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Adipocyte-Induced Inflammation In Prostate Tumor Progression In Bone: Role Of Cxcr2 And Osteopontin

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ADIPOCYTE-INDUCED INFLAMMATION IN PROSTATE TUMOR PROGRESSION IN BONE: ROLE OF CXCR2 AND OSTEOPONTIN SIGNALING AXES

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DEDICATION

This work is dedicated to my grandfather John Baker and my aunt Sandy Hardaway. Both of you have left an imprint on my life that drives my desire to understand how cancer claimed your lives before I was ready to say goodbye. This work and sacrifice was done in love and I hope that I made you proud. I will continue to stand in the gap and fight the battle that you were unable to finish on your own.

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TABLE OF CONTENTS

List of Tables

List of Figures

LIST OF ABBREVIATIONS

ARG1 – Arginase 1

BAX – BCL2-Associated X Protein

BCL-2 – B-cell Lymphoma-2

BMM – Bone Marrow Macrophages

CA9 – Carbonic Anhydrase IX

CD163 – Cluster of Differentiation 163

CD44 – Cluster of Differentiation 44

CD44v6 – Cluster of Differentiation Variant 6

HFD – High Fat Diet

LFD – Low Fat Diet

M-CSF – Monocyte Colony Stimulating Factor

NOS-2– Nitric Oxide Synthase 2

OPN – Osteopontin

PCa – Prostate Cancer

RANKL – Receptor Activator of NFκB Ligand

VEGF – Vascular Endothelial Growth Factor

XBP-1 – X-Box Binding Protein 1

αVβ3 – alpha variant beta 3

CHAPTER 1: OVERVIEW OF PROSTATE CANCER AND OBESITY

Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed cancers in men [1]. Because this disease is normally associated with aging, long-term exposure to environmental agents, chronic inflammation, genetic alterations, and defective androgen receptor signaling have all been speculated as causal factors in tumor initiation and support [2, 3]. Within the last decade, new research has suggested that increased adiposity represents another important factor contributing to overall poor prognosis, shorter time to disease recurrence, and increased mortality [4]. However, the data that directly link adiposity and tumor initiation and metastasis have so far been conflicting [5]. A meta-analysis of 17 cohort studies reported that obese men have increased risk of aggressive prostate cancer and mortality compared to normal weight men [5]. The same study also noted that there were mixed conclusions linking obesity with tumor initiation. A retrospective study of 2210 patients that received radical prostatectomy revealed no significant differences in disease progression with increased body mass index (BMI) [6]. Other studies reported a strong correlation between obesity and benign prostatic hyperplasia (BPH) [7, 8]. Clearly, conflicting clinical findings warrant further investigation to determine the driving forces that promote PCa aggressiveness in patients. The studies presented here examined the effects of bone marrow adiposity on inflammation and metastatic tumor growth in bone with the hope of uncovering novel mechanisms associated with disease progression.

1.1 Tumor Dissemination out of the Prostate to Bone

During normal development, prostate cells rely on androgen signaling to mediate cell growth and function [9]. However, defective androgen signaling is often the result of genetic mutations that accumulate with repeated environmental exposures and factors such as infection, diet, environmental carcinogens as well as race, family history and age [10]. The road from prostate cancer initiation to androgen independence and advanced disease is lengthy and if PCa is detected early, the 5-year survival is almost 100% [11]. The first stage of prostate cancer is enlargement of the prostate resulting from augmented androgen signaling, or benign prostatic hyperplasia (BPH) [12]. However, it is important to keep in mind that men with enlarged prostates do not always go on to develop cancer [12]. If the disease progresses to form prostatic intraepithelial neoplasia (PIN), abnormal growths form around prostatic ducts [13]. Studies demonstrated that cells in PIN lesions acquire several genetic alterations including telomere shortening, overexpression of oncogenes, and expression of the TMPRSS2-ERG fusion gene [13]. Eventually, tumors progress to prostate adenocarcinoma, but they are still responsive to androgens in a majority of patients [14]. The first line of treatment for such cancers is androgen ablation and tumors often shrink because androgen withdrawal disrupts the unequal balance between cell proliferation and apoptosis [15]. However, this initial response is often followed by the development of androgen-independent signaling resulting in uncontrolled growth and eventual resistance to androgen deprivation and classic chemotherapy [16].

Androgen-independent tumor growth has been an area of intense research in attempts to understand the molecular mechanisms associated with disease recurrence [17, 18] and to

develop novel therapeutics to treat androgen-independent PCa [19, 20]. Ineffective therapies result in disease recurrence and more aggressive disease [14]. Evidence has demonstrated that gain-of-function mutations in tumor cells promote anchorage-independent growth and stem-like characteristics that support dissemination out of the prostate and into the circulatory and lymphatic systems [21]. Several studies have tried to determine why prostate cancer prefers certain anatomical sites over others for secondary growth. A large post-mortem study of 1,589 patients with prostate cancer reported that 35% of patients had some form of metastasis and 90% of those examined had tumor lesions in bone compared to other common metastatic sites such as liver (25%) and lung (46%) [22]. Early ground-breaking work by Roy et al. demonstrated that prostate cancer cells cultured with bone marrow conditioned media exhibited extensive proliferation compared to cells exposed to conditioned media from other common metastatic sites [23]. Moreover, these proliferative effects were stimulated by tumor-supplied hematopoietic factors fibroblast growth factor (FGF), GM-CSF, as well as transforming growth factors α and β [23]. Since these early studies, several groups have identified other chemotactic factors that stimulate prostate tumor homing and seeding in bone including growth factors [24], stromal factors [25] and cytokines [26]. As more studies uncover the roles of the bone microenvironment in tumor metastasis, we now realize tumor cells have a supporting cast that aids in tumor progression.

1.2 Contributions of Adipocytes and Macrophages to Prostate Tumor Progression

During the aging process the bone is continuously remodeled, but with time the density of trabecular bone is decreased resulting in reduced bone mass and osteoporosis [27]. 'Inflamm-aging' or chronic low-grade inflammation is an underlying consequence of reduced

bone formation associated with age and it results in production of inflammatory factors, such as IL-6, TNF-α, and IL-1β [28]. Interestingly, these factors are direct or indirect stimulators of osteoclastogenesis and chemoattractants for prostate cancer cells [28-30]. The preferred sites for prostate tumor growth in the bone is the axial skeleton known to be under active remodeling [31]. Therefore, accelerated bone remodeling and chronic inflammation may be the perfect combination tumor cells need to thrive outside the prostate. Although disseminated tumor cells have some innate ability to enhance metabolic functions needed for accelerated growth, they also recruit stromal cells to aid in these processes including bone marrow adipocytes and macrophages. Together, they provide the factors necessary for tumor growth, invasion, immune evasion, angiogenesis that often lead to eventual patient mortality [32].

1.2.1 Bone marrow adipocytes

Tumor promotion and aggressive disease have been well-linked with visceral adiposity [33]; however, little is known concerning the roles of bone marrow adiposity in progression of metastatic disease. With age and obesity, a shift in bone marrow composition occurs toward formation of fat cells, parallel with increased osteoclast function and resulting bone loss [34, 35]. In fact, the bone marrow of newborn infants is composed primarily of hematopoietic cells whereas adult bone marrow contains as much as 7% of total body fat [36]. Other factors that can induce marrow adiposity in addition to obesity and age include alcoholism, inactivity, anorexia nervosa, and paralysis [37]. This is of importance, as evidence has suggested that bone marrow adipocytes are a unique subset of fat different from other depots, particularly in their distribution and fatty acid composition [38, 39]. Since these fat cells produce potent signaling molecules such as adiponectin and leptin, hormones, cytokines and growth factors,

they are capable of modulating bone metabolism [36, 40].

Magnetic resonance imaging of the skeleton demonstrated that fat is unequally distributed throughout bone [41]. The differential accumulation of fat in the proximal and distal region of long bones may have functional roles in normal physiology and metastatic bone disease. Some studies suggest that proximal fat may be supplying energy and adipokines necessary for bone remodeling while adipocytes in the less actively remodelled distal bone may be inactive cells that hinder new bone formation [40]. New evidence is beginning to uncover a possible role of bone marrow adiposity and tumor progression in bone (as reviewed in [42]) . Therefore, it is important to understand how adipocytes promote tumor homing to bone and what factors aid in tumor growth in the skeletal microenvironment.

Adipocytes secrete lipids that modulate cell signaling pathways of different cell types and may aid in altering the bone milieu in favor of tumor progression. Our lab previously reported that prostate cancer cells readily take up lipids from bone marrow adipocytes and long-term exposure to adipocyte-derived factors leads to increased growth and invasiveness [43]. Bone marrow adiposity has also been shown to evoke cancer-promoting effects in multiple myeloma. Studies demonstrated that myeloma cells are attracted to adipocytes and factors released by this interaction promote tumor cell proliferation and migration while inhibiting apoptosis [44]. Together, these data suggest that tumor cells not only have the capabilities of drawing lipids from surrounding adipocytes, but they also can utilize factors that stimulate lipid formation to supply their own fat stores.

In addition to lipids, tumor cells also depend on adipocyte-supplied adipokines and cytokines to drive tumor growth and survival. One such well-studied adipokine is leptin, a

hormone that responds to changes in nutritional status and energy utilization [45]. Interestingly, reduced expression of leptin is correlated with obesity, abnormal development of long bones, and cancer [42, 46, 47]. Leptin stimulates expression of genes associated with epithelial-to-mesenchymal transition (EMT) [48, 49] and estrogen-independent cell growth in breast cancer [49]. It promotes tumor growth, migration, and invasion in prostate and ovarian cancers as well [50-52]. Leptin is also important in prostate tumor growth in bone; however, some data suggest that it does this indirectly through leptin-stimulated bone resorption [53, 54]. Others suggest that this adipokine exerts mitogenic effects in prostate cancer cells directly by activating MAPK [55] and JNK pathways [56]. Although the specific functions of leptin are still being investigated, these data demonstrate that tumor cells rely of adipocyte-supplied factors for tumor growth and metastasis.

Adiponectin, another well-characterized adipokine, is also expressed in adipocytes and functions with leptin to regulate energy utilization and insulin sensitivity [57]. Interestingly, this protein also serves in bone remodeling by inhibiting osteoblast differentiation and subsequent bone formation [58]. Adiponectin is expressed at much higher levels in marrow adipose tissue compared to other fat depots and is secreted in response to caloric restriction, particular anorexia and cancer treatment [59, 60]. Levels of secreted adiponectin have been extensively investigated in the context of tumorigenic activation or inhibition; however, studies have demonstrated that its roles may be unique in different malignancies [61]. Reports show that tissue isolated from patients with prostate cancer expressed lower levels of adiponectin compared to benign prostatic hyperplasia tissue samples, result associated with increased cellular proliferation, EMT, and overall poor prognosis [62]. Other studies have reported similar

findings demonstrating that low adiponectin levels are also linked with increased grade of disease in prostate cancer patients and may serve as a marker of progression [63]. Despite compelling evidence that adiposity is associated with tumor progression, the role of individual adipokines in these processes has yet to be fully investigated.

1.2.2 Bone marrow macrophages and inflammation

Inflammation in bone plays an equally important role in tumor progression as adiposity. As previously mentioned, adipocytes secrete factors associated with chronic inflammation [42]; however, adipocytes may also work in concert with resident bone marrow macrophages (BMMs) to enhance inflammation that contributes to metastatic disease. BMM numbers, phenotype and function are influenced by changes in bone remodeling [64], increased adiposity [65], as well as tumor-driven alterations in the bone marrow milieu [66]. Because of the overwhelming exposure to a number of environmental signals, macrophages only respond to particular factors to prevent continuous inflammation associated with chronic inflammatory disorders [67]. Macrophages also have inherent programming that prevents them from attacking host tissue and resulting in autoimmune disorders such as Crohn's disease and rheumatoid arthritis [67]. This ability for programmed response may present a difficult and unique challenge for PCa therapy because, as studies have shown, macrophages can be recruited by tumor cells to aid in angiogenesis and cell survival instead of targeting malignant cells for phagocytosis [68].

To add to the complexity of the immune response in bone, in addition to serving as resident inflammatory mediators, BMMs, also aid in bone remodelling events. Specifically, they

contribute to recalcification to maintain homeostatic bone remodeling and secrete factors that aid in osteoblastogenesis [69]. Studies utilizing macrophage Fas-induced apoptosis (MAFIA) mouse model of macrophage depletion demonstrated that mice devoid of macrophages develop an osteopetrotic bone phenotype [70]. These mice also express lower levels of bone remodeling genes and have reduced bone volume compared to normal controls [70]. Because BMMs have diverse roles in bone, they are among the most manipulated stromal cell type that aids in immune evasion, angiogenesis, and tumor promotion [71]. Recent attention has focused on the roles of bone marrow adiposity and adipose-associated macrophages in chronic inflammation, metabolic alterations, and osteolysis [72, 73]. An area of intense investigation in tumor-driven inflammatory response is the ability of tumors to alter the behavior of macrophages to aid in tumor progression: specifically, phenotypic switching from a tumoricidal M1 to tumorigenic M2 macrophage phenotype [74, 75].

1.2.2 M1 and M2 Macrophages

M1 macrophages are activated by interferon-γ (IFN-γ) and foreign bacterial antigens and produce potent anti-microbial factors that promote bacterial cell death [76]. They are generally characterized by expression of cell surface proteins and cytokines such nitric oxide synthase-2 (NOS-2), tumor necrosis factor α (TNFα), IL-12, and IL-23 and their accepted function is to initiate the Th1 anti-tumorigenic immune response [76, 77]. Particularly, TNFα can sensitize prostate, gastric, and lung cancers to chemotherapy agents, making it a potent driver of immune-mediated cancer death [78]. On the other end of the spectrum, M2 macrophages produce factors that promote wound healing, cell proliferation, and ultimately conclude the inflammatory response [79]. These cells are characterized by the expression of arginase-1,

mannose receptor, and CD163 among other markers and they mediate a T-helper 2 inflammatory response that counteracts the T-helper 1 M1 macrophage response [79, 80]. M2 macrophages have been positively associated with increased BMI and have similar molecular characteristics to tumor-associated macrophages, suggesting that adiposity can promote macrophage phenotype switching [81]. This evidence may provide a valuable link between aggressive disease, inflammation, and adiposity in order to develop preventative measures to slow disease progression.

In addition to interactions with adipocytes, macrophages can also communicate with tumor cells to promote tumor growth and progression. In fact, patients treated with androgen deprivation therapy show surprisingly significant infiltration of tumor-associated macrophages into the prostate, event that has been associated with disease recurrence [82]. Reports suggested this influx is mediated by CCL2 signaling as the number of macrophages in the prostate can be reduced using an antagonist to the CCL2 receptor [82]. This suggests the importance of macrophage involvement in tumor progression. Notably, CCL2 is known to be secreted by tumor cells, promotes macrophage skewing from M1 to M2 phenotype to facilitate growth and progression, and may account for reduced poor prognosis [83].

The ability of tumor cells to overcome obstacles and grow efficiently in the primary site is particularly challenging in the secondary, complex microenvironments such as bone. Vascularization is one of the key components that tumor cells must acquire to effectively colonize in bone. Indeed, upon intratibial injection of prostate cells into canine model increased vascularity of tumor bone was observed compared to control bone, and interestingly $CD68^{+}$ macrophages lined the periphery of the tumor aiding in bone remodeling and tumor outgrowth

[84]. Proteolysis and matrix degradation are additional factors aiding in tumor progression in bone. Specifically, cathepsin K (CTSK), a bone remodeling cysteine protease that degrades collagen I plays a role in tumor growth and inflammation in bone marrow [85], and is one of the critical factors in bone marrow macrophage-derived inflammation [86]. Studies in our lab have shown that macrophages that express CTSK infiltrate prostate tumors more efficiently than CTSK knockout macrophages resulting in reduced inflammatory response and tumor growth [86].

