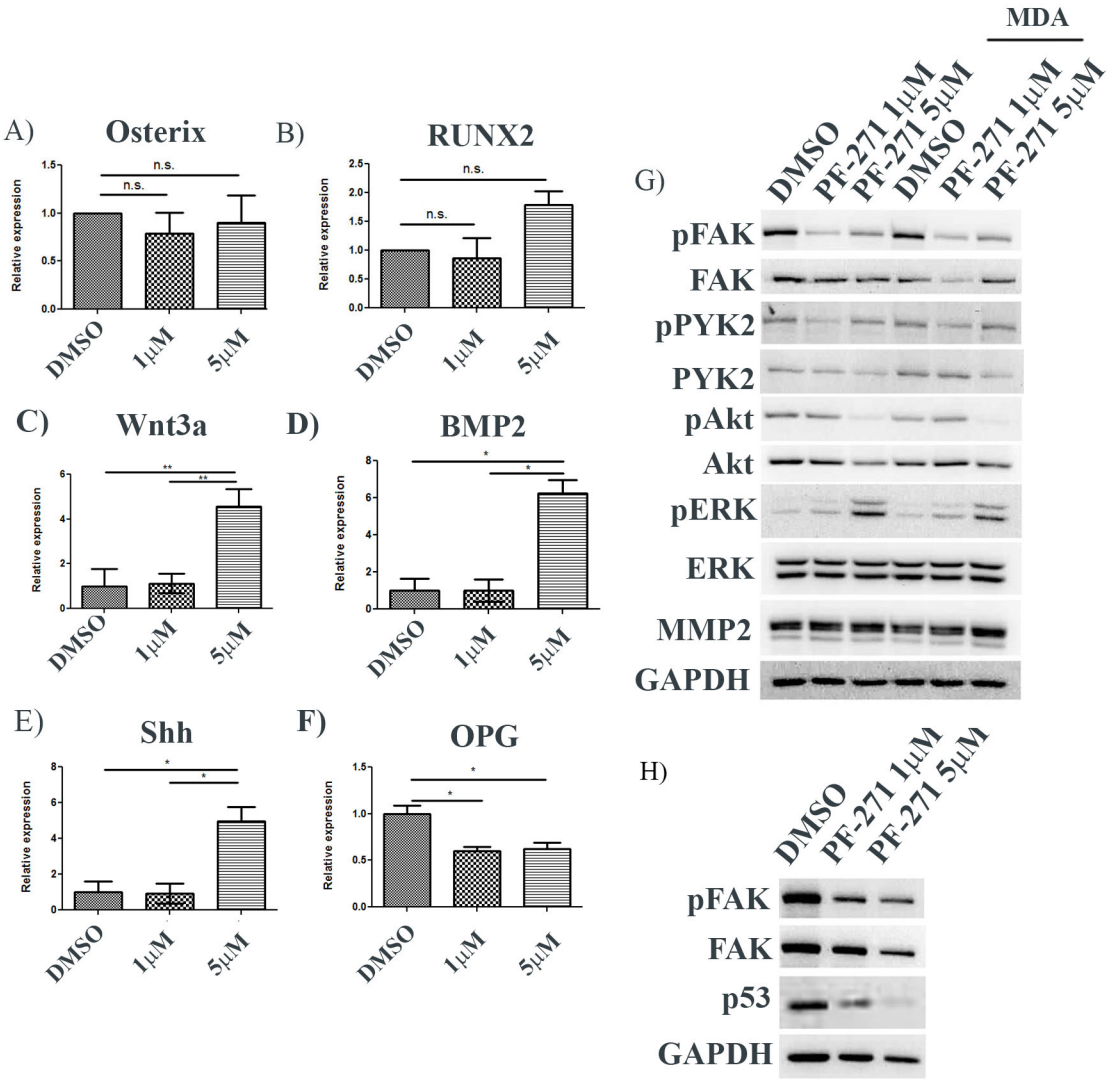

A)
Osterix
B)
RUNX2
C)
Wnt3a
D)
BMP2
E)
Shh
F)
OPG
Relative expression
n.s.
DMSO
1μM
5μM
G)
MDA
DMSO
PF-271 1μM
PF-271 5μM
pFAK
FAK
pPYK2
PYK2
pAkt
Akt
pERK
ERK
MMP2
GAPDH
H)
p53

Figure 13. PF-271 results in the upregulation of multiple pathways involved in osteoblast differentiation through depletion of p53. MC3T3-E1 cells were differentiated with ascorbic acid and β-glycerophosphate for 21 days, changing the media every 2-3 days. Cells were then treated for 96 hours with DMSO as a vehicle control or various doses of PF-271, changing the media every 2 days. RNA was then collected, and RT-PCR was performed to assess the expression of A) Osterix, B) RUNX2, C) Wnt3a, D) BMP2, E) Shh and F) OPG (n=2 * $p<0.05$, ** $p<0.01$, n.s. non-significant, one-way ANOVA was performed with a Bonferroni post hoc test.). Total protein was also collected and used to perform Western blots with equal amounts of protein as described in the materials and methods. G) Blotting for phospho-FAK Y397 was performed first, followed by phopsho-PYK2 and phosphor-Akt. Blotting for relevant total proteins followed, namely for FAK, Akt, MMP2. Phopsho-ERK and ERK were then performed, before blotting for GAPDH was performed as a loading control. H) Blotting for phospho-FAK was performed first, followed by FAK and p53. GAPDH blotting was performed as a loading control.