Other factors with promoting effects on tumor growth in bone include monocyte chemoattractant protein-1 (MCP-1) and cyclooxygenase-2 (COX-2). PC3 prostate tumor cells overexpressing MCP-1 injected intravenously into SCID mice display an accelerated rate of metastatic bone lesion formation due to the recruitment of resident macrophages in bone [87]. Cyclooxygenase-2, an inducible protein implicated in normal and tumor-induced inflammatory response, was shown to be highly expressed by macrophages and to affect bone metabolism by altering prostaglandin E_2 (PGE_2) synthesis and macrophage migration through cooperation with CCL2 [as reviewed in [42]]. Indeed, nude mice injected intratibially with PC3 cells lacking the functional receptor for PGE_2, E-prostanoid receptor had reduced tumor burden, more intact bone, and decreased production of COX2, IL-1β, and IL-6 cytokines [88], likely due to the disruption of prostate tumor paracrine interactions with inflammatory mediators in the bone. Collectively, these findings suggest that the supportive cells in the tumor microenvironment are clearly playing critical roles in tumor seeding and metastasis and more directed studies investigating these specific interactions may prove important for drug development and modulating the immune response to tumoricidal attacks.

1.3 Bone Remodeling and Metastasis

Tumor metastasis to bone has plagued mankind since the beginning of time. The oldest recorded evidence of bone metastasis dates back circa 1200 BC based on the remains of an ancient Egypt male that presented with osteolytic bone lesions in vertebrae pelvis, and long bones [89]. The bone tends to be a preferential site of metastasis for cancers of the prostate, breast, uterus, bladder, kidney, lung, and thyroid [8]. There have been many hypotheses why these cancers spread to a site that is very rigid in nature and hard to colonize due to its naturally hypoxic state. Although there has been a lot of progress in understanding the attraction of prostate tumor cells to the bone, the role of bone microenvironment in supporting the progression of metastatic cells in the skeleton is not well-understood. Once the tumor cells invade into bone prostate cancer becomes incurable. Therefore, it is important to understand the function of bone remodeling cells and the mechanisms used to promote bone turnover and thrive in bone.

1.3.1 Normal Bone Remodeling

Normal bone remodeling is a complex process that involves the synchronous effort of cytokines, growth factors, proteases and various cell types. The skeletal remodeling machinery is made up of three major cell types: osteoblasts, osteoclasts, and osteocytes. Osteoblasts are bone forming cells derived from mesenchymal stem cells, as are adipocytes, chondrocytes (collagen-forming cells) and myocytes (muscle cells) [90]. Once osteoblasts have differentiated, a fraction of them become osteocytes by being enclosed in the matrix they secrete which ultimately forms hardened bone [91]. The role of osteocytes in the regulation of bone health has recently become an area of more intense investigation and studies have uncovered the

critical role of this cell type in initiating and orchestrating bone remodeling. In response to changes in mechanical loads, osteocytes release prostatglandin E_2, nitric oxide, COX2, as well as insulin growth factor-1 to activate osteoblasts that line the bone to promote secretion of bone matrix [91]. Conversely, these cells also have the ability to prevent bone remodeling namely by the expression and secretion of sclerostin, a glycoprotein that inhibits the formation of cortical bone and dysregulated bone mineralization [92]. Notably, sclerostin expression is enhanced as a result of increased inflammation and activation of NF-κB and tumor necrosis factor alpha (TNF-α), resulting in reduced bone formation, increased receptor activator of nuclear factor kappa-B ligand (RANKL), and accelerated bone resorption [93].

Both osteoblasts and osteocytes function in concert with bone degrading osteoclasts, which are derived from hematopoietic stem cells similarly to immune cells such as T cells, B cells, natural killer cells, and macrophages [94]. Osteoclasts are located near the surface of the bone and they degrade old or damaged tissue. Their differentiation occurs by the fusion of monocytes followed by the induction by haematopoietic factors macrophage-colony stimulating factor (MCF) and RANKL, both of which are required for formation of mature osteoclasts [95, 96]. During bone degradation, osteoclasts communicate with osteoblasts to stimulate the production of new bone matrix through several signaling factors. Complement component 3a (C3a) was identified as a factor secreted by mature osteoclasts that stimulates osteoblast differentiation and increased bone turnover both *in vitro* and *in vivo*. Osteoblastogenesis was significantly reduced when C3a was knocked down in osteoclasts or the C3a receptor on osteoblasts was blocked utilizing a receptor antagonist [97]. Transforming growth factor-β1 (TGF-β1) is another factor highly secreted by osteoclasts actively degrading

bone matrix [98]. Studies have demonstrated that TGF-β1 supports the migration of bone marrow stromal cells to sites of active bone remodeling by triggering downstream SMAD2/3 pathway, formation of osteoblasts and increased bone mineral density [99].

Once osteoblasts are activated, they release both non-collagen components (e. g., osteopontin, alkaline phosphatase, osteonectin), mineral components such as hydroxyapatite and magnesium that provide tensile strength, and collagen components including collagen I (most abundant in bone) and IV (very low amounts detected) that aid in plasticity [100]. Osteoblasts depend on the expression of several pro-osteoblastic proteins for effective production of these factors. Studies have demonstrated that knockdown of fibroblast growth factor-2 partially inhibits osteoblast differentiation *in vitro* [101]. *In vivo* studies verified that FGF-2 null mice had reduced trabecular bone density and reduced osteoblast differentiation [101]. Other studies have implicated other classical pathways that stimulate osteoblastogenesis such as ERK and AKT signaling while hepatocyte growth factor/c-MET signaling abrogates these effects [102] and this may prove important depending on changes in the bone microenvironment resulting from age, disease, and inflammation.

Bone is a dynamic organ that changes with aging, physical activity, weight, and diet. Studies have shown that bone marrow adiposity in particular affects bone function by promoting osteoclastogenesis via stimulating the pre-osteoblasts to secrete higher levels of RANKL while repressing genes associated with osteoblastogenesis such as RUNX2, osteocalcin, and alkaline phosphatase [103]. Other studies have linked enhanced osteoclastogenesis and accelerated bone resorption with induction of hypoxia. This is specifically evident when pre-cursor cells are cultured in hypoxic conditions and these effects are partly driven by activation

of hypoxia-inducible factor alpha (HIF1α) and autophagy pathways [104]. Indeed inhibition of autophagy reduces both HIF1α expression and osteoclast formation suggesting that osteoclasts utilize this mechanism when oxygen levels are reduced [104]. Recent evidence further suggests that HIF1α induces vascular endothelial growth factor (VEGF), a factor recently implicated as an alternate protein that stimulates osteoclast fusion independent of MCSF [105]. Understanding the involvement of the abovementioned pathways in bone may prove important depending on changes in the bone microenvironment resulting from age, disease, and inflammation.

1.3.2 Modifications to Bone in Tumor Metastasis

Tumor-driven changes in bone morphology vary upon the type of tumor. There are three types of lesions that can form in bone as a result of metastatic disease: osteolytic, osteoblastic, and mixed lesions. Osteolytic bone lesions are the result of overactive osteoclast-mediated bone degradation found mostly in lung [106], thyroid [107], kidney [108] and breast [109] metastases. Osteoblastic lesions are formed in prostate cancer and are characterized by the presence of a dense, fibrous network of newly woven bone that surrounds the tumor [110]. Interestingly, although bone scans have generally classified prostate cancer as an osteoblastic disease, these lesions have usually mixed osteolytic-ostaoblastic phenotype with osteoblastic appearance dominating over lytic phenotype [111]. This suggests that osteoclastic bone destruction is necessary to make room for new bone formation [112].

Metastatic bone disease involves extensive remodeling to accommodate rapidly dividing tumor cells that disseminated to bone. Tumors accomplish this in part by altering their genetic profile to express proteins that induce bone remodeling [113]. One such group of proteins are the bone morphogenic protein (BMP) family responsible for new bone and cartilage formation

but also widely expressed in various tissues regulating proliferation, cell cycle, and calcium levels among other functions [114]. Studies have also shown that the transcription factor zinc finger E-box binding homeobox 1 (ZEB1) is expressed in breast cancer cells and promotes the expression and secretion of inhibitors of BMPs resulting in osteoclastogenesis and bone destruction to make room for tumor seeding [115]. It is not fully understood what factors in the bone marrow microenvironment contribute to the gain-of-function mutations acquired by tumor cells to alter the bone milieu to promote metastatic bone disease.

While evidence suggests that tumor cells can directly contribute to bone remodeling, it is well-accepted that they predominantly accomplish this by paracrine signaling mechanisms that accelerate osteoclastogenesis and osteoblastogenesis, although the exact mechanisms are not well understood [111]. Various tumor-secreted factors expressed by tumor cells have been implicated in bone remodeling including vascular endothelial growth factor (VEGF) [111]. VEGF is an important survival and growth factor that initiates endothelial cell proliferation and survival and regulates vascular formation needed for normal development of long bones, inflammation and wound healing [116]. VEGF is also expressed and secreted by tumors cells to promote neovascularization and bone remodeling [117]. Interestingly, VEGF expression by prostate tumor cells was shown to promote bone mineralization and secretion of osteocalcin, a marker of late osteoblast differentiation [118]. Studies have reported that blocking signaling with selective antagonists to VEGF reduces prostate tumor growth in bone and prevents formation of osteoblastic lesions [118]. Specifically, *in vitro* studies demonstrated that BMPs stimulate the ability of prostate cancer cells to enhance osteoblast maturation and function in part by increasing tumor-derived VEGF [119]. Additional evidence clearly showed that newly

disseminated prostate cancer cells prefer osteoblast-rich areas of bone [120]. These studies highlight the deleterious effects of prostate tumor cells colonizing the bone, and underline the need for understanding the molecular mechanisms behind this process.

As previously mentioned there is an osteolytic component associated with bone seeking tumors, which makes the metastatic bone disease even more complex. This is evidenced by both clinical and preclinical studies that demonstrate osteoclast involvement in osteoblastic bone formation [121]. Primary blood monocytes collected from prostate cancer patients with osteoblastic lesions spontaneously form more osteoclasts *in vitro* and serum levels of osteoclastic markers IL-7 and RANKL are elevated in prostate cancer patients compared to healthy controls and patients without metastatic disease [121]. Further evidence suggested that tumor cells that seed in bone early in metastatic disease express dickkopf-1 (DKK-1), a soluble tumor-derived factor that inhibits WNT-dependent osteoblast function [122]. Additional evidence showed that mice intratibially injected with C4-2B cells that overexpress DKK-1 produce osteolytic lesions compared to mixed lesions formed by empty vector controls [123]. This work suggests that DKK-1 may serve as a switch needed to promote initial tumor colonization before osteoblastogenesis is activated [122], and provide a potential mechanism for underlying osteolysis in prostate cancer. Other studies have simply attributed the osteolytic aspect of metastatic bone disease to failed compensatory effort of the bone to repair damage caused by excessive bone formation [124]. Understanding the molecular mechanisms used by tumor cells to drive both osteolytic and osteoblastic bone remodeling may reveal novel therapeutic targets to slow prostate tumor growth in the bone marrow niche.

1.4 CXCL1 and CXCL2 Signaling Axis is Inflammation and Cancer

1.4.1 CXCL1 and CXCL2 Structure and Functions

CXCL1 (Groα) and CXCL2 (Groβ) are members of the CXC subtype of chemokines, classified as such by the possession of two conserved cysteine residues separated by an amino acid (X) [125]. Members of this subtype are characterized by the absence or presence of an ELR (Glu-Leu-Arg) motif located on the amino terminus immediately before the first cysteine residue [126]. ELR+ chemokines include CXCL1, 2, 3, 5, 6, 7, and 8 and all serve as potent angiogenic factors and chemoattractants for neutrophils [125]. Similar to human CXCL1 and CXCL2, the murine homologs, keratinocyte chemoattractant (KC/CXCL1) and macrophage inflammatory protein 2 (MIP-2/CXCL2), have 90% homology and bind to the CXC receptor 2 (CXCR2) [127] [128]. Notably, although both chemokines are CXCR2 ligands, CXCL1 seems to have greater binding affinity [129], which may play a role in modulating inflammatory responses. CXCL1 and CXCL2 are secreted predominantly by granulocytes, macrophages and mast cells during bacterial infection to recruit neutrophils and aid in clearance [130]. These chemokines are also expressed by epithelial cells and evidence suggest they may play a role in cancer progression [131].

Studies have demonstrated that CXCL1 and CXCL2 are modulated by two signaling pathways: NFκB and Signal Transducers and Activators of Transcription 1 (STAT1) [132]. Both NFκB and STAT1 have recognition sites near the promoter regions of CXCL1 and CXCL2 and regulate their transcription [132]. Others have suggested that JNK mediates CXCL1 expression as well [133]. More studies are needed to determine the exact mechanism of transcriptional regulation of CXCL1 and CXCL2 chemokines to better understand how it may be best targeted.

It is noteworthy that ELR+ chemokines, including CXCL1 and CXCL2, have also been recently shown to be modified by proteases. In fact, CXCL1 and CXCL2 are cleaved at the amino terminus by cathepsins K, L, and S, process that markedly enhances their chemotactic functions [134]. Together, transcriptional and post-translation regulation of CXCL1 and CXCL2 may have important consequences in immunity and disease.

1.4.2 CXCL1 and CXCL2 Signaling Axis in Cancer

CXCL1- and CXCL2-mediated inflammatory responses play roles in tumor cell invasion and cancer-associated vasculature formation [135]. Fluorescence in situ hybridization (FISH) analyses of CXCL1 and CXCL2 in cancerous breast tissue revealed that both chemokines are amplified in 7.5% of primary breast tissue and 19.9% of metastatic tissue compared to surrounding non-malignant tissue [131]. *In vivo* studies also demonstrated that tumor volume and lung metastases are reduced, and caspase 3 cleavage is increased in breast cancer cells depleted of CXCL1 and CXCL2 [131]. Additional investigations also demonstrated that CXCL1 is expressed in bladder cancer cells and regulates tumor invasiveness *in vitro,* and its expression directly correlates with tumor grade *in vivo* [136]. Moreover, melanoma cells express and secrete CXCL1 to support tumor-driven angiogenesis and colony formation and these effects are inhibited by blocking CXCL1 signaling [137].

Although several studies demonstrated that tumor cells can produce CXCL1 and CXCL2 to drive metastasis and tumor growth, evidence shows that supporting macrophages are a significant source of these factors. Previous reports revealed that macrophages co-cultured with breast cancer cells undergo an M1 to M2 phenotype switch and levels of pro-angiogenic

chemokines, such as CXCL1, are increased [138]. This suggests that M2 macrophages in the tumor microenvironment promote tumor growth in part by supplying vascular factors that stimulate vessel formation [138]. Unfortunately, there have only been causal associations between prostate cancer and increased levels of CXCL1 and CXCL2 [139, 140]. Overwhelming evidence demonstrates that CXCL1 and CXCL2 are potent drivers in malignancy; however, studies are needed to determine the effects of CXCL1 and CXCL2 on prostate tumor invasion.

CXCL1 and CXCL2 ligands bind CXCR2 to mediate cellular effects in neutrophils [141] and cancer cells [142]. CXCR2 is highly expressed on the surface of neutrophils and it binds chemotactic factors such as CXCL1 to initiate pro-inflammatory responses [143]. Additionally, CXCR2 overexpression is correlated with tumor growth, invasion, migration and poor patient prognosis in several cancers [144]. CXCR2 expression is enhanced in kidney [145], prostate [146], and colon cancers [147]. *In vivo* studies utilizing TRAMP mice that spontaneously form prostate tumors, showed that CXCR2 deletion results in smaller tumors and reduced tumor vasculature [148]. These studies suggest that CXCR2 expression and signaling aid in tumor growth and progression. More investigations may reveal the potential for novel therapeutics that target CXCR2 to slow cancer growth.

1.5 Osteopontin Signaling Axis

1.5.1 Osteopontin Expression and Functions

Osteopontin (OPN) also known as secreted phosphoprotein 1 (SPP1) belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) family [149]. OPN is a multifunctional protein that is involved in several cellular processes that mediate bone

homeostasis, angiogenesis, inflammation, and cancer [150]. This protein is not very conserved between species, sharing only 63% homology between human and mouse, and is subject to extensive translational and post-translational modifications (i.e. glycosylation, phosphorylation, and sulfation) [151]. There are currently three known isoforms of OPN: OPNa, the full length isoform, and OPNb and OPNc that lack exons 5 and 4, respectively [149]. Interestingly, these modifications appear to have opposing effects in vascular tube formation. Studies have shown that OPNa and OPNb both stimulate new vessel formation and tissue repair while OPNc has inhibitory effects on tube formation and VEGF expression [150]. Alternative modification of OPN may prove to be an advantageous target in diseases that rely on osteopontin signaling to stimulate angiogenic responses in the tumor microenvironment.

OPN is one of the main non-collagenous matrix proteins in bone [151]. It is expressed by several cell types, including osteoblasts during bone mineralization and induces the expression of bone forming markers alkaline phosphatase and osteocalcin [152]. Studies demonstrated that osteoclasts treated with OPN show calcium-dependent activation of nuclear factor of activated T cells 1 (NFAT1), a factor critical for osteoclastogenesis [153]. Moreover, in the absence of OPN, NFAT1 signaling is inhibited and prevents successful differentiation of precursor cells while inducing apoptosis in mature osteoclasts [153]. Similar to osteoclasts, macrophages depend on osteopontin signaling for survival and function. Specifically, mice lacking osteopontin display reduced ability to clear debris from tissue following injury [154]. Evidence also suggested that OPN null macrophages have increased expression of mannose receptor, a marker of M2 phenotype, further suggesting that their pro-inflammatory response may be impaired [154]. This suggests that osteopontin may be playing a significant role in

tissue repair and remodeling, particularly in a context of tumor progression.

The many functions of OPN would not be possible without the numerous receptors that it binds including integrin receptors αVβ3 and α4β1, as well as CD44 and its variant CD44v6, just to name a few [151]. *In vitro* studies have shown OPN binds αVβ3 on HUVEC cells to support cell survival and angiogenesis via increased expression of Akt and ERK1/2 target genes Bcl-xL and VEGF [155]. CD44-mediated osteopontin signaling is important in insulin resistance and obesity [156]. Clinical reports showed that levels of OPN and CD44+ macrophages are enhanced in subcutaneous fat isolated from insulin-resistant patients [157], suggesting adipose tissue inflammation is partly driven by OPN signaling in macrophages. CD44 and integrin receptor activation also regulate cell adhesion, motility, and signal transduction via transcription factors and kinases [150]. The OPN receptor CD44v6, specifically, has been linked to increased metastatic potential in gastric cancer cells and overall poor prognosis [158]. This underlines the breadth and diversity behind the OPN signaling in both normal and disease processes [159], possibly making it a difficult therapeutic target.

1.5.2 Osteopontin Signaling in Cancer

Because OPN has diverse functions in bone remodeling, cell survival, and angiogenesis, it is no surprise that tumor cells utilize this protein for the very same reasons. In fact, OPN overexpression was shown to result in the formation of larger colonies *in vitro*, enhancement of tumor volume *in vivo*, and activation of cell survival by regulating HIF-1α and Akt pathways [160]. Some studies have shown that the splice variant OPNc is the main culprit that mediates increased Akt signaling, invasion and tumor growth rather than the full length OPNa isoform

[161]. Clearly, different isoforms have unique functions in tumor progression depend on the type of cancer. Evidence has also demonstrated that osteopontin may be a predictor of survival and risk of metastatic disease. Specifically, histological examination of primary and metastatic bone tissue samples from patients with nasopharyngeal cancer revealed an inverse association between OPN levels and bone metastasis-free survival [162].

Although tumor-derived OPN has been implicated in tumor progression, its secretion by tumor-associated cells in the microenvironment has also been suggested as a culprit in this process. Increased expression of osteopontin in gastric tumors has been mainly attributed to its secretion by the M2 macrophages and correlated with overall reduced patient prognosis [163]. This result was in line with *in vivo* observations demonstrating gastric tumors, surrounding vasculature, and M2 macrophage infiltration were all reduced in OPN null mice [163]. Increased OPN expression was also observed in macrophages surrounding vascular and necrotic regions of glioblastomas [164], suggesting that this protein may be promoting tumor survival by stimulating neovasculature in hypoxic regions of tumors. As previously mentioned, some tumors are capable of expressing endogenous OPN to stimulate vascular formation and immune cell infiltration [165]; however, not all tumors have this capability and must depend on circulating OPN from the tumor microenvironment. This was demonstrated in a study that showed OPN knockdown in hepatoma cells decreased tumor cell motility, invasiveness, and reduced foci formation [166]. Interestingly, when these cells were co-cultured with OPN-positive macrophages, foci formation, tumor invasion and motility were all rescued [166]. These and other studies are a testament that osteopontin signaling in the microenvironment is a key component of tumor-mediated inflammation and growth.

There have been intense investigations that have attempted to identify downstream targets of OPN signalling in tumor cells. Studies have particularly focused on CD44 and its variant CD44v6 in disease progression [167]. In fact, CD44v6 expression is increased in prostate cancer cells and supports tumor sphere formation [168]. Moreover, knock down of CD44v6 greatly sensitizes prostate cancer cells to chemotherapy agents such as methotrexate and docetaxel [168]. In addition, CD44v6 regulates Wnt and Akt activation as well as stem cell markers Slug, Twist, Snail, and Vimentin [168]. The alternative OPN receptor, αVβ3 has also been implicated in tumor cell adhesion and migration to bone matrix [169]. Specifically, αVβ3 signaling has been linked to Akt activation via focal adhesion kinase (FAK) phosphorylation to enhance prostate cancer cell migration and survival [170]. This suggests that OPN-αVβ3 binding might be an important factor in tumor colonization in bone. All three OPN receptors, CD44, CD44v6, and αVβ3, seem to have reported roles in tumor cell survival and investigations are needed to determine other targets of osteopontin that promote tumor progression in bone.

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

We hypothesized that growth and aggressiveness of metastatic prostate cancer in the bone is driven by the cooperative effort between bone marrow adipocytes, macrophages, osteoclasts and metastatic tumor cells in bone. Adipocyte-driven inflammation involving CXCL1/CXCL2/CXCR2 axis, and macrophage-supplied osteopontin perpetuate the vicious cycle of bone metastasis by 1) stimulating osteoclastogenesis and bone degradation; 2) promoting macrophage recruitment and invasiveness; 2) changing the cytokine profiles to those that drive metastatic events in skeleton; and, consequently 3) increasing tumor cell proliferation, invasion, and survival in the metastatic niche.

Our project was composed of three specific aims:

Aim 1 was to investigate contributions of marrow adipocyte-supplied CXCL1 and CXCL2 to tumor-driven osteolysis.

Aim 2 was to determine the role of CXCL1/CXCL2/CXCR2 axis in bone marrow macrophage phenotype and function in the bone tumor microenvironment.

Aim 3 was to examine the role of macrophage-derived osteopontin in tumor cell survival in bone.

Overall, these studies were designed to discover novel molecular mechanisms and pathways behind aggressiveness and survival of metastatic tumors in bone in hopes of discovering new therapeutic targets for presently incurable metastatic PCa.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEMα), tartrate resistant acid phosphatase (TRAcP) staining kit, and other chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA). Protease inhibitor was from MBL International (Woburn, MA). Rabbit anti-human/mouse cathepsin K antibody was from Abcam (Cambridge, UK). Rabbit anti-human/mouse ß-actin antibody was from Novus Biologicals (Littleton, CO). Monoclonal mouse anti human CXCR2 antibody, recombinant mouse CXCL1 and CXCL2, goat anti-mouse CXCL1 and CXCL2 neutralizing antibodies, macrophage-colony stimulating factor (M-CSF), receptor activator of NFκB ligand proteins (RANKL) mouse anti human integrin αVβ3 antibody, goat anti mouse osteopontin, mouse recombinant osteopontin and Quantikine mouse CXCL1 and CXCL2 ELISA kits were from R&D Systems (Minneapolis, MN). Immunoblotting "Western Lightning ECL Plus" and "Luminata Forte Western HRP Substrate" detection kits were from Perkin Elmer LLC (Waltham, MA) and Millipore (Billerica, MA), respectively. RNeasy Mini Kits were from Qiagen (Valencia, CA). Mouse anti human CD44v6 antibody was from Millipore (Temecula, CA). Mouse polyclonal to GAPDH and rabbit anti human survivin antibodies were from Novus Biological. Phosphatase inhibitor, rabbit anti human phosphorylated Akt serine 473 (^{pAktS473}), total Akt, BiP, Bcl-xL, and CD44, were from Cell Signaling (Danvers, MA).

3.2 Animals

All experiments involving mice were performed in accordance with the protocol approved by the institutional Animal Investigational Committee of Wayne State University and NIH guidelines. *In vivo* xenograft studies were performed in 8- to 10-week old male mice in the FVB/N background with homozygous null mutations in the Rag-1 gene (FVB/N/N5, Rag-1−/−). Mice were bred in-house. Osteopontin KO macrophages were isolated from B6.129S6(Cg)-Spp1 -/- mice and aged-matched C57BL/6J controls purchased from Jackson Laboratories.

3.3 Diets

At 5 weeks of age, mice caged in the groups of 4 were started on either a low-fat (LFD; N = 9) diet (10 % calories from fat; Research Diets no. D12450Bi) or a high-fat (HFD; N = 11) diet (60 % calories from fat; Research Diets no. D12492i) as previously described [43]. Mice were maintained on diets for 8 weeks prior to and 6–8 weeks following the tumor implantation into bone (total of 16 weeks).

3.4 Cell lines

PC3, an androgen-independent osteolytic cell line derived from a bone metastasis of a high-grade adenocarcinoma [171], and DU145, an androgen-independent osteolytic line derived from a brain metastasis [172], were purchased from American Type Culture Collection (Manassas, VA, USA). ARCaP(M), androgen-repressed metastatic prostate cancer cells M ('Mesenchymal' Clone) were purchased from Novicure Biotechnology (Birmingham, AL, USA). C4-2B human prostate were derived from LNCaP isolated from lumbar spine of athymic mice [173], were a kind gift from Dr. Leland Chung. L929 cells (source of M-CSF for osteoclast precursors), were cultured in DMEM containing 10 % FBS until confluent and conditioned media

was collected, centrifuged, and stored at −80 °C until ready for use. PC3 and DU145 cells were cultured in DMEM supplemented with 10 % FBS. ARCaP(M) and C4-2B cells were cultured in RPMI supplemented with 10 % FBS. All cells were maintained in a 37°C humidified incubator ventilated with 5 % CO2.

3.5 Intratibial injections of tumor cells

Intratibial tumor injections were performed under isoflurane inhalational anesthesia according to previously published procedures [86]. Briefly, a cell preparation containing 5×10^5 of PC3 or ARCaP(M) cells in PBS (20 µl, right tibia), or PBS alone (control, 20 µl, left tibia) was injected into the bone marrow. 6 or 8 weeks post-injection (for PC3 and ARCaP(M) cells, respectively) mice were euthanized, and control and tumor-bearing tibiae were removed and imaged *ex vivo*. X-ray images were obtained using a Carestream XVivo Multimodal Animal Imager. Half of the intratibial tumor samples from each group were then fixed in Z-fix, bone tumors were decalcified, and all samples were embedded in paraffin. The 5 µm longitudinal sections from tibiae were deparaffinized, and stained with tartrate resistant acid phosphatase (Sigma Aldrich) according to the manufacturer's instructions. Digital images were captured under ×5 and ×10 magnification using a Zeiss Scope A.1 conventional light microscope with CCD camera. Remaining tissues were snap-frozen in liquid nitrogen, powderized using a tissue pulverizer and RNA was isolated using Trizol and RNeasy Mini Kit.

3.6 Bone marrow adipocyte-conditioned medium

Primary mouse bone marrow stromal cells (mBMSC) were isolated from femurs and tibiae of 6- to 8- week old FVB/N mice and induced to become bone marrow adipocytes as previously described [43]. Briefly, mBMSC cells were plated in 3D collagen I gels, grown to confluency for

48–72 h and treated with adipogenic cocktail (30 % StemXVivo Adipogenic Supplement, 1 µM insulin, 2 µM Rosiglitazone; DMEM and 10 % FBS) for 8–10 days. Differentiated bone marrow adipocyte cultures were cultured in serum-free DMEM for 12–16 h and medium was collected, centrifuged, and stored at −80 °C. Prior to use, serum-free medium collected from adipocyte cultures was diluted 1:1 with MEMα appropriate for osteoclast treatment and designated 'Adipo CM'. Cells were maintained in a 37 °C humidified incubator ventilated with 5 % CO2.

3.7 Isolation of BMMs

Bone marrow macrophages (BMMs) were differentiated from primary murine bone marrow cells. Bone marrow was flushed from femurs and tibiae of 10 to 12-week-old FVB/N male mice with BMM growth medium (MEMα containing 20 % FBS and 30 % L929 conditioned medium as the source of M-CSF [86]). For comparison of BMMs in OPN WT versus KO mice, B6.129S6(Cg)-Spp1 -/- mice aged-matched C57BL/6J were used. The cell suspension was plated on petri dishes and incubated for 4–5 days to obtain differentiated bone marrow macrophages (BMMs).

3.8 Adipocyte and BMM co-culture with PC3 tumor cells

For indirect adipocyte-tumor cell co-cultures, mBMSC cells were seeded in collagen I-coated 6-well plates, differentiated into adipocytes, and 200,000 tumor cells were seeded on top of a Transwell filter (0.2 µm pore size) to allow sharing of soluble factors between the two cell types. Cells were cultured as described for 48 h and serum-starved for additional 12–16 h prior to sample collection for analyses. For ELISA analyses, media were concentrated through 3 K Millipore centrifugal filters, and all samples were stored at −80 °C for future use.

3.9 BMM co-culture with PC3 and ARCaP(M) tumor cells

For indirect BMM-tumor cell co-cultures, primary BMMs were seeded in 6-well plates, at a density of 300,000 cells per well and 200,000 tumor cells were seeded on top of a Transwell filter and cultured in the absence or presence of Adipo CM. To determine if CD44v6 and αVβ3 receptors promoted survival and ER stress in co-culture, blocking antibodies to CD44v6 and αVβ3 (0.8µg/mL) were also added. To determine changes in tumor survival in BMM-PCa co-culture, PC3 cells we plated at a density of 300,000 cells on 6-well plates and cultured with 5 and 10 nM of docetaxel for 48 hours. Macrophages were seeded at a density of 300,000 cells per well on Transwell filters and cells were allowed to recover. For RNA isolation, cells were washed with PBS cells and collected into RLT buffer and RNA purified according to the RNeasy Mini Kit instructions. For lysate and media, cells were cultured as described for 48 h and serum-starved for additional 12–16 h prior to sample collection of lysates and media for analyses. Cells were washed three times with PBS, collected in SME lysis buffer (250 mM sucrose, 25 mM 2-(N-morpholino) ethanesulfonic acid (MES), 1 mM EDTA, 0.1 % Triton-X 100 pH 6.5) containing protease and phosphatase inhibitors. All samples were then stored at −80 °C for future use. To induce hypoxia, co-cultures were treated with 100uM cobalt chloride for 48 hours.