5.7 Assessing the differences of siRNA mediated FAK knockdown and PF-271 treatment in breast cancer cells

Given recent literature suggesting that PF-271 treatment may cause FAK to accumulate in the nuclear compartment (142,143), and our findings that p53 levels appear decreased in osteoblasts treated with PF-271, it remains possible that PF-271 treatment could lead to alternative biological outcomes as compared to a complete FAK knockdown at the protein level, possibly due to its effects on p53. If PF-271 decreased p53 expression in breast cancer cell lines as it did in the osteoblasts, the outcome may not be beneficial for inhibiting tumor growth. We thus decided to interrogate the differences between FAK protein depletion as compared to blockade of its activity and possible nuclear accumulation with PF-271 further. A Western blot was performed on protein collected from 48-hour treated PF-271 and siRNA FAK knockdown MDA-MB-231 cells to assess p53 levels between the treatments. Neither the PF-271 treatment nor siRNA mediated FAK knockdown caused a change in p53 expression in MDA-MB-231 cells (Figure 14g). Although we did not see similar effects on p53 levels as we saw following PF-271 treatment of osteoblasts, MDA-MB-231 cells have a p53 mutation, which may make them insensitive to FAK mediated p53 degradation. However, the full effects of nuclear FAK accumulation due to PF-271 have not been assessed. Thus, it was still important to assess the differences between pharmacological FAK inhibition and siRNA mediated FAK knockdown. MDA-MB-231 cells were treated with PF-271 for 48 hours in order to compare to previous data obtained following siRNA-mediated FAK depletion in these same breast cancer cells (figure 7). RNA collected from PF-271 treated cells was

similarly assessed for expression of known osteolytic factors. Interestingly, PTHrP was significantly increased in the 5μM dose compared to DMSO control (figure 14a) whereas PTHrP was significantly decreased with siRNA mediated FAK depletion (figure 7). M-CSF also had a trend to an increase with increasing doses of PF-271, which was also opposite of the siRNA data (figure 14b). The three interleukins assessed, IL-6, IL-8 and IL-11 showed no change in expression (Figure 14c, d, e), consistent with what was observed for siRNA mediated depletion of FAK (figure 7). This data provides evidence that blockade of FAK activity via PF-271 treatment and FAK knockdown via siRNA result in differential regulation of osteolytic targets in tumor cells. Moreover, although p53 levels were not affected, MDA-MB-231 cells did respond differently to PF-271 than siRNA mediated FAK depletion, suggesting a mechanism independent of p53.

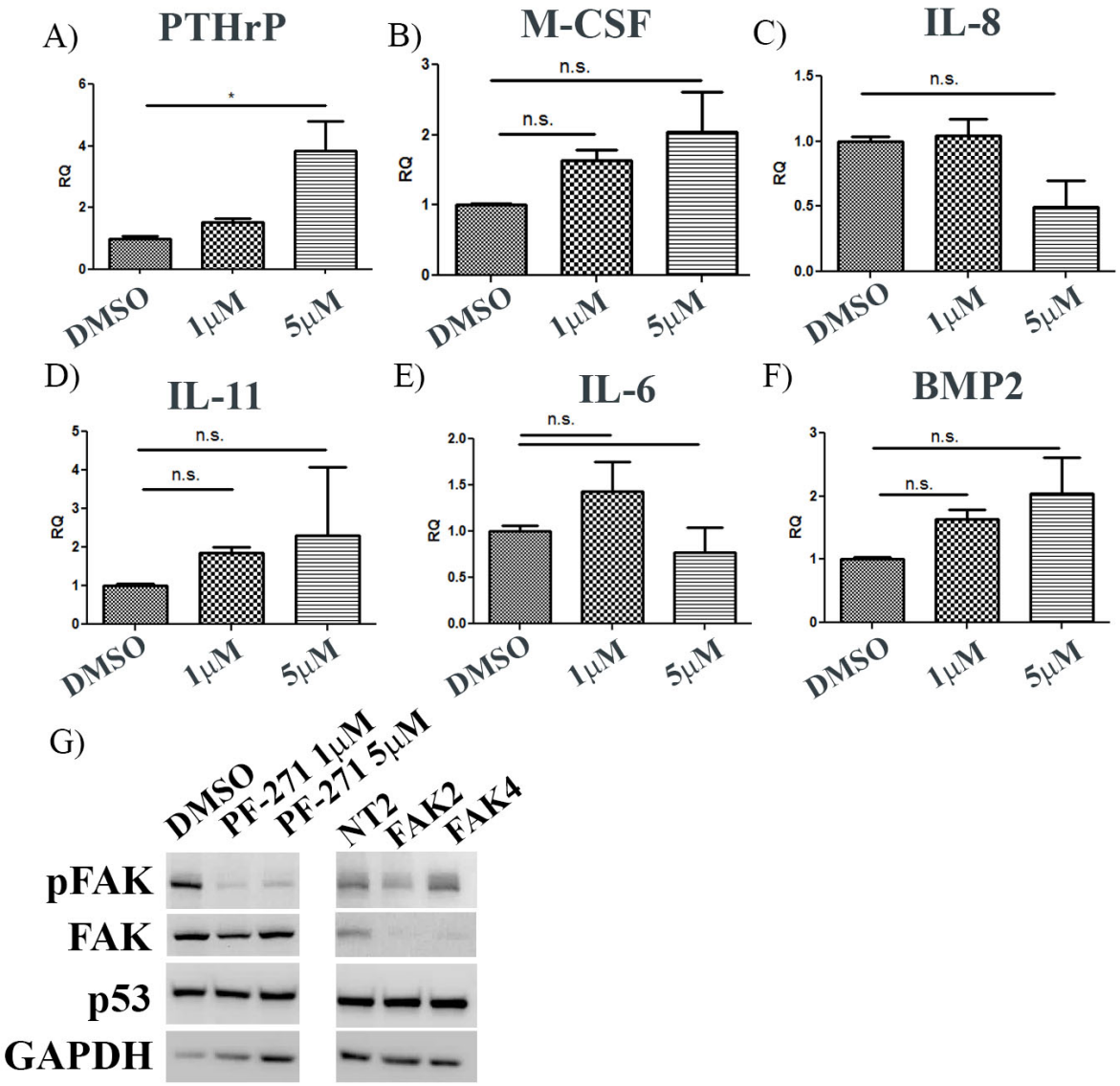

A)
PTHrP
RQ
*
DMSO
1μM
5μM
B)
M-CSF
n.s.
n.s.
C)
IL-8
n.s.
D)
IL-11
n.s.
n.s.
E)
IL-6
n.s.
F)
BMP2
n.s.
n.s.
G)
DMSO
PF-271 1μM
PF-271 5μM
NT2
FAK2
FAK4
pFAK
FAK
p53
GAPDH