3.10 Osteoclast formation for tartrate resistant acid phosphatase staining

BMMs were seeded at a density of 250,000 cells per well in a 24-well dish on glass coverslips in BMM growth medium as described above and allowed to attach overnight. For osteoclastogenesis assays, cells were cultured with 1:1 ratio of MEMα and DMEM or Adipo CM containing 10 % FBS, 20 ng/mL M-CSF and 10 ng/mL RANKL. Assays were performed in the absence or presence of recombinant proteins to CXCL1 (0.5 µg/mL) and CXCL2 (0.25 µg/mL),

neutralizing CXCL1/CXCL2 antibodies (3 µg/mL), CXCR2 neutralizing antibodies (5 µg/mL) or CXCR2 antagonist SB225002 (2.5 µM; from Cayman Chemical, Ann Arbor, MI). Every 48 h, half of the medium was removed and replenished with fresh medium supplemented with M-CSF, RANKL and appropriate treatment reagents. Data were collected from at least three independent experiments, each performed in duplicate. Osteoclasts were formed within 4–6 days and TRAcP staining was performed according to manufacturer's instructions.

3.11 Quantification of TRAcP-positive cells

TRAcP-positive cells that contained three or more nuclei were considered osteoclasts and counted. Six representative images per coverslip were captured using a Zeiss Scope A.1 conventional light microscope at 5x magnification to evaluate the number and surface area of osteoclasts per field. For each experimental condition, the total number and cell surface area of osteoclasts were manually counted using ImageJ software.

3.12 RNA and lysate isolation of osteoclasts

BMMs were seeded at a density of 500,000 cells per well in a 12-well dish for 24 h as described above. BMMs were then treated with RANKL and M-CSF in the presence or absence of Adipo CM. When indicated, cells were treated with recombinant CXCL1 and CXCL2 proteins, CXCL1/CXCL2 neutralizing antibodies, CXCR2 blocking antibody, or CXCR2 antagonist SB225002 as described above. Following formation of mature osteoclasts, cells were washed three times with PBS, collected in SME lysis buffer (250 mM sucrose, 25 mM 2-(N-morpholino) ethanesulfonic acid (MES), 1 mM EDTA, 0.1 % Triton-X 100 pH 6.5) and stored at −80 °C for future use. For RNA isolation, cells were washed with PBS cells and collected into RLT buffer

and RNA purified according to the RNeasy Mini Kit instructions.

3.13 Taqman RT-PCR analyses

The cDNA from cells and *in vivo* samples was prepared from 1–2 µg of total RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) as previously described [86]. Analyses of genes associated with bone remodeling and inflammation were performed using mouse-specific TaqMan® Individual Gene Expression assays for cathepsin K (Mm00484039), matrix metalloproteinase-9 (Mm00442991), calcineurin (Ms00432282), CXCL1 (Mm01354329), CXCL2 (Mm00436450), and DC-STAMP (Mm04209236). Murine genes were normalized to HPRT1 (Mm00446968) and 18S (Mm03928990). Genes associated with ER stress, hypoxia, and cell survival in PCa cells were analysing using probes for BCL2 (Hs00608023), BAX (Hs00180269), and CA9 (Hs00154208), and VEGF (Hs00900055) and were normalized to HPRT1 (Hs99999909). Analysis of mRNA expression of XBP-1 in tumor cells was performed by reverse transcription using Taqman Master Mix (Qiagen). PCR for XBP1 expressions was performed using forward and reverse primers XBP1a (5'CCTGGTTGCTGAAGAGGAGG 3') and XBP1b (5' CCATGGGGAGATGTTCTGGAG 3') and normalized to β actin a (5' GGATGCAGAAGGAGATCACTG 3') and β actin b (5' CGATCCACACGGAGTACTTC 3'). Human osteopontin (Hs Hs00959010) was also assessed and normalized to human 18S (Hs03003631). M1 and M2 markers assessed were arginase-1 (Ms00475988), IL-10 (Ms00439614), NOS2 (Ms004404851), and CD163 (Ms00474091). Human specific gene expression assays of CXCR2 were performed using Taqman probes Hs01891184 (recognizes amplicon length 64bp) and Hs00174304 (recognizes amplicon length of 95bp). Primary human neutrophil cDNA was kindly provided by Dr. Kingsley Osuala, Wayne State University to serve as a positive control for CXCR2 expression. Assays were done

on three biological replicates using TaqMan® Fast Universal PCR Master Mix and 25 ng of cDNA/well for RNA isolated from cells and 50 ng cDNA/well for in vivo samples. All reactions were run on an Applied Biosystems StepOnePlus™ system. DataAssist™ Software (Applied Biosystems) was used for all analyses.

3.14 Immunoblot and ELISA analyses

Lysates were equally loaded based on DNA concentrations as previously described [85, 86]. Proteins were electrophoresed on 12 % SDS-PAGE gels, transferred to PVDF membranes and immunoblotted for cathepsin K (1:500) and β-actin (1:5000), $pAkt^{S473}$, total AKT, Bcl-xL, BiP, survivin, CD44, CD44v6, αVβ3, and OPN (1:1000). Secondary antibodies labeled with horseradish peroxidase were used at 1:10,000. Quantification and analyses of bands were performed using a Luminescent Image Analyzer LAS-1000 Plus from Fujifilm (Stamford, CT) and expressed as arbitrary units (AU) per square millimeter. For ELISA assays, media from each condition were diluted based on DNA concentrations in cell lysates and were run in duplicate according to manufacturer's instructions (R&D Systems). Optical density of each well was determined at 450 nm with correction wavelength set to 540 using TECAN-Infinite M200 PRO plate reader (Männedorf, Switzerland). The data were analyzed based on the standard curve values using a four parameter logistic (4-PL) curve-fit.

3.15 Cathepsin K activity assay

Enzymatic activity of cathepsin K was measured in cell lysates utilizing the fluorescent substrate Z-Glycine-Proline-Arginine-7-amido-4-methylcoumarin-HCl (Z-Gly-Pro-Arg-AMC) from Bachem Chemical (100 µM; Torrance, CA). The reaction was performed in the presence of the selective inhibitor to cathepsin B, CA074 (1 µM) to eliminate the activity due to cathepsin B-mediated

cleavage of Z-Gly-Pro-Arg-AMC [85]. The progress of the reaction was monitored every minute for a period of 30 min on a Tecan SpectraFluor Plus plate reader. Results of activity assays are expressed as maximum fluorescence units formed per minute. Equal amounts of cell lysate were used based on DNA concentrations in cell lysates.

3.16 MTT assay

BMMs were seeded at a density of 20,000 cells per well in a 96-well in MEMα medium with 15% L929 conditioned medium and 10 % FBS. After 24 h, cells were treated with DMSO (control) or increasing concentrations of SB225002 or CXCR2 neutralizing antibody. Cells were retreated after 48 h and Vybrant® MTT Cell Proliferation Assay kit (Life Technologies) was performed after 4 days. Conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan by viable tumor cells was measured at 540 nm according to manufacturer's instructions using Infinite® F200 Pro plate reader.

3.17 Immunofluorescence Analysis

Cytoplasmic and surface expression of CD44v6 were examined by immunofluorescence staining using goat anti-human CD44v6 (1:50) primary antibody and Alexa 488 secondary antibodies. Fluorescent images were captured with a Zeiss LSM510 META NLO confocal microscope using 40x oil immersion lens. Controls were run in the absence of primary antibodies.

3.18 Flow cytometry analysis of CD44v6 and αVβ3

ARCaP(M) tumor cells were seeded at a density of $1.3x10^6$ cells per well in 100mm dishes and allowed to settle overnight. Half of the growth medium was removed and replaced with DMEM containing 10% FBS (Control) and 200 ng/mL OPN for 48 hours. Cells were trypsinized and washed 2x with PBS. Cells from control and OPN treated conditions were counted and at least

$1x10^6$ cells were used for antibody labelling. Cells were labelled with CD44v6 (1:100), CD44 (1:50) and αVβ3 (1:20) in 100ul PBS and incubated at RT for 1 hour and washed 2x with PBS. Cells were resuspended in 100ul PBS and Alexa Fluor 488 secondary anti mouse and anti-rabbit secondary antibodies and incubated for 30 minutes protected from the light. Cells were washed 2x with PBS and pellet was resuspended in 1ml PBS and kept on ice. Analyses of CD44v6 and αVβ3 were performed using BD LSR II Analyzer.

3.19 Invasion Assays

To determine the effects of BMM-secreted OPN in tumor invasion, BMMs were seeded at a density of .1 x 10^6 cells per well in a 24-well plate and allowed to settle overnight. Tumor cells were serum starved overnight and seeded on top of the BD invasion filter (8 µm pore size) coated with 3-D Culture Matrix™ reduced growth factor basement membrane extract (Trevigen, 1mg/ml). Tumor cells were then seeded at the density of 5×10^4 cells/filter in serum-free medium in the presence or absence of blocking antibodies to αVβ3 (0.8µg/mL), CD44v6 (0.8µg/mL), or recombinant OPN protein (500ng/mL) and allowed to invade to BMM media containing 10% FBS and MEMα (control) or BMMs to serve as a chemoattractant. Cells were allowed to invade for 48 hours and filters were stained with Kwik Diff Staining kit (Thermo Scientific) according to manufacturer's instructions. Five representative images per coverslip were captured using an Olympus BX43 conventional light microscope at 10X magnification to visualize invading cells and counted using ImageJ software. Data were collected from at least three independent experiments performed in duplicate.

3.20 OPN mRNA Expression in Primary vs Metastatic Sites Using Oncomine™ Database

Gene expression of osteopontin in primary prostate compared to metastatic prostate cancer was performed using publicly available microarray datasets in Oncomine™ (www.oncomine.org). Data were graphed using Box and Whisker plots and fold increase in primary and metastatic prostate tissue were compared to normal prostate tissue. Changes in gene expression were represented as $\log_2$ median-centered intensity.

Statistical analyses

All data analyses were performed using GraphPad Software version 6.05. Data were presented as mean +/− SEM and statistically analyzed using Student's t test. For three or more groups, one-way analysis of variance was used.

CHAPTER 4: MARROW ADIPOCYTE-DERIVED CXCL1 AND CXCL2 CONTRIBUTE TO OSTEOLYSIS IN METASTATIC PROSTATE CANCER

4.1 Introduction

Bone has critical functions in haematopoiesis, metabolism, inflammation, and structural support [42, 174]. Multiple proteins and cell types in the bone marrow maintain and preserve normal bone homeostasis [42]; however, this balance is often disrupted by diseases involving chronic inflammation and changes in bone remodeling induced by rheumatoid arthritis [175], diabetes, osteoporosis [35, 176, 177], and metastatic cancers of the breast and prostate [178]. One important part of the bone marrow is the adipocyte, a cell type that not only stores and secretes lipids and fatty acids to promote metabolic functions in the bone, but also secretes adipokines, cytokines, and other inflammatory factors [176, 177, 179]. It is already known that adiposity of the bone marrow increases greatly with age, obesity, and metabolic disorders [42, 176, 177]. There is also growing evidence that the number of adipocytes in the bone is inversely correlated with bone mineral density [34, 42, 176, 180-182].

Bone marrow fat is particularly localized to trabecular areas of the bone, sites of active remodeling, suggesting that it may have some involvement in bone degradation [176]. Different factors have been reported to be associated with increased bone marrow adiposity and altered bone health. They include a shift in mesenchymal cells from osteoblasts to adipocytes resulting in reduced bone mass [179, 180, 183], fatty acid-driven promotion of osteoclastogenesis and prolonged survival, enhanced PPARγ expression [184, 185], and increased levels of osteoclastic factors: macrophages colony-stimulating factors (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) [176, 180]. It is less well known if

adipocytes supply other factors that promote normal M-CSF/RANKL-driven osteoclastogenesis.

Studies of bone marrow adiposity in mouse models fed a high fat diet (HFD) have linked decreases in trabecular bone volume and bone marrow density with enhanced expression of the bone-degrading protease cathepsin K, pro-inflammatory cytokines, IL-6 and tumor necrosis factor α (TNF-α) and decreased expression of IL-10, a negative regulator of osteoclast maturation [181, 182, 186-188]. We have recently shown that COX-2 and CCL-2, two host-derived inflammatory factors, are increased in bones of mice on HFD [43]. We also reported that increased marrow adiposity has stimulating effects on growth of skeletal prostate tumors [43], similar to previous studies indicating that fatty marrow is a storage depot for pro-tumorigenic factors important for tumor colonization [189, 190].

Two such factors are members of the CXC chemokine family, CXCL1 and CXCL2, potent chemotactic proteins that promote inflammation and support tumor growth [191]. CXCL1 and CXCL2 bind to CXCR2 (IL-8RB), a $G_{\alpha i}$ protein coupled receptor expressed on macrophages, epithelial cells, and neutrophils [130, 131, 141, 192] and their normal function is to attract neutrophils to sites of injury [191]. Although the role of CXCR2 in the inflammatory response is well characterized, the functions of CXCL1 and CXCL2 signaling in osteoclast precursor cells and its function in bone remodeling is not well understood [193]. Only recently a possible involvement of CXCL1 and CXCL2 in migration and differentiation of osteoclast precursor cells was suggested by some studies [194, 195]. CXCR2 and its ligands have been linked to the progression of several malignancies including melanoma, non-small cell lung cancer, and pancreatic cancer (reviewed in [196]); however, to date there have been no studies that have

determined the role of CXCR2 in adipocyte-associated deregulation of bone remodeling and the deleterious consequences on tumor progression in bone.

The objective of the present study was to investigate the role of CXCL1 and CXCL2 and their receptor CXCR2 in adipocyte-induced osteoclast differentiation and in prostate tumor-driven bone degradation. Utilizing diet-induced obesity model, a well-documented approach to induce bone marrow adiposity [42, 177, 181, 188], we demonstrate a positive association between bone marrow adiposity, augmented levels of CXCL1 and CXCL2, and bone degradation by ARCaP(M) and PC3 prostate tumor cells. By *in vitro* osteoclastogenesis assays, we also show that media conditioned by bone marrow adipocytes accelerates osteoclast maturation and increases gene expression of proteolytic factors important for osteoclast formation and function. We also show that bone marrow adipocytes are a significant source of CXCL1 and CXCL2, chemokines levels of which are potentiated by adipocyte-tumor cell interactions. In addition, we demonstrate that neutralization of the CXCR2 receptor or CXCL1/CXCL2 ligands effectively abrogates osteoclast formation. Together, our results demonstrate a mechanism of the contribution of bone marrow adiposity to tumor-stimulated osteolysis.

4.2 Results

4.2.1 Bone remodeling is increased in tibiae of mice with increased marrow adiposity

Bone marrow adiposity has been linked to reduced bone density, increased presence of osteoclasts, fewer osteoblasts, and accelerated growth of prostate cancer in bone [197, 198]. We previously demonstrated that mice fed HFD have more adipocytes in marrow compared to LFD control mice [43]. We also showed that the increased adiposity promotes growth of PC3 prostate tumors in bone [43]. Based on these findings we investigated the effects of marrow adiposity in intratibially implanted ARCaP(M) cells. These cells have a mixed osteoblastic and osteolytic phenotype *in vivo*, and better reflect the metastatic phenotype in humans [199, 200]. Under LFD conditions, ARCaP(M) tumors remained contained within the bone matrix with X-ray and histological evidence of both bone destruction and new bone acquisition **(Figure 4.1 A–E)**, whereas HFD tumors exhibited extensive destruction of the bone **(Figure 4.1 F–J)**.

We validated these results by measuring the area of each tibia that is not occupied by tumor and showed significant reduction in intact bone in tumor-bearing mice fed HFD as compared to LFD mice **(Figure 4.2)**. These data confirm our previous report of adiposity-stimulated bone destruction in mice bearing PC3 tibial tumors [43]. To better visualize the effects of bone marrow adiposity-driven effects of tumor growth on osteolysis and the location of osteoclasts in these models, we performed TRAcP staining of tibial cross-sections of PC3- and ARCaP(M)-bearing mice. Our results verified our histological and X-ray analysis showing LFD tumors are confined to the bone and surrounded by TRAcP-positive cells at the bone-tumor [43] interface **(Figure 4.1 D, E, Figure 4.3 C, E)**. In comparison, HFD tumors resulted in

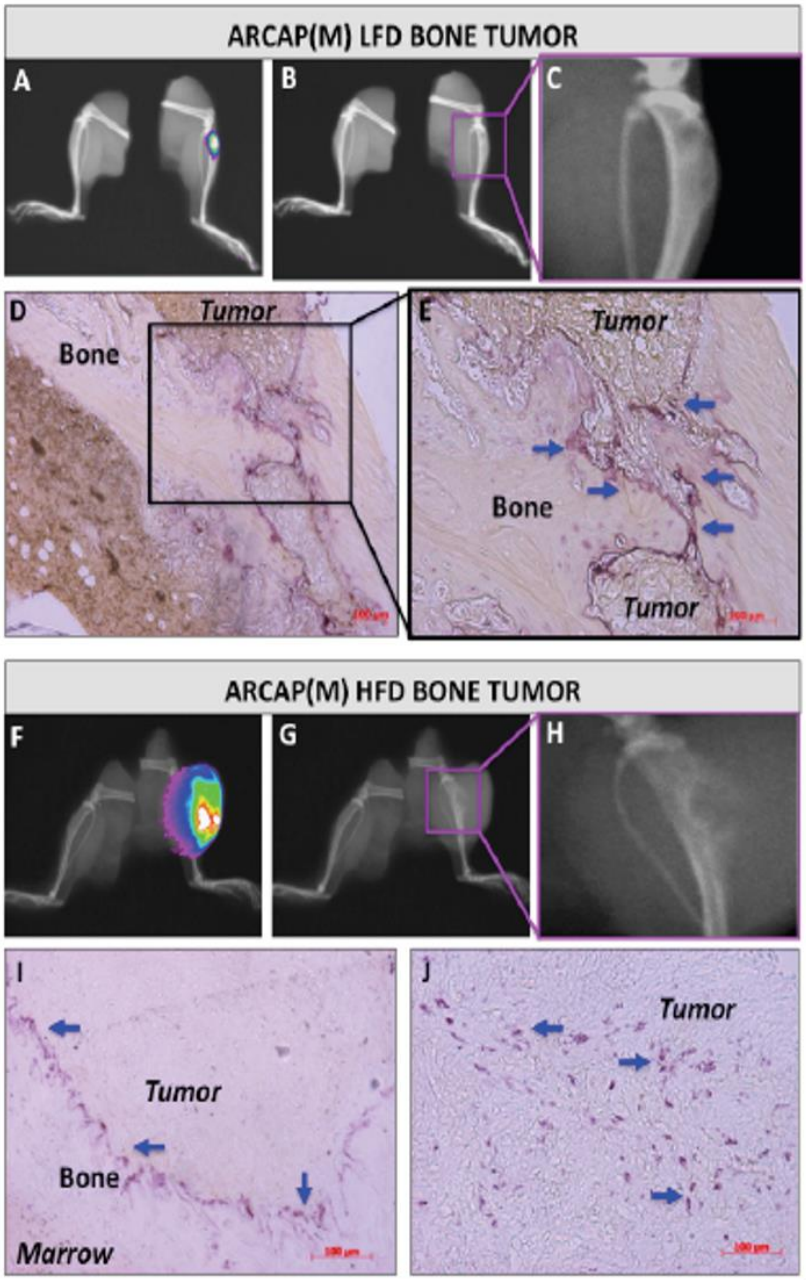


Figure 4.1 Diet-induced marrow adiposity correlates with increased osteolysis in ARCaP(M) bone tumors. FVB/N/N5, Rag-$1^{-/-}$ mice were fed a normal (LFD; **A-E)** or high fat (HFD; **F-J**) diets for 8 weeks followed by intratibial injections of ARCaP-DsRed cells into the right tibia. Tumors were imaged at 8 weeks post injection (N = 9 mice/LFD group and N = 11 mice/HFD group). The overlay of X-ray and 600 nm RFP fluorescence (**A, F**). **B**, **G** X-ray images depicting osteolytic changes in the bone occupied by tumor (magnified in **C, H**). TRAcP-positive osteoclasts (dark staining indicated by arrows) in tibial cross sections of tumor-bearing mice on LFD (**D, E**) and HFD (**I, J**). TRAcP data are representative of at least three individual sections from three separate LFD and HFD tumors.

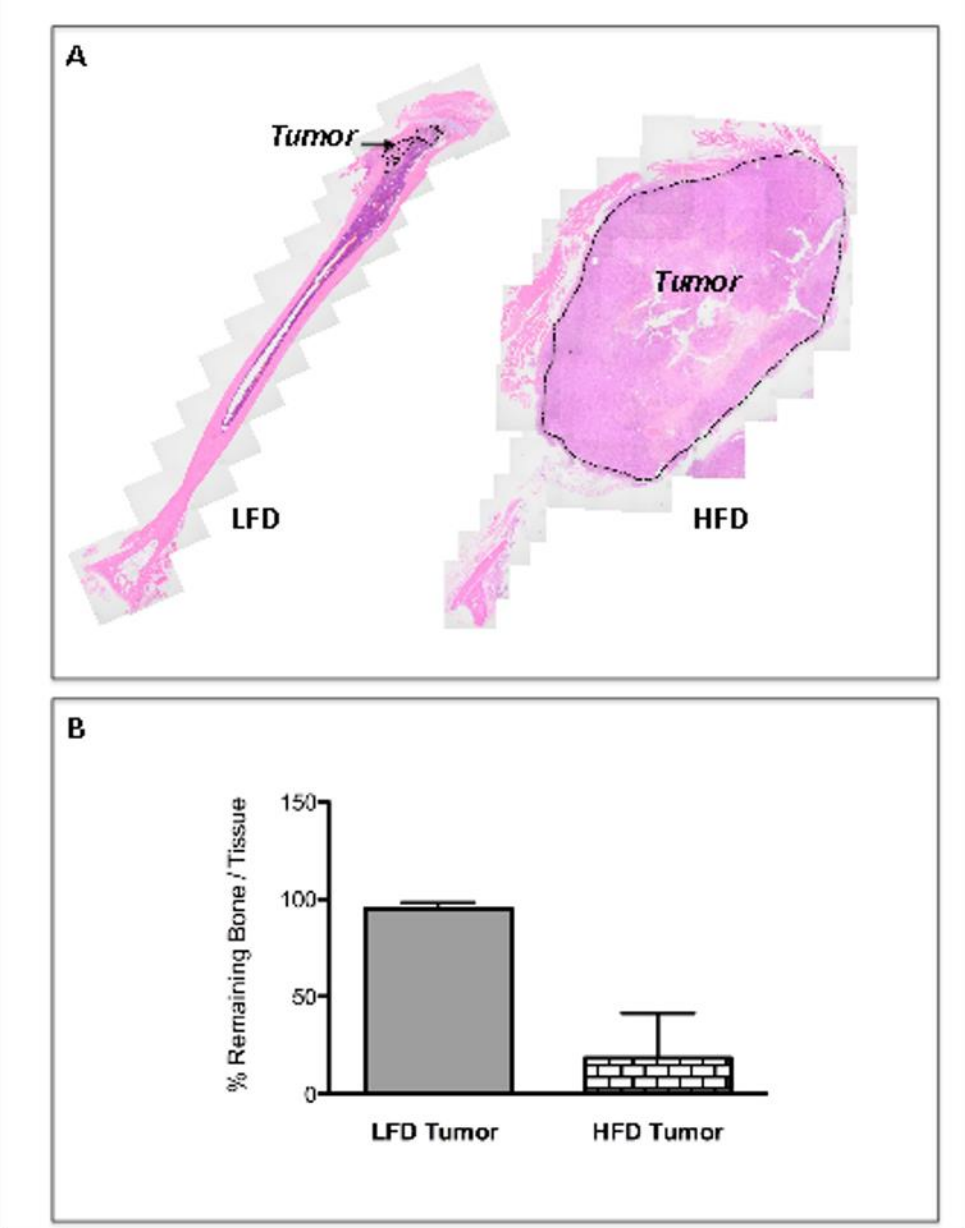


Figure 4.2: HFD-induced marrow adiposity correlates with bone destruction in ARCaP(M) tumors. A: H&E staining of ARCaP(M)-bearing tibiae of LFD (left panel) and HFD (right panel) mice. **B:** Quantification of bone (non-tumor) tissue in tumor bearing tibiae from LFD and HFD mice. Data are shown as a mean ratio of the area of remaining bone to total area of the tibiae +/-SD. Extensive bone degradation is observed in tumors from HFD mice after 8 weeks of growth.

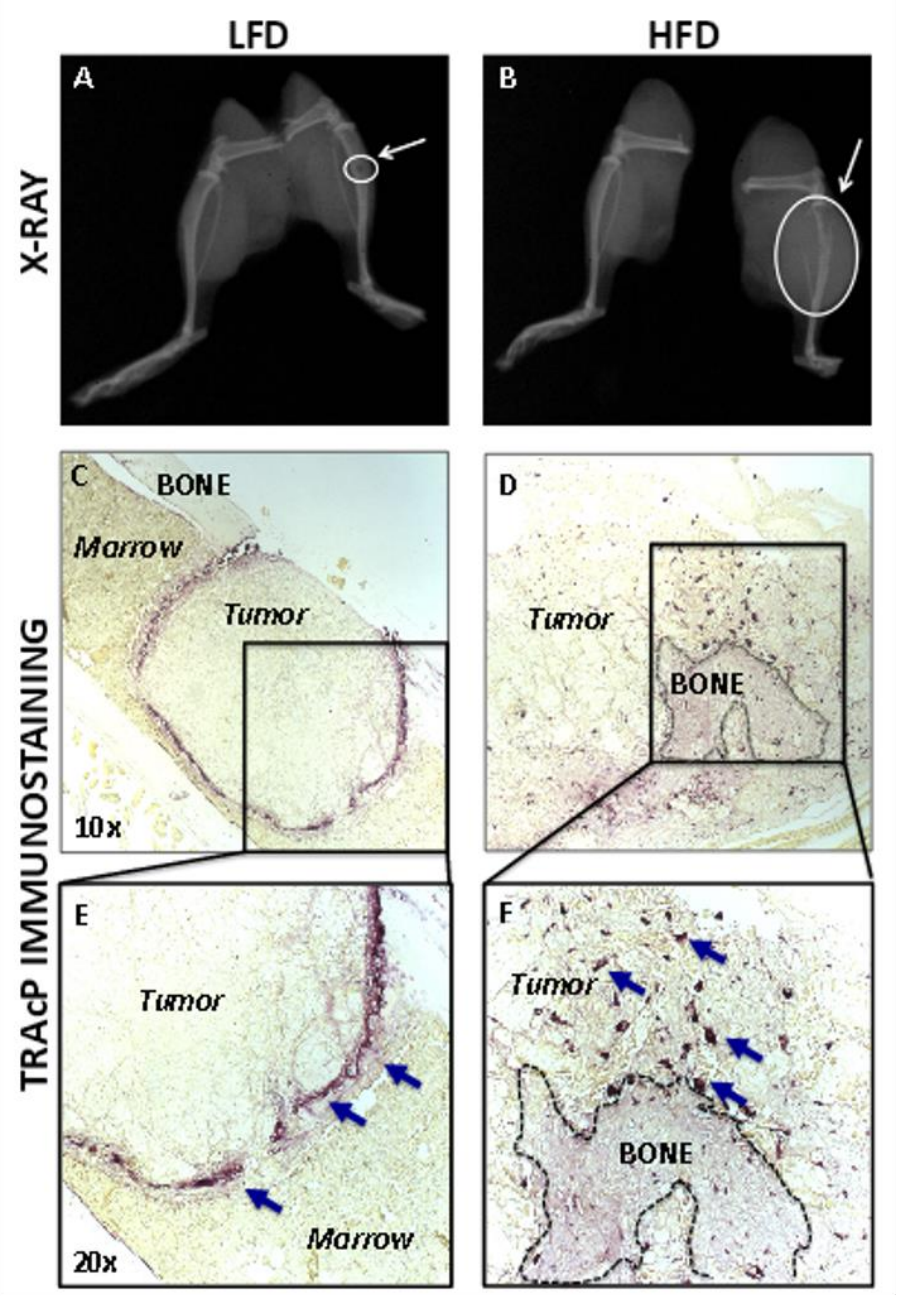


Figure 4.3: HFD-induced osteolysis in PC3-bone tumors. FVBN/N/N5 Rag-/- mice were fed a normal (LFD) or high fat diet (HFD) for 8 weeks followed by intratibial injections of PC3 cells into the right tibia. Left tibiae were PBS controls. Ex vivo X-ray images of LFD **(A)** and HFD **(B)** mice 6 weeks post-tumor implantation. TRAcP staining (purple) of osteoclasts (blue arrows) in the tibial tumor cross sections from LFD **(C,E)** and HFD mice **(D,F)**.

substantial bone destruction and large osteoclastic clusters were observed both around bone remnants as well as in the tumor **(Figure 4.1 I, J, Figure 4.3 D, F)**. Together, these data suggest that bone marrow adiposity accelerates tumor-derived osteolysis *in vivo*.

4.2.2 Adipocyte-derived factors enhance osteoclastogenesis and proteolytic activity of osteoclast-derived cathepsin K *in vitro*

To determine the effects of adipocyte-derived factors in osteoclastogenesis, we differentiated osteoclasts *in vitro* in the absence or presence of conditioned medium from bone marrow adipocytes (Adipo CM). Assays were performed in the presence of M-CSF and RANKL, both required for the induction of osteoclastogenesis [201]. As shown in **Figure 4.4**, treatment with Adipo CM resulted in increased osteoclast maturation compared to cultures treated with RANKL and MCSF alone **(Figure 4.4 A, B)**. Upon closer examination, we observed there were significantly larger TRAcP-positive cells formed in addition to an increase in the total number of osteoclasts differentiated in the presence of Adipo CM compared to control **(Figure 4.4 C, and D).** Osteoclast formation was due to enhanced BMM fusion, as indicated by the formation of multi-nucleated cells and increased expression of the osteoclast fusion marker DC-STAMP [202] by osteoclasts differentiated in the presence of Adipo CM **(Figure 4.5 A, B)**. Bone marrow adiposity-driven osteoclastogenesis was further demonstrated by significant increases in genes correlated with osteoclastogenesis and bone remodeling including calcineurin, cathepsin K, and matrix metalloproteinase-9 (MMP-9) [203-205] **(Figure 4.6 A)**.