Figure 14. Pharmacological FAK inhibition results in differential regulation of osteolytic factors compared to siRNA-mediated FAK depletion in MDA-MB-231 cells. MDA-MB-231 cells were plated, and the next day treated for 48 hours with PF-271 or DMSO as a vehicle control. RNA was extracted, and RT-PCR was performed to assess: A) PTHrP, B) MCSF, C) IL-11, D) IL-6, E) IL-8, and F) LOX with GAPDH used as a loading control. N.s. non significant, $p<0.05$*, $p<0.01$**. Statistical analysis performed was a one-way ANOVA with a Bonferroni post hoc test. G) In parallel experiments, protein was extracted and a Western blot was performed to confirm the efficacy of the PF-271mediated inhibition of FAK activity and siRNA mediated FAK knockdown through probing with phospho-FAK Y397 followed by FAK and p53. GAPDH was used as a loading control.

6. DISCUSSION

In order to test the hypothesis that FAK plays a role in regulating breast cancer mediated osteolysis, it was important to ensure that our *in vitro* models mimic what occurs in patients. In order to model the bone microenvironment, a coculture of MC3T3 E1 pre-osteoblasts and RAW 264.7 pre-osteoclast cells was used which together with conditioned media from breast tumor cells lines, would represent the 3 main players in the vicious cycle of bone metastasis. Breast cancer conditioned media from different cell lines was then used to assess their ability to induce osteoclastogenesis. Within our *in vitro* coculture model, MDA-MB-231 breast cancer conditioned media had the greatest effect on inducing osteoclastogenesis compared to MCF7 and T47D cell lines (Figure 6a). This confirms what others have shown *in vitro* and also suggests that conditioned media from MDA-MB-231 cells contains more osteolytic factors compared to the other cell lines tested (138). This also replicates what has been shown *in vivo* where MDA-MB-231 cells injected intracardially into mice form osteolytic lesions within 3-4 weeks, while MCF7 cells are less aggressive and only form lytic lesions after 6-8 weeks (144). Moreover, all breast cancer cell lines tested were able to stimulate osteoclastogenesis, and inhibit osteoblasts, which mimics the phenotypes leading to osteolytic lesions that occur in breast cancer patients. This is in contrast to prostate cancer bone metastases, which display both osteoblastic and osteolytic lesions by increasing the activity of both osteoblasts and osteoclasts (145). Thus, this coculture of MC3T3 E1 and RAW 264.7 cells treated with conditioned media from MDA-MB-231 resulted in an osteolytic phenotype, which mimics *in vivo* models and thus could be used for further testing in the

context of bone metastases, such as evaluation of putative novel combination treatments on inhibiting bone lytic phenotypes.

Interestingly, our coculture data also suggested that it is important to have differentiating osteoblasts in order to most effectively induce osteoclastogenesis. While treating the coculture with breast cancer conditioned media alone induced low levels of osteoclastogenesis, the addition of both of the differentiating osteoblast factors, AAB, and breast cancer conditioned media together had a more robust effect on inducing osteoclastogenesis (figure 6a). This increase in osteoclastogenesis is mediated by an effect of breast cancer conditioned media on differentiating MC3T3 E1 cells, as the addition of AAB alone, or MDA-MB-231 conditioned media alone did not induce the same level of osteoclastogenesis. This is likely a result of AAB inducing the expression of factors within the osteoblast that are not expressed in the untreated pre-osteoblasts which may render them more sensitive to factors in breast cancer cell conditioned media, however the mechanism of this in our model system remains unknown at this time. Interestingly, there are many hypotheses as to what factor is responsible for the osteolytic nature of bone metastases caused by breast cancer cells. While Cox *et al.,* (2015) show that LOX causes osteolytic bone metastases, others provide evidence for the effects of PTHrP, TGF-β, sclerostin and many others (72,146,147). This is not surprising, as many factors are known to contribute to normal osteoclastogenesis and as such it is likely that all of these factors play a role in breast cancer cells being able to induce a shift in osteoblast homeostasis towards osteolysis. Thus breast cancer conditioned media may provide the stimulus to change the expression profile of osteoblasts to continuously express osteolytic factors. In addition, given the fact that there are multiple factors

capable of promoting osteoclastogenesis, and our results suggested that FAK in tumor cells could affect some, but not all of these factors, it is perhaps not surprising that we did not see major effects on osteoclastogenesis in our assay system upon FAK depletion in tumor cells.

While much of the breast cancer bone metastasis literature focuses on the effects of tumor on osteoclasts given the lytic nature of the disease, our data suggests that the effects of tumor on bone homeostasis are compounded by its effects on the osteoblast compartment as well. This gives importance to studying the effects of breast cancer conditioned media on osteoblasts as they play a large role in mediating osteolysis. To assess the differentiation of MC3T3 E1 cells treated with AAB and breast cancer conditioned media, the expression of ALP, a marker of osteoblast differentiation was assessed. All three breast cancer cell lines tested showed a decrease in ALP by PCR, which was confirmed by ALP staining (figure 6d & e). This confirms what others have shown, that breast cancer inhibits osteoblast differentiation (73,74,138,147). When OPG, the inhibitor of osteoclastogenesis, was assessed in MC3T3 cells treated with conditioned media from all three breast cancer cell lines, it showed a two-fold decrease compared to AAB differentiated osteoblasts. This result confirms what others have shown with regards to breast cancer cells decreasing osteoblast OPG expression (74,138). However, because all of the cell lines showed the same decrease in OPG, but showed variable induction of osteoclastogenesis this is not the sole factor responsible for the osteolytic phenotype. Thus breast cancer may induce osteolysis by affecting OPG expression in osteoblasts, as

well as other factors, which may work directly on osteoclasts, or on osteoclasts via indirect effects on osteoblasts.

Having confirmed that breast cancer conditioned media induces osteoclastogenesis, the role of FAK in mediating the expression of factors secreted by breast cancer cells that may contribute to this phenotype could be assessed. Both MDA-MB-231 and MCF7 cells were assessed further to provide data from tumors which appear to have more and less osteolytic potential respectively. Utilizing siRNA mediated FAK depletion, MDA-MB-231 cells had a decrease of M-CSF, the osteoclastogenic factor, at the message level with FAK knockdown (Figure 7c). This is similar to the study of another group, which showed that periodontal ligament cells transfected with FAK siRNA showed a mild decrease in M-CSF expression in response to compressive stress (148). Interestingly, only MDA-MB-231 conditioned media had detectable levels of M-CSF by ELISA, which correlates with its increased ability to form osteolytic lesions (figure 7d). This breast cancer secreted M-CSF has been shown to be sufficient to induce osteoclastogenesis with addition of RANK-L in pre-osteoclast differentiation models (68). However, while soluble protein levels of M-CSF showed a trend to decrease with FAK knockdown by ELISA, it did not reach significance (figure 7d). This may be caused by the limitations of siRNA, where FAK levels may not be completely reduced, allowing for low levels of protein expression to function as well as levels being transiently lowered which could allow for subsequent accumulation of M-CSF as FAK expression levels return. Lastly, it is possible that M-CSF protein levels are stable and may not be rapidly turned over, and as such there may be detection of residual M-CSF that was formed prior to the siRNA knockdown. Thus through further optimization, FAK knockdown may be beneficial in

reducing osteolysis in part through regulating M-CSF expression, however due to the experimental limitations of our approaches, we were unable to confirm a role for FAK-regulated M-CSF in this setting.

Although reduced mRNA levels of M-CSF through FAK knockdown was promising in our desire to decrease osteolysis, we could not confirm a significant decrease in M-CSF protein levels. Therefore, we moved forward testing other osteolytic factors to evaluate whether they may be altered under conditions of depleted FAK levels. Among breast cancer secreted factors that have a known role in osteolysis are the interleukins IL-6, IL-8 and IL-11. However, none of these showed significant changes with FAK knockdown as detected by RT-PCR or ELISA (figure 7f-h). Another factor that is widely studied in regards to breast cancer mediated osteolysis is PTHrP. Knockdown of FAK resulted in a significant decrease of PTHrP at the mRNA level (7j). This is interesting, as PTHrP has only been documented to function upstream of FAK (135,149), and our findings suggest that a feedback loop for FAK-PTHrP interactions may exist. Moreover, studies have shown that PTHrP depletion or inhibition can be used to inhibit breast cancer bone metastasis (66,70,72,146). As such, decreasing PTHrP production by breast cancer cells through FAK depletion may result in breast cancer cells having a decreased ability to induce osteoclastogenesis and this could explain our findings.

As our data suggested that FAK might regulate the secretion of osteolytic factors by tumor cells, the ability of conditioned media collected from cells with decreased FAK

expression was assessed. In addition, given the complex nature of the coculture system, we also decided to test the various effects of FAK depletion in each individual cell type. In a monoculture of RAW 264.7 pre-osteoclasts, conditioned media from FAK-depleted cells showed no change in the ability to induce osteoclastogenesis when used in combination with RANKL stimulation (figure 8a). Although this result was surprising given that M-CSF is required for osteoclastogenesis, M-CSF levels were not dramatically reduced in FAK-depleted cells and so it is possible the levels of M-CSF that were produced were sufficient to induce osteoclastogenesis. However, multiple studies have also suggested that RAW 264.7 cells do not require M-CSF for osteoclast differentiation in the presence of RANKL stimulation (150). As we were unable to see differentiation in this model system in the absence of RANKL stimulation, we could not address the role of the modest reduction of M-CSF in inducing osteoclastogenesis in this model. However, other groups have shown that mouse bone marrow cells are more dependent on co-stimulation with both RANKL and M-CSF, and thus similar experiments in this context may more definitively show whether or not a role of FAK-induced M-CSF exists. In addition to possible issues with the model system, FAK knockdown did not show a decrease in LOX mRNA expression (figure 6i). LOX was recently identified as a mediator of osteoclastogenesis shown to mediate its effects directly on pre-osteoclasts (64). Thus, although there was no difference in their ability to induce osteoclastogenesis between control and FAK depleted breast cancer cell conditioned media in monoculture, it is possible that the factors regulated by FAK mediate osteoclastogenesis through osteoblasts. As such, our next experiment was to assess the effects of control and FAK depleted breast cancer conditioned media on our coculture system.

In a homeostatic bone environment, osteoblasts are the major mediator of osteoclastogenesis. Our data in Figure 6 suggesting that AAB differentiating osteoblasts induce more osteoclastogenesis than un-stimulated pre-osteoblasts confirms this. Moreover, as PTHrP levels, a factor known to regulate osteoblast differentiation and function (149,151), may be regulated by FAK (Figure 7j), a coculture of osteoblasts/pre-osteoclasts may provide better evidence for the role of FAK in breast cancer production of osteolytic factors. When conditioned media from FAK depleted breast cancer cells was tested for its ability to induce osteoclastogenesis in this osteoblast/osteoclast coculture system, a slight decrease in osteoclastogenesis was seen with the inclusion of AAB-differentiated osteoblasts, although this did not reach statistical significance (figure 8b). However, generally our data suggested a significant role of tumor-induced effects on osteoblasts that led to the osteoclastogenesis observed. As such, we investigated the effects of tumor-expressed FAK on osteoblast phenotypes directly. To assess this, osteoblasts were treated in monoculture with conditioned media from FAK-depleted or expressing MDA-MB-231. While ALP and OPG levels, factors expressed by mature osteoblasts, showed a trend to increase, neither reached significance. Given that PTHrP has been found to have varying effects on the bone, both anabolic and catabolic (reviewed in 152), it is possible that its reduction in the FAK-depleted conditioned media did not successfully restore ALP and OPG levels. Thus we cannot conclude that the tumor-derived factors that are regulating the osteoblast phenotype are FAK-controlled. However, again these experiments are confounded by the limitations of siRNA-mediated depletion of FAK. While siRNA mediated knockdown of FAK was chosen due to the non-viability of FAK null tumor cells, these non-significant results support the limitations