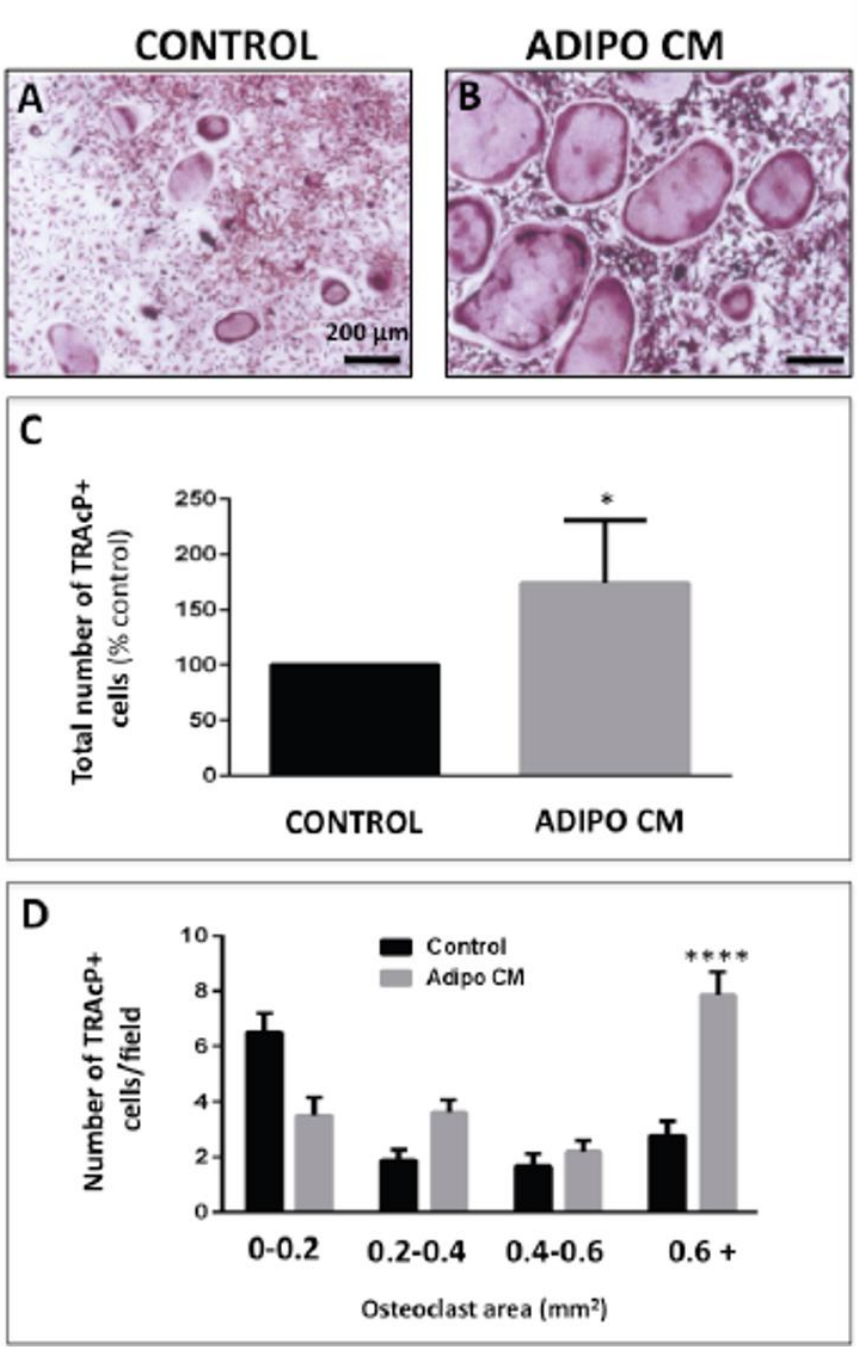


Figure 4.4: Bone marrow adipocyte-secreted factors promote osteoclastogenesis *in vitro*. TRAcP staining of osteoclasts differentiated in the absence (**A** ; Control) or presence of Adipo CM (**B**). **C:** Quantification of total number of TRAcP positive cells using ImageJ software shown as percent of control (% control). **D:** The areas of osteoclasts (in mm^2) were measured using ImageJ software and separated based on size from smallest (0-0.2mm^2) to largest (0.6mm^2+). Graph represents the total number of osteoclasts/field in each size group. Experiments are representative of at least three replicate experiments and shown as mean ± s.e.m; Values indicated by ****($p<0.0001$) and * ($p<0.05$) are considered statistically significant.

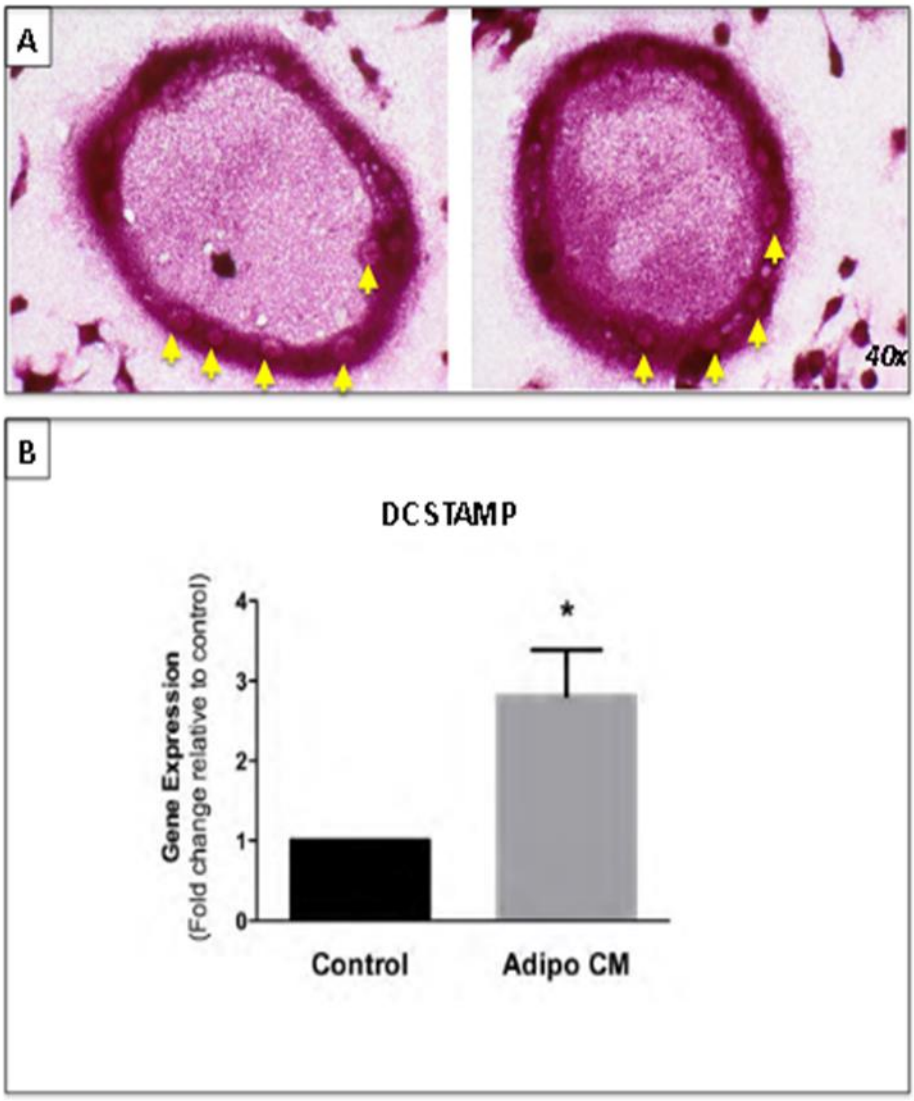


Figure 4.5: Adipocyte-derived factors stimulate osteoclast fusion. A: High magnification (40x) TRAcP staining images depicting multinucleated cells formed in the presence of Adipo CM. Yellow arrows indicate nuclei. B: Taqman RT PCR analysis for DC-STAMP (an osteoclast fusion marker) in osteoclasts differentiated under control conditions or in the presence of Adipo CM. Data are normalized to ribosomal 18S and shown as increase relative to control cultures.

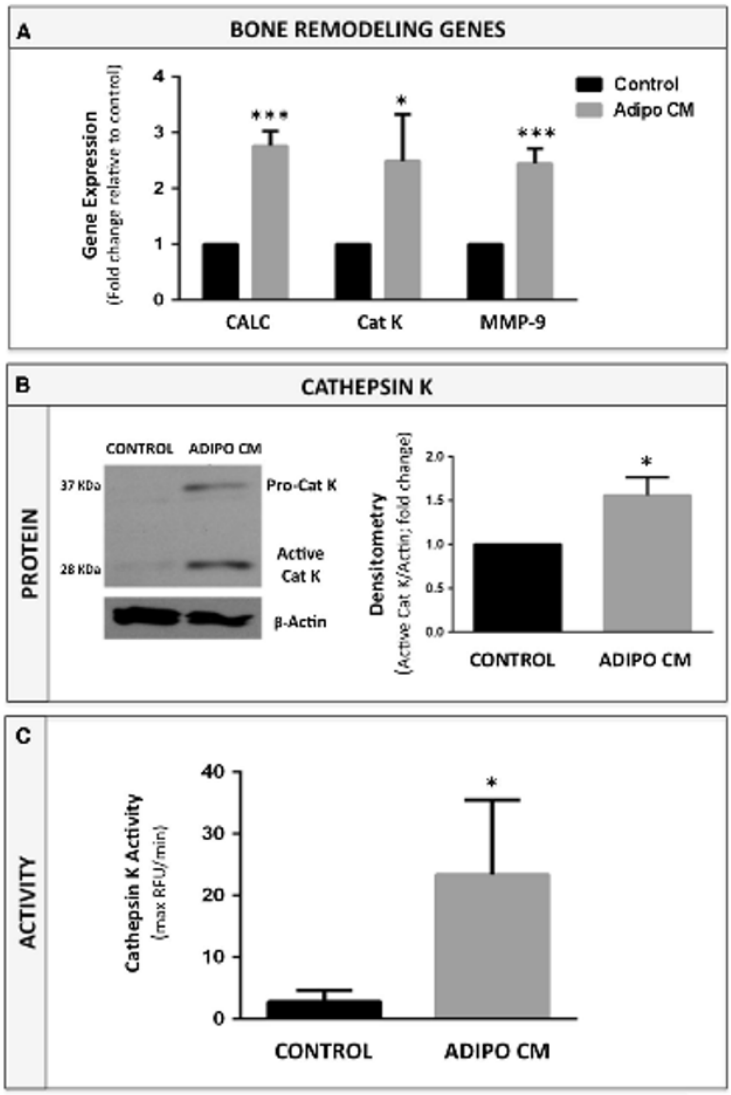


Figure 4.6: Cathepsin K expression and activity are increased in Adipo CM-treated osteoclasts. A: Taqman RT-PCR analysis of bone remodeling genes: calcineurin (CALC), cathepsin K (CAT K), and matrix metalloproteinase-9 (MMP-9) in osteoclasts differentiated in the absence (CONTROL) or presence of Adipo CM (ADIPO CM). Data are normalized to 18S and are graphed as fold increase relative to control. **B:** Western blot of cathepsin K in osteoclasts (left panel). Levels of pro-cathepsin K (37 kD) and active cathepsin K (28 kD) are increased in osteoclasts treated with Adipo CM. Densitometric analysis of active cathepsin K levels in osteoclasts (right panel) measured as a ratio of 28 kDa band to β-actin (in AU/mm^2) and represented as % control. **C:** Proteolytic activity of cathepsin K in cell lysates from osteoclasts differentiated in the absence and presence of Adipo CM. Assays were run against fluorescent cathepsin K substrate Z-Gyl-Pro-Arg-7-amido-4-methylcoumarin (Z-Gly-Pro-Arg-AMC) in a presence of 1 μM. cathepsin B inhibitor Ca074. Fluorescence was measured in maximum relative fluorescent units per minute (Max RFU per min). Data are representative of three replicate experiments. All values are shown as mean ± s.e.m. (*** $p<0.001$, * $p<0.05$ are considered statistically significant).

We further investigated changes in cathepsin K protein expression and activity in Adipo CM-treated osteoclasts since it is one of main proteases expressed by osteoclasts, and known to degrade collagen, a matrix protein that makes up 90% of the bone matrix [206]. Consistent with our RT-PCR analyses, total protein expression of the full-length and mature active forms cathepsin K were increased in lysates of Adipo CM-treated osteoclasts **(Figure 4.6 B)**. To determine if Adipo CM-treated osteoclasts expressed active cathepsin K, we also performed proteolysis assays and measured the amount of cleavage of cathepsin K substrate, Z-Gly-Pro-Arg-AMC. Indeed, osteoclasts differentiated in the presence of Adipo CM had 5 times more activity than control cells **(Figure 4.6 C)**. These results suggest that adipocyte-derived factors accelerate osteoclastogenesis beyond the RANKL- and M-CSF-induced stimulation.

4.2.3 Marrow adiposity is associated with increased levels of CXCL1 and CXCL2

Previously, we showed that adiposity promotes increased production of COX2 and CCL2, inflammatory factors implicated in bone metabolism, osteoclast differentiation, and metastatic tumor growth in bone [42]. This suggested bone marrow adiposity induces changes in the microenvironment to promote tumor progression in bone, and we investigated if other adipocyte-derived factors may also be involved in this process. Of the various factors we found to be increased in our ARCaP(M) and PC3 HFD tumor models, two that stood out were CXCL1 and CXCL2 **(Figure 4.7 A)**. Notably, CXCL1 and CXCL2 have been implicated in several malignancies such as endometrial and ovarian cancer and have been reported to regulate tumor invasiveness [207], and, most recently, to stimulate migration of osteoclasts pre-cursor cells and promote differentiation [194, 195]. We performed RT-PCR analyses of CXCL1 and

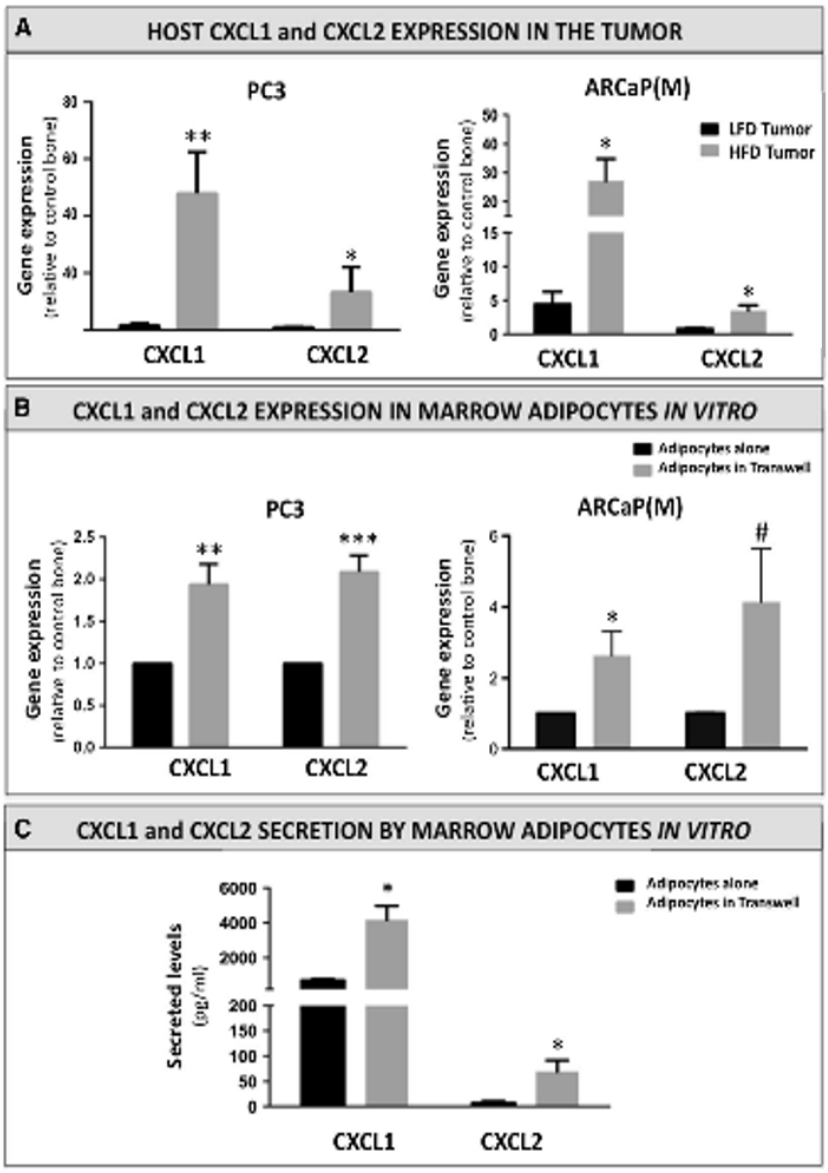


Figure 4.7: CXCL1 and CXCL2 expression and secretion are increased in adipocytes interacting with tumor cells *in vivo* and *in vitro*. A: Taqman RT-PCR analysis of host CXCL1 and CXCL2 expression in LFD and HFD mice bearing PC3 (left panel) and ARCAP(M) (right panel) tumors. Data are normalized to murine (host) HPRT1 and shown as fold increase relative to control bone. **B:** Taqman RT-PCR analysis of CXCL1 and CXCL2 expression in bone marrow adipocytes cultured alone (black bar) or in a transwell co-culture (grey bar) with PC3 (left panel) or ARCaP(M) cells (right panel). Data are normalized to HPRT1 and shown as fold increase relative to adipocytes alone. **C:** ELISA assay results for CXCL1 and CXCL2 secreted by the bone marrow adipocytes grown alone or in a transwell system with PC3 cells. Media samples were diluted based on DNA concentrations in cell lysates. Data are expressed in pg/mL. All data are representative of three replicate experiments. All values are shown as mean ± s.e.m. (*** $p<0.001$ and * $p<0.05$ are considered statistically significant, # $p = 0.0792$).

CXCL2 in bone marrow adipocytes cultured in Transwell with PC3 and ARCaP(M) cells, and there was in fact increased expression of these pro-inflammatory chemokines in response to PCa-derived factors **(Figure 4.7 B)**. We also performed ELISA analysis of conditioned media from the Transwell co-cultures to verify that secretion of CXCL1 and CXCL2 corresponded with gene expression (**Figure 4.7 C).** Indeed, both factors were secreted by adipocytes (399.2 pg/mL for CXCL1 and 3.111pg/mL for CXCL2) and secretion was further increased during interaction with PCa tumor cells (7.5- fold increase for CXCL1 and 54-fold increase for CXCL2). These data show that bone marrow adipocytes contribute to tumor-induced inflammation in bone.

4.2.4 CXCL1 and CXCL2 chemokines stimulate osteoclast differentiation *in vitro*

To investigate the role of CXCL1 and CXCL2 in osteoclastogenesis, we differentiated osteoclast precursor cells in the absence or presence of recombinant proteins to each of the chemokines. TRAcP staining showed that CXCL1 and CXCL2 markedly increased osteoclast differentiation compared to control conditions **(Figure 4.8 A-C)**. Interestingly, although the total number of osteoclasts did not change significantly between conditions, both chemokines promoted the formation of substantially larger osteoclasts compared to those differentiated in the absence of CXCL1 and CXCL2 **(Figure 4.8 D-E)**. Likewise, increased mRNA levels of cathepsin K paralleled the clear presence of larger osteoclasts as established by TRacP staining **(Figure 4.9).** Conversely, we determined whether osteoclastogenesis can be inhibited by neutralizing antibodies to CXCL1 and CXCL2 **(Figure 4.10 A)**. We observed a considerable reduction in both the total number of osteoclasts formed **(Figure 4.10 B)** and size of osteoclasts formed compared to Adipo CM alone **(Figure 4.10 C)**. These results demonstrate that CXCL1 and CXCL2 are in part involved in adipocyte-driven osteolysis.

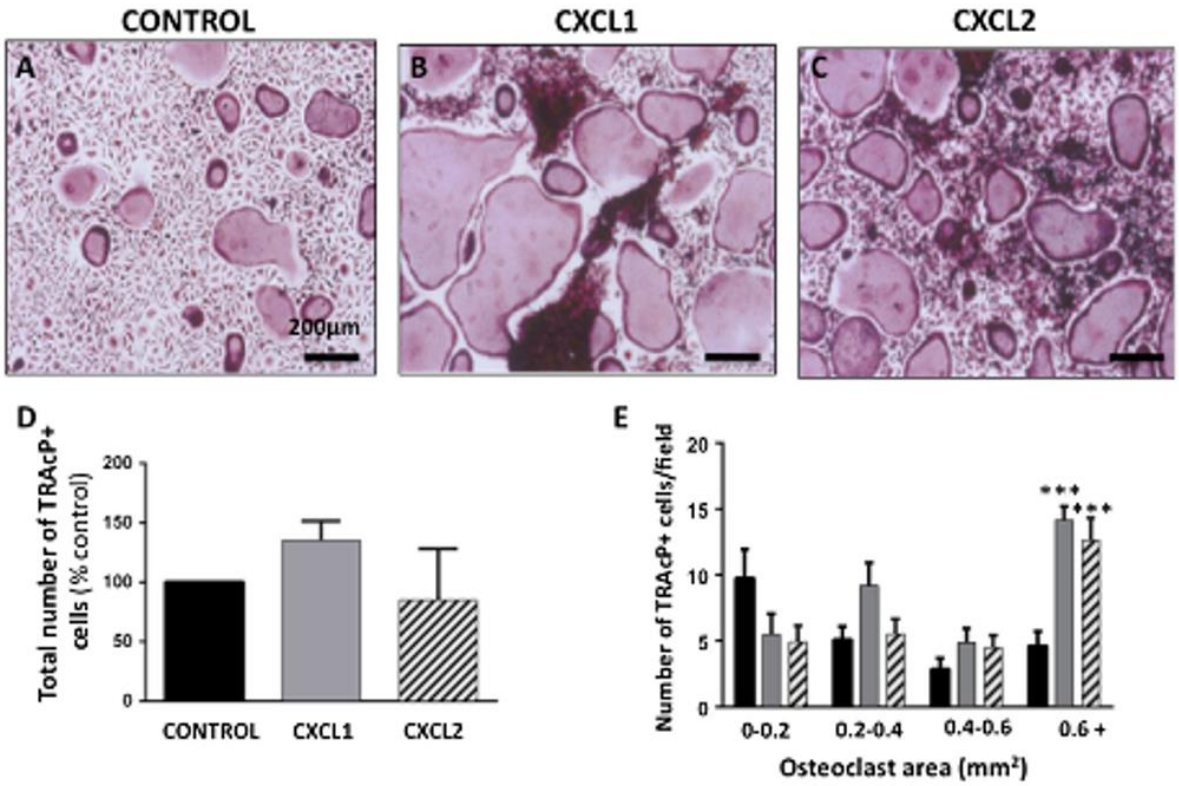


Figure 4.8: Recombinant CXCL1 and CXCL2 proteins accelerate osteoclastogenesis. A-C: TRAcP staining of osteoclasts differentiated in the absence (**A;** control) or presence of recombinant CXCL1 (**B**) and CXCL2 (**C)** proteins. **D:** Quantification of total number of TRAcP positive cells shown as percent of control (% control). **E:** Total number of osteoclast/field categorized based on size from the smallest (0-0.2mm^2) to largest (0.6mm^2+). Significantly larger osteoclasts were formed in the presence of CXCL1 and CXCL2. Values are shown as mean ± s.e.m and are representative of three replicate experiments. (*** p<0.005considered statistically significant).

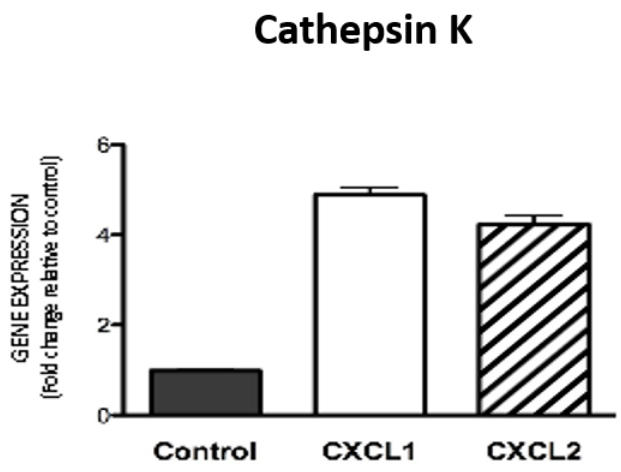


Figure 4.9: Cathepsin K gene expression increases in osteoclasts differentiated in the presence of recombinant CXCL1 and CXCL2. Taqman RT PCR analysis for Cathepsin Kin osteoclasts differentiated under control conditions or in the presence of recombinant CXCL1 or CXCL2. Data are normalized to 18S and shown as increase relative to control conditions.

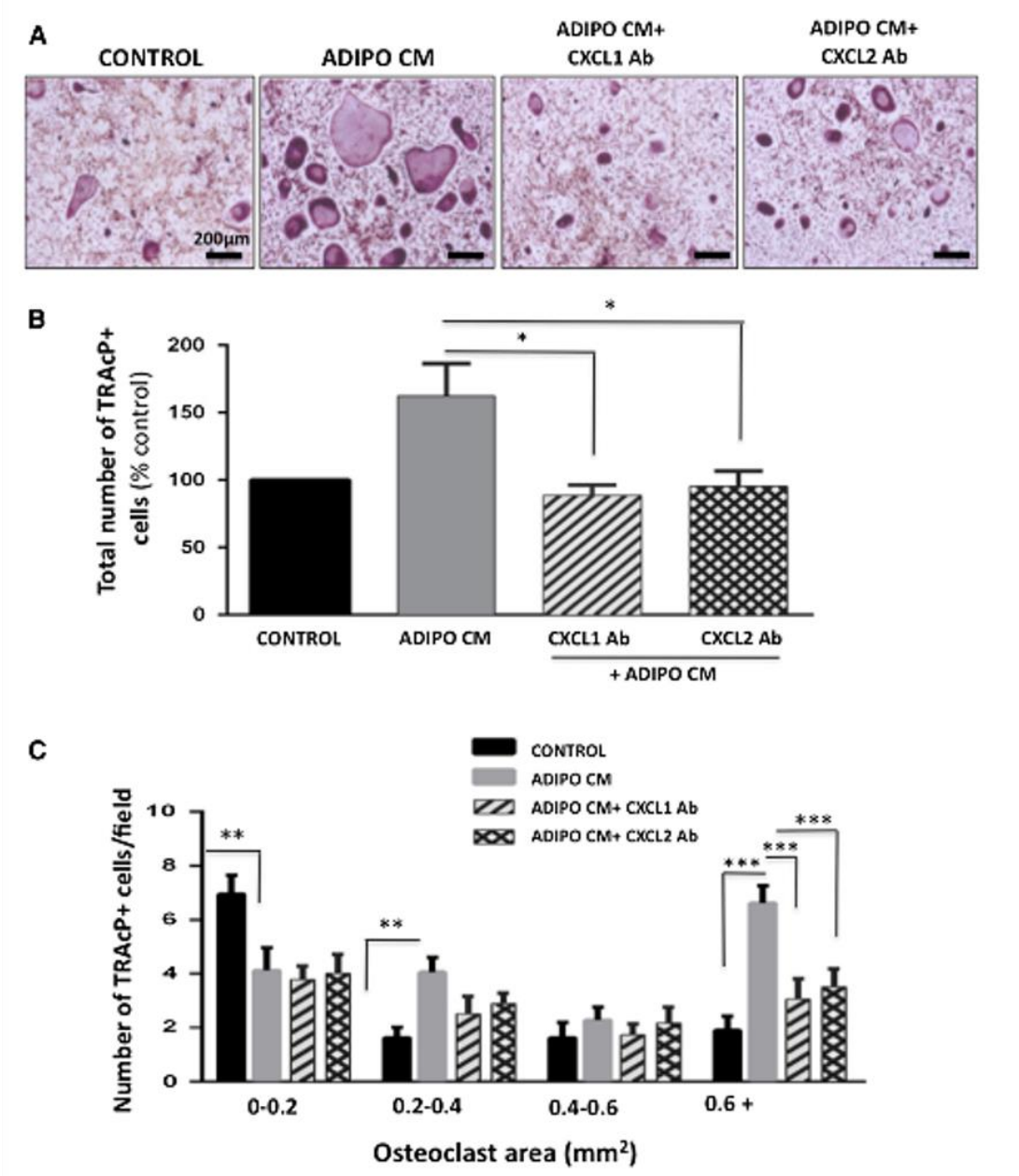


Figure 4.10: Adipocyte-driven osteoclastogenesis is inhibited by neutralizing CXCL1 and CXCL2. **A:** TRAcP staining of osteoclasts differentiated under control conditions (far left panel), with Adipo CM (left middle panel) or with Adipo CM in the presence of neutralizing antibodies to CXCL1 and CXCL2 (right panels). **B:** Total number of TRAcP positive cells in each experimental condition shown as % control. **C:** Total number of osteoclasts/field categorized based on size for each experimental condition. Neutralization of CXCL1 and CXCL2 ligands inhibits Adipo CM-stimulated osteoclastogenesis. Data are representative of at least three replicate experiments. (****p<0.0001; ***p<0.001; **p<0.01 and *p<0.05 are statistically significant).

4.2.5 Osteoclastogenesis is partially regulated by the CXCR2 signaling axis

As previously discussed, CXCL1 and CXCL2 bind to the G-protein coupled receptor CXCR2 [130]. Importantly, CXCR2 is highly expressed in osteoclast precursor cells [208] and studies have shown that binding of another CXCR2 ligand IL-8 in human monocytes promotes osteoclastogenesis [193]. To date, however, the role of CXCL1 and CXCL2 signaling through CXCR2 in promoting osteoclast formation has not been well-characterized. Since we established a role of CXCL1 and CXCL2 in osteoclastogenesis, we determined whether blocking CXCR2 and subsequent downstream signaling of the ligands would suppress this process. We treated osteoclast precursors with Adipo CM in the presence or absence of blocking antibodies to CXCR2 or an antagonist to CXCR2, SB225002. We determined effective doses of CXCR2 antibody (5µg/mL) and the CXCR2 antagonist (2.5µM) by TRAcP and MTT assays and showed that both the blocking antibodies and antagonist are not toxic to osteoclast precursors (data not shown).