of siRNA, and also suggest that any remaining levels of FAK expression in the breast cancer cells may be sufficient to induce expression of factors that can alter osteoblast and osteoclast function. Moreover, there are a multitude of factors that regulate osteoclastogenesis. Although FAK may regulate the secretion of some osteolytic factors, others may compensate and provide enough stimulus to stimulate osteoclastogenesis. Thus while FAK may play a role in mediating breast cancer induced osteolysis through changing the expression profile of osteoblasts, alternative methods of confirming this are limited. We have found that we are unable to rescue FAK targeted shRNA cell lines, and FAK null transgenic mice show embryonic lethality (92), and thus it is difficult to rescue FAK null osteoblasts or osteoclasts. As such, it was imperative to test the inhibition of FAK within each cell type individually. Clues from these sort of experimental results, would help establish a role of FAK in breast cancer mediated osteolysis, while also providing a more accurate understanding of what could happen in an *in vivo* setting, where therapeutics would be given systemically.

In order to be an effective treatment for osteolytic bone metastases anti-FAK treatment should ideally inhibit tumor growth directly, but also decrease osteolytic factors within the osteoclast to prevent further bone degradation. Because FAK regulates many pathways within the cell and has previously established roles in regulating chemokine and MMP expression, it was hypothesized that FAK would also regulate osteolytic factors (107,108,115). siRNA mediated knockdown of FAK within RAW 264.7 cells resulted in the decrease of TRAP expression by both RNA and protein in early differentiating osteoclasts (figure 9c&f). Although the role of TRAP has not been fully

defined, its circulation in serum correlates with osteolysis (13). Thus, decreasing TRAP expression through FAK may decrease osteolysis in this manner. Although this defect in TRAP expression was seen in early differentiating osteoclasts, FAK depletion had no effect on the ability to form TRAP positive multinucleated cells by day 7 (figure 9e). This suggests that although there was a defect early on, the cells are able to overcome decreased FAK expression. This may be due in part to the transient nature of the siRNA-mediated knockdown of FAK. However, these findings are also in line with the work of others, where it was shown that the FAK family member PYK2, but not FAK, was more important for osteoclastogenesis (125). The Cathepsin K data, where only one FAK siRNA sequence resulted in a significant reduction in their mRNA levels, also highlights the limitations of the use of siRNA (figure 9d). Taken together, these results suggest either a limitation of siRNA or a more prominent role for PYK2 in osteoclasts. With the siRNA being specific to FAK knockdown, PYK2 would still be able to function within these cells, and efficiently induce differentiation. Thus, while FAK may play a small role, osteoclast differentiation may be more dependent on PYK2, and thus osteoclast differentiation is not observed to be affected by FAK knockdown in this model system.

While we observed some modest effects following FAK depletion using siRNA strategies, we observed more significant changes following treatment of osteoblasts or osteoclasts with the inhibitor PF-271. Given, the previously published data suggesting a major role for PYK2 in regulating osteoclast function (116,117,125), and that PYK2 plays a compensatory role for FAK in FAK null cells, it is perhaps not surprising that the dual FAK/PYK2 inhibitor seemed to have more significant effects than siRNA mediated

depletion of FAK alone (121). Treatment of mature osteoclasts with PF-271 caused a decrease in the number of osteoclasts with increasing doses of PF-271 (figure 10a), suggesting that minimally dual inhibition of FAK/Pyk2 may affect osteoclast viability. Not surprisingly, as FAK and PYK2 are known to play a role in mediating cell survival (86,98,113), an increase in cleaved PARP, indicative of induction of apoptosis was also seen with increasing doses of PF-271 treatment in osteoclasts (figure 10b). This suggests the decrease in the number of osteoclasts was due to PF-271 inducing apoptosis in these osteoclasts. This is in line with the work of others, in many cell types, that FAK inhibition results in apoptosis (86,154-156). We also observed that treatment of mature osteoclasts with PF-271 inhibited their osteolysis activity, as evidenced by decreased pit formation of differentiated osteoclasts in a Corning Osteo Assay plate. Although one could argue that this is simply due to the reduced number of osteoclasts as a result of the apparent increased apoptosis resulting from PF-271 treatment, we saw effective inhibition of pit formation even at the low 1μM dose of PF-271 (figure 10c), where the number of mature osteoclasts remained constant and similar to levels found in untreated controls under similar differentiation conditions (figure 10a). This suggests that although there were mature osteoclasts remaining after treatment with the 1μM PF-271 dose, their ability to form pits and lyse mineralized matrix was inhibited by PF-271. This may be a result of the decrease in enzyme production as a result of FAK inhibition, as evidenced by its ability to regulate MMP production (107,108), and our data suggesting it may control levels of TRAP in osteoclasts which would be effectively reduced by FAK inhibition.

With osteoblasts playing a large role in mediating both osteoclastogenesis and bone formation, it was important to assess the effects of PF-271 on these cells. Moreover, although studies have been done using knockdown experiments targeting FAK, the role of PF-271 has not yet been shown in osteoblasts. In an ideal treatment for osteolytic bone metastases, the treatment would not only result in inhibition of osteoclast function, but also promote osteoblast differentiation and restore bone formation. Compared to treatment with vehicle control, treatment of mature osteoblasts with the 5μM PF-271 dose greatly decreased the intensity of ALP (figure 11a), suggesting either induction of apoptosis of mature osteoclasts, or induction of their dedifferentiation. This result is perhaps not surprising, as it has been shown that β1-integrin, an upstream mediator of FAK activation, regulates osteoblast differentiation following binding of its ligand collagen I which is the predominant matrix in bone (50-53). Downstream of collagen-β1 integrin engagement, this leads to the activation of FAK and the subsequent induction of ALP expression (123,124). It was further shown that inhibiting β1-integrin by antibody or the use of antisense FAK in pre-osteoblasts resulted in decreased ALP expression and impaired osteoblast differentiation (52,124). Our data would suggest that FAK inhibition can also inhibit ALP expression in mature osteoblasts and not just ALP expression during differentiation. Interestingly, within 96 hours of PF-271 treatment, osteoblast mineralization function was also inhibited (figure 11b and c). This could be due in part to the fact that ALP is reduced in PF-271 treated mature osteoblasts. It is thought that ALP functions to aid in bone mineralization by providing inorganic phosphate (reviewed in 56), thus the decrease in ALP caused by PF-271 treatment may lead to impaired mineralization as a result of impaired ALP expression. The inability of the 5μM PF-271

treated osteoblasts to mineralize would suggest a possible unbeneficial effect for osteolytic bone metastases patients, as they already have impaired bone mineralization caused by the breast cancer cells. However, the 1μM dose of PF-271 showed a less drastic effect, where mineralization, as detected by Alizarin Red stained calcium deposits, was still visualized. This suggests PF-271 causes dose-dependent effects in osteoblast maturity and function, and therefore the doses must be further assessed to ensure osteoblast function is not completely inhibited. This is comparable to the work of Horikiri *et al*. (2013), who found that depletion of 70-84% of FAK using shRNA in MC3T3 cells reduced ALP and Alizarin red stain. Interestingly, they also found that FAK depleted MC3T3 cells had reduced RANKL expression in response to PTHrP stimulation, and induced less TRAP positive cells compared to control cells in a coculture stimulated with Shh and PTHrP (149). However, when Kim *et al*. (2007), generated FAK null calvarial osteoblasts from crossing $FAK^{fl/fl}$ $p53^{-/-}$ and $FAK^{fl/-}$ $p53^{-/-}$ mice and removed remaining FAK with cre, they found that only 30% of the clones were able to undergo osteoblast differentiation compared to control FAK expressing cells (122). Interestingly, in these FAK null cells they found that PYK2 had redistributed from the perinuclear region to the focal adhesions, where it could partially compensate for the lack of FAK (122). Taken together, the use of the dual FAK/PYK2 inhibitor on differentiated osteoblasts may inhibit osteoblast differentiation and function as it blocks the activity of both FAK and its compensator PYK2. Despite the possible detrimental effects on the osteoblasts ability to induce bone mineralization, our data suggests that PF-271 treatment may still benefit patients as a result of its ability to block osteolytic functions of osteoclasts directly, thereby at least preventing further bone loss.

High doses of PF-271 FAK inhibitor resulted in substantial inhibition of osteoblast mineralization ability, a novel finding. To determine the putative mechanism by which FAK inhibitors could block this function, we initially focused on factors know to regulate ALP and mineralization as these were the two most significantly affected osteoblast characteristics observed following drug treatment. Osterix and RUNX2 were examined as both are known to regulate osteoblast differentiation (36-39,47,48), however FAK inhibition following PF-271 treatment did not result in changes in their expression, at least at the message level (figure 13a & b). FAK depletion has been shown to decrease ALP expression through a decrease in Osterix and RUNX2 function through decreasing ERK phosphorylation (157). However, our data shows that high doses of PF-271 resulted in an increase in ERK phosphorylation (figure 13g), which would not be consistent with this proposed mechanism. As such, we sought to investigate another major pathway involved in osteoblast differentiation, the Wnt signaling pathway. High doses of PF-271, which also resulted in significantly reduced ALP expression and inhibition of mineralization, resulted in a 5-fold increase in Wnt3a expression at the mRNA level (figure 13c). Wnt3a, a Wnt/β-catenin agonist, is known to induce ALP expression and osteoblast differentiation (45,140). Our observations do not correlate with the literature findings suggesting that FAK positively regulates Wnt3a expression, with RNAi FAK depletion resulting in a decrease of Wnt3a levels in the neural plate as well as MDA-MB-231, MCF7 cells, suggesting a possible difference between depletion of FAK and use of a pharmacological drug (158), which is a phenomenon we have also observed in our studies. Nonetheless, based on these findings we would thus predict that if FAK inhibition using PF-271 results in an increase in Wnt3a, we should actually see increased

ALP expression, however we saw the opposite and observed reduced ALP expression. However, it has been shown that Wnt3a secretion in conjunction with BMP2, both inducers of osteoblast differentiation, results in the opposite effect and osteoblast inhibition (140). As such, we examined the levels of BMP2 expression in PF-271 treated osteoblasts. We found that treatment with the 5μM PF-271 dose induced a 6-fold increase in BMP2 expression (figure 13d). This result was not seen in the 1μM dose. These findings would then be consistent with the overall finding of inhibition of osteoblasts. Shh, which was also upregulated following treatment with the 5μM dose of PF-271 has also been shown to function with BMP2, with Shh pretreatment of osteoblasts enhancing BMP2 induced ALP (46). Although the 1μM dose of PF-271 did not induce an upregulation of these factors, it is possible that although levels of RUNX2 do not change, its phosphorylation and thus its activity is reduced by FAK inhibition as others have shown, allowing for some reduction in ALP expression and mineralization (157). Taken together our data suggests that FAK inhibition using PF-271 treatment leads to increased expression of both Wnt3a and BMP2 in osteoblasts effectively resulting in inhibition of ALP expression and osteoblast differentiation.

Interestingly, p53 has links to osteoblast differentiation through Wnt, and BMP, while also having links to FAK. P53 has been shown to repress canonical Wnt, with knockdown of p53 resulting in an increase of β-catenin, as well as WNT1 and LRP6 (159,160). This regulation was shown to be through mir-34, which transactivates in response to p53 to repress canonical Wnt genes (160). Moreover, p53 has been shown to regulate BMP2 expression, however the findings were inconsistent, with normal levels