Similar to the effects of blocking CXCL1 and CXCL2, blocking the CXCR2 receptor with antibodies or an antagonist substantially abrogated osteoclast formation **(Figure 4.11 A)**. Likewise, the total number and size of the osteoclasts formed was reduced compared to Adipo CM treatment alone **(Figure 4.11 A-C)**. To further establish the effects of the CXCR2 signaling axis in this process, we assessed gene and protein expression of osteoclast markers calcineurin, cathepsin K and MMP-9 in osteoclasts treated with Adipo CM in the presence or absence of CXCR2 antibodies. mRNA levels of all three genes were reduced in the presence of blocking antibodies **(Figure 4.12 A)**. Additionally, both protein expression and activity of cathepsin K were reduced with antibody treatment **(Figure 4.11 B-C)**. These results confirm the role of

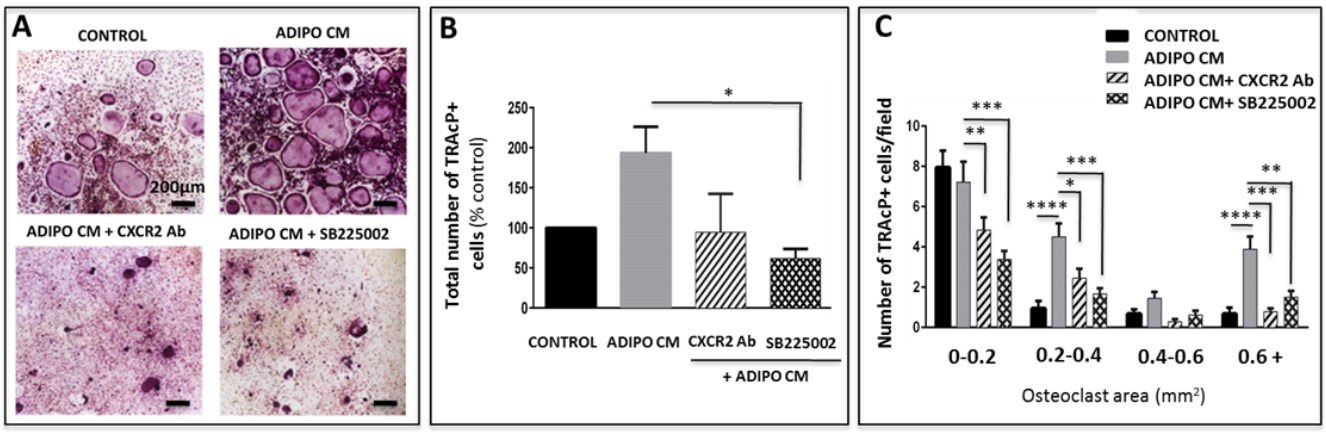


Figure 4.11: Blocking the CXCR2 receptor partially inhibits adipocyte-driven osteoclastogenesis. A: TRAcP staining of osteoclasts differentiated under control conditions (Control), with Adipo CM (Adipo CM) or with Adipo CM in the presence of neutralizing antibodies to CXCR2 (bottom left panel) or CXCR2 antagonist SB225002 (bottom right panel). **B:** The total number of TRAcP positive cells corresponding to each treatment; **C:** Total number of osteoclasts/field categorized based on size for each experimental condition: Control, Adipo CM, Adipo CM + CXCR2 Ab, Adipo CM + SB225002. Data are representative of three replicate experiments (****P<0.0001; ***P<0.001; **P<0 .01; *P<0.05 are statistically significant).

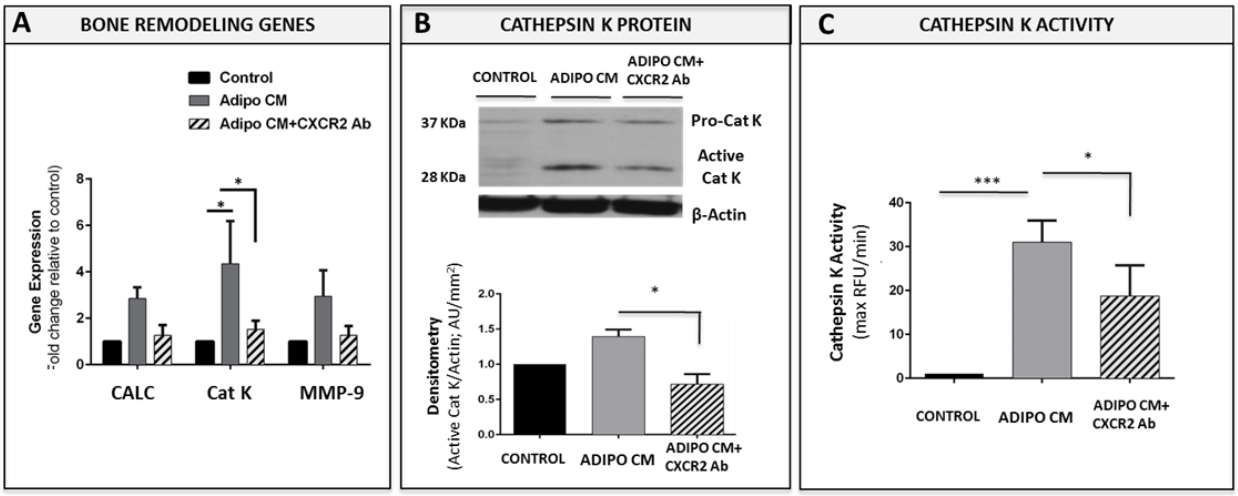


Figure 4.12: Blocking the CXCR2 receptor partially inhibits expression of osteoclasts markers and cathepsin K activity. A: Taqman RT-PCR analysis of bone remodeling genes: calcineurin, cathepsin K, and MMP-9 in osteoclasts differentiated under control conditions, with Adipo CM, or with Adipo CM in the presence of neutralizing antibodies to CXCR2. Data are normalized to 18S and shown as fold increase relative to control. **B:** Immunoblot of pro- and active cathepsin K in osteoclasts differentiated under control conditions or with Adipo CM in the absence or presence of CXCR2 neutralizing antibody (top panel); Densitometric analysis of active cathepsin K levels in osteoclasts measured as a ratio of 28 kDa band to β-actin (in AU/mm^2) and represented as % control (bottom panel). **C:** Proteolytic activity of cathepsin K in cell lysates from osteoclasts differentiated in the absence and presence of Adipo CM, or with Adipo CM plus CXCR2 neutralizing antibody. Assays were run against fluorescent cathepsin K substrate Z-Gly-Pro-Arg-AMC in a presence of 1 μM cathepsin B inhibitor Ca074. Fluorescence was measured in Max RFU per min. Data are representative of three replicate experiments ***P<0.001; *P<0.05 are statistically significant).

CXCR2 signaling in bone marrow adipocyte-induced osteoclastogenesis.

4.3 Discussion

Accelerated bone destruction is a debilitating condition associated with metastatic tumor progression in bone [42, 198, 209, 210]. Almost 80% of men with metastatic prostate cancer present with bone lesions [211], and though most lesions appear osteoblastic, strong evidence supports these lesions are formed by the cooperative dysregulation of osteoclastic and osteoblastic activity [121, 212-214]. Notably, increased bone marrow adiposity and altered adipocyte metabolism are features associated with aging and accelerated osteolysis [34, 42, 179-182]. Moreover, bone marrow adipocytes are usually concentrated in areas of active bone remodeling, such as the trabecular bone [176], and areas rich in red marrow within the axial skeleton [189, 215]. Interestingly, these areas of bone are also preferred sites of metastatic tumor cell seeding and growth [31, 216]. We previously demonstrated that HFD-induced bone marrow adiposity accelerates growth of PCa prostate tumors in bone [43]. In line with previous findings reported by others [189, 215, 217, 218], we demonstrated lipid transfer between bone marrow adipocytes and prostate tumor cells has effects on stimulating growth and invasion. These interactions result in significantly larger skeletal tumors as well as extensive bone destruction [43].

Emanating from our previous findings, the present study focused on the role of bone marrow adiposity on tumor-driven osteolysis of the bone. We showed that HFD-fed mice exhibit more pronounced bone degradation when intratibially injected with either PC3 or ARCaP(M) tumors (Figure 4.1- 4.3). We also demonstrated that gene expression of host-derived

CXCL1 and CXCL2 is significantly higher in tumor-bearing mice on HFD compared to LFD mice and both chemokines are highly secreted by bone marrow adipocytes *in vitro* (Figure 4.7). Moreover, Adipo CM accelerated osteoclast differentiation, a process that can be simulated by treating osteoclast precursors with recombinant CXCL1 and CXCL2 ligands (Figure 4.8). Conversely, the inhibition of adipocyte-driven osteoclast differentiation by the neutralizing antibodies or agents that block the CXCR2 receptor strengthened the importance of CXCR2 signaling in this process (Figure 4.10).

The role of the CXCR2 and its ligands CXCL1 and CXCL2 is osteoclastogenesis has only recently been investigated. Limited reports suggested these chemokines play a role in migration and/or differentiation of osteoclasts [194, 195] and promoting bone loss in periodontal disease [195], rheumatoid arthritis [208], and oral squamous cell carcinoma[219]. Much less is known about the activities of adipocyte-derived CXCL1 and CXCL2 in this process. CXCL1 has been shown to be highly expressed by 3T3-L1 adipocytes co-cultured with macrophages [220], and has also been implicated in inducing inflammation in white adipocytes [221]. Other studies demonstrated that obese individuals have increased levels of CXCL2 in pre-adipocytes, mature adipocytes, and peripheral blood mononuclear cells [222, 223]. Together, these studies suggest a possible link between CXCL1 and CXCL2 in adipocyte-derived inflammation and its relevance to changes in bone tumor milieu. Our lab has previously reported evidence of BMM-driven inflammation in prostate tumors grown in bone, proposing cooperative relationship between tumor cells, bone marrow adipocytes, and osteolysis in bone [86]. We have also shown that augmented levels of bone marrow macrophages result in overexpression of host COX-2 and MCP-1 in skeletal prostate tumors [42]. This present work highlights CXCL1 and CXCL2 as two

additional host-derived pro-inflammatory factors that are augmented in metastatic bone tumors with increased bone marrow adiposity. This inflammatory response in the tumor bone microenvironment may be due to enhanced presence of bone marrow fat as our data show that HFD mice have higher expression of CXCL1 and CXCL2 compared to LFD mice (data not shown).

In addition to bone marrow adipocytes, there are other inflammatory cells in the bone that may be sources of CXCL1 and CXCL2 including tumor cells, endothelial cells, and macrophages [130, 191, 224], which may possibly explain why CXCL1 and CXCL2 levels are upregulated in HFD mice (Figure 4.7 A). Several groups have shown that macrophage- and endothelia-secreted CXCL1 and CXCL2 function as mediators in neutrophil recruitment to sites of tumor growth [130, 191, 216]. Importantly, evidence suggests that tumor cell-derived CXCL1 and CXCL2 may be important in chemoresistance and metastasis [131]. Various sources of these chemokines secreted into the bone microenvironment may have direct effects on bone marrow adipocytes; however, it is unknown if this promotes osteolysis and tumor growth in bone. Regardless of the multiple sources of CXCL1 and CXCL2 in the bone, our results clearly uncover specific roles for these adipocyte-supplied chemokines in osteoclast differentiation. This was clearly demonstrated in this study when neutralizing antibodies to CXCL1 and CXCL2 abrogated adipocyte-induced osteoclastogenesis (Figure 4.10). These effects were mirrored by blocking ligand binding to the CXCR2 receptor (Figure 4.11), highlighting the important function of CXCR2 signaling in osteoclast maturation. It is noteworthy that additional ligands of CXCR2 could possibly contribute to adipocyte-driven osteoclastogenesis including CXCL8 (IL-8) and CXCL5. Both chemokines have been reported as stimulators of osteoclast differentiation and

function [193, 225, 226] and are present in adipose tissue; however, CXCL5 and CXCL8 expression is principally associated with the stromal portion of adipose tissue [227-229]. This suggests that bone marrow adipocyte-derived CXCL1 and CXCL2 chemokines, in part, drive CXCR2 signaling in osteoclasts. However, further studies are necessary to fully understand function and expression of CXCR2 ligands in bone marrow adipocytes, which has been shown to have different features than white adipose tissue [42, 230].

Although diet-induced adiposity is a well-documented and acceptable method to induce bone marrow adiposity [43, 177, 181, 187], we cannot discount possible consequences of the diet on bone degradation and tumor growth. Further studies using genetic models of obesity as well as age-induced models of bone marrow adiposity will provide greater insight into the function of fat-derived CXCL1 and CXCL2 in tumor-driven bone degradation. Increased bone marrow adiposity promotes changes in the bone marrow microenvironment that prove deleterious to bone health with increased age, obesity, and metabolic disorders. Although, the work presented herein did not investigate the effects of diet on the bone milieu, our results do suggest that adipocytes have stimulatory effects on tumor-driven osteolysis through secretion of CXCL1 and CXCL2 and activation of CXCR2 on osteoclast precursors. To our knowledge, this is the first study that directly attributes adipocyte-supplied CXCL1 and CXCL2 with accelerated osteolysis to aid in prostate tumor progression in bone.

This work contributes to the understudied mechanisms associated with the involvement of marrow adiposity in the maintenance of bone health and regulating tumor behavior in bone. Unfortunately, therapies for metastatic bone disease such as bisphosphonate zoledronic acid and the monoclonal antibody denosumab remain palliative [213, 214]. Moreover, no studies

have assessed the possible effects of CXCR2 targeted-therapy as an option in bone disease because of its important roles in normal immunity and other biological functions. The evidence provided in this study and previous reports on the roles of macrophages and adipocytes in tumor progression in bone [42, 43, 86], demonstrate that multi-targeted and combinational therapies may be necessary to effectively reduce the incidence and growth of tumors in metastatic bone disease. Particularly, therapies that target adipocyte, osteoclast, and macrophage pathways may be advantageous in improving quality of life, prolonging survival, reducing bone pain, and eradicating the disease.

CHAPTER 5: Bone Marrow Adiposity Promotes Secretion of Macrophage-Supplied CXCL1 and CXCL2 to Support Tumor Invasion

5.1 Introduction

Inflammation plays a significant role in various diseases including metabolic syndrome, obesity, arthritis, and cancer [231, 232]. Studies have shown that prostate cancer progression and aggressiveness are positively associated with obesity and inflammation and result in increased mortality [233]. Increased subcutaneous adiposity promotes infiltration of macrophages, which can take up as much as 40% of adipose tissue in high fat diet-fed mice [65]. Numerous studies have focused specifically on the role of visceral adiposity in prostate tumor growth and aggressiveness [234, 235]; however, the effects of bone marrow adiposity and inflammation in tumor growth in bone are not well studied. Bone is a primary site of metastasis of prostate cancer and obese men have a three-fold higher risk of progression to metastatic disease compared to normal-weight men receiving the same treatment [236]. Emerging literature evidence suggests that conditions including obesity and inflammation, known to disturb homeostasis in the bone microenvironment [35, 182], may be contributing factors to colonization and growth of prostate tumors in the bone (reviewed in [42]).

Bone marrow macrophages (BMMs) are mediators of inflammatory processes suggested to play distinct functions within tumor microenvironment [86, 237, 238]. In conjunction with adipocytes, BMMs release various cytokines that can aid in tumor homing and growth at metastatic sites such as bone. Two such inflammatory chemokines are CXCL1 and CXCL2 that share 90% sequence homology and bind to the same G-protein coupled receptor, CXCR2 [131]. Both chemokines play a significant role in neutrophil recruitment during normal immune response; however, they have also been linked to tumor growth and malignancy. A beautiful

study conducted by Acharyya and colleagues demonstrated that CXCL1 and CXCL2 are amplified in 20% of human metastatic breast tumors and knockdown of CXCL1 and CXCL2 *in vivo* reduced tumor growth and the incidence of lung metastasis and relapse of metastatic lung tumors [131]. This was also functionally significant because tumor secretion of CXCL1 and CXCL2 activated infiltrating myeloid cells to secrete calcium-binding proteins that protect tumor cells from death, even after chemotherapy [131]. Others have shown that CXCL1 transcripts are enhanced in stromal fibroblasts in aged mice and this supply of CXCL1 potentiates epithelial proliferation of prostate tissue in benign prostatic hyperplasia [239]. Moreover, studies have suggested that HFD-fed mice have increased circulation of CXCL1 and CXCL