causing an increase in BMP2 expression, but overexpression of p53 inhibiting BMP2 expression (161). Loss of p53 also resulted in the hyperactivation of BMP-Smad1 pathway in neural stem cell proliferation (162), suggesting a role for p53 in regulation of BMPs. In one study, p53 was found to negatively regulate osteoblastogenesis, through repressing the essential transcription factor Osterix (49). This, taken together with data suggesting that FAK TKIs can lead to nuclear accumulation of FAK (142,143), and with FAK's documented role in regulating Mdm2-mediated p53 degradation (86), suggested that a FAK-p53 axis might be the link between these proteins. Thus, it was hypothesized that FAK inhibition using PF-271 treatment could alter p53 expression, resulting in an increase in Wnt3a and BMP2. When p53 levels were assessed in PF-271 treated osteoblasts, its expression was decreased with increasing doses of PF-271. This is contrary to what has been previously suggested, whereby Lim *et al.* (2008) showed that loss of FAK promotes p53 accumulation (86). However, there is evidence that PF-271 does not translate to a loss of FAK, but functions to promote nuclear accumulation of FAK (86,143). This may be caused by PF-271 binding to the FAK kinase domain, which contains the nuclear export signal (NES), thereby masking the NES and preventing FAK from being exported from the nucleus (143). Thus, FAK can accumulate in the nucleus and can function to decrease p53 levels, which may cause the up-regulation of both Wnt3a and BMP2 as observed in our studies. Although the 1μM dose of PF-271 did not induce an upregulation of these factors, it is also had more p53 compared to the 5μM dose, which was almost completely inhibited. Thus it is possible that the residual p53 levels in the 1μM dose conditions are sufficient to suppress canonical Wnt and BMP expression. Moreover, Fujita *et al.* (2007) also show that while Wnt3a is responsible for

production of OPG, BMP2 inhibits Wnt3a-induced OPG expression. This is in line with our findings that FAK inhibition resulted in an overall decrease in OPG (figure 13f). However, Fujita *et al.* (2007) show that the use of either a Noggin inhibitor to inhibit BMP2 or a Dikkopf related protein (Dkk) inhibitor to inhibit Wnt3a (canonical Wnt), could alleviate the inhibition of ALP expression by these two proteins. Thus, the use of a Noggin inhibitor in conjunction with FAK inhibition could result in an increase of OPG production, while also increasing ALP production, and as such allow new bone formation while still decreasing the survival of osteoclasts. This remains to be tested in our system.

Taken together, all of the data using PF-271 treated osteoblasts suggest that there might be a multitude of regulated factors that result in a reduction of differentiated osteoblast markers and function. Firstly, as collagen I plays an important role in regulating osteoblast differentiation through binding of β1 integrin, with subsequent activation of FAK (50-53), it is possible that through inhibition of FAK, osteoblasts begin to dedifferentiate as a result of a loss of contact with collagen I. This may be through decreasing the function of RUNX2 or Osterix, as others have seen (157), although there are differences between our work and others, namely the induction of ERK at higher doses of the PF-271 inhibitor. As a result of pharmacological FAK inhibition, FAK may also accumulate in the nucleus and result in Mdm-2 mediated p53 degradation (figure 13h). With p53 having documented roles in inhibiting osteoblast differentiation, its degradation may result in the cells trying to overcome the loss of collagen I/β1 integrin binding. As a result, p53 degradation causes removal of its suppressive effects on Wnt3a and BMP2, causing their upregulation, two stimulators of osteoblastogenesis. However,

when both expressed they can result in a reduction of ALP and OPG expression and ultimately inhibit osteoblast differentiation (140). This process is depicted in figure (15). As such, pharmacological inhibition of FAK may inhibit their differentiation and function by hitting multiple targets. However, the use of a Wnt3a or BMP2 inhibitor may restore ALP expression and thus increase their function.

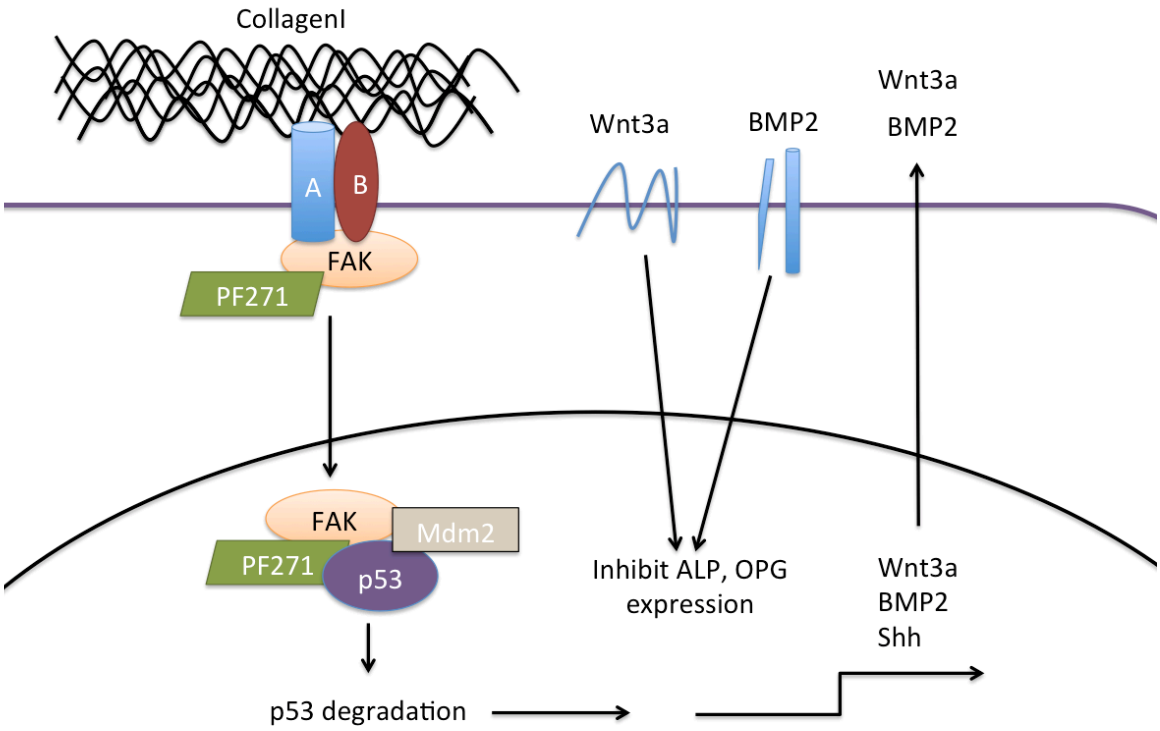

CollagenI
A
B
FAK
PF271
Wnt3a
BMP2
Wnt3a
BMP2
FAK
Mdm2
PF271
p53
Inhibit ALP, OPG
expression
Wnt3a
BMP2
Shh
p53 degradation

Figure 15. The use of PF-271 may inhibit osteoblast differentiation through p53/Wnt3a/Bmp2. The use of a pharmacological FAK inhibitor results in FAK accumulation in the nucleus as a result of the NES being blocked due to steric interference from the small molecule binding the kinase domain. This allows for FAK to function as a scaffolding protein for Mdm2 mediated degradation of p53 (86). Loss of p53 alleviates its suppressive effects on canonical Wnt, and BMP2 resulting in an increase in their expression. However, this dual inhibition results in a decrease of ALP and OPG levels, favouring an osteolytic environment.

Throughout my research project, it has become clear to us that there are many phenotypic differences observed between the use of siRNA mediated knockdown of FAK, and the pharmacological FAK inhibition that would occur in patient use. As seen with evidence that FAK knockdown results in a reduction of Wnt3a (158), which was opposite to our findings that PF-271 resulted in an increase in its expression. As such, use of pharmacological inhibitors could lead to detrimental, unexpected or off target effects, and as such pharmacological inhibitors should be further investigated in additional putative cell targets aside from tumor cells before their clinical use. It is possible that while FAK inhibition through PF-271 caused p53 degradation in osteoblasts, FAK knockdown using alternative depletion strategies such as the work of Horikiri *et al.,* (2013), who used shRNA to deplete FAK, could result in an increase p53 levels through decreasing its FAK mediated degradation (86). This could be of importance, especially for regulation of tumor cells. Other factors may also be differentially regulated by the pharmacological inhibition and the siRNA mediated knockdown. Thus, p53 expression levels were compared in FAK knockdown and PF-271 treated MDA-MB-231 cells. Interestingly, p53 levels were equal among treatment groups. MDA-MB-231 cells are p53 mutant and express high levels of p53 compared to wild-type p53 MCF7 cells (163) or to osteoblasts and osteoclasts which are also p53 wildtype. Thus, FAK inhibition or knockdown may not have an impact on p53 expression or activity due to the high levels of mutated p53 expressed in MDA-MB-231 cells, leading to different observed effects on various targets in mutant p53 tumor cells compared to wild type expressing cells. This is noteworthy in the context of predicting patient response to these agents clinically, as differences may be observed in patients whose breast cancers harbor p53 mutations as compared to those

who do not. Despite this, other factors or pathways that are dependent on other FAK activities, such as its kinase activity or its scaffolding properties may still be affected by pharmacological kinase inhibition as these would be effectively blocked regardless of p53 status. When M-CSF levels were tested in PF-271 treated MDA-MB-231 cells, its expression trended towards increasing (figure 14b), while siRNA mediated FAK depletion trended towards a decrease in mRNA levels, although neither reached significance (Figure 7d). Moreover, the expression of PTHrP was significantly increased with high doses of FAK inhibition, while it was significantly reduced with siRNA (figure 14a). Thus, the pharmacological inhibitor PF-271 and FAK knockdown cause different effects in cells. This may provide evidence towards the limited success of clinical trials involving pharmacological FAK inhibition, as unexpected factors, such as PTHrP, a protein heavily involved in promoting cancer progression become upregulated. It is therefore imperative that drugs such as these be further tested *in vivo* in preclinical models and in vitro in relevant non-tumor cell types in order to assess the full role of FAK inhibition, and better predict patient outcome following their use.

7. SUMMARY

Our data suggests that FAK plays an important role in mediating survival and function of all 3 cell types involved in breast cancer mediated osteolysis. While FAK played a role in regulating the expression of PTHrP, it did not change the expression of all secreted factors known to play a role in osteoclastogenesis, and as such did not significantly impair osteolysis in an osteoblast/osteoclast coculture. However, FAK inhibition using PF-271 resulted in the apoptosis and decreased capacity for osteolysis in mature osteoclasts, the mediators of osteolysis. This was consistent with the work of Bagi *et al.*

(2008), who found that PF-271 reduced intra-tibial tumor growth and restored some structural bone parameters, as detected by CT scan (136). Moreover, and perhaps unexpectedly, our work showed that FAK inhibition resulted in a dose dependent inhibition of osteoblast maturation and mineralization. Interestingly, FAK inhibition also resulted in decreased expression of p53, possibly through PF-271 mediated nuclear accumulation of FAK resulting in Mdm2 mediated degradation. P53, a negative regulator of osteoblast differentiation then results in the upregulation of osteoblast differentiation factors Shh, BMP2 and Wnt3a, which in combination results in the inhibition of osteoblast differentiation. While overall these findings would suggest that PF-271 treatment would not be beneficial for bone repair following osteolysis, it is possible that with concurrent inhibition of BMP2, osteoblast mineralization could be restored. Taken together, PF-271 has a known role in inhibiting breast cancer tumor growth, and we have shown that it also induces apoptosis in mature osteoclasts ultimately leading to a reduction in bone matrix degradation. As such, the use of PF-271, possibly in conjunction with a BMP2 inhibitor to further restore bone formation, should be further assessed *in vivo* for treatment of breast cancer mediated osteolysis.

8. FUTURE EXPERIMENTS

Future experiments should assess the mechanism by which pharmacological FAK inhibition results in osteoblast inhibition. This would include assessing if PF-271 does in fact cause nuclear accumulation of FAK and mediate p53 degradation through performing nuclear fractionation. These experiments should focus on assessing dual

inhibition of FAK and BMP2, as it has a known role for suppressing OPG levels, in order to restore bone formation. Lastly, *in vivo* analysis should be performed on a breast cancer bone metastases model such as an intra-tibial injected bone model, in order to assess the effects of PF-271 alone or in combination with the bisphosphonate zoledronic acid in order to assess its efficacy against the current standard treatment.

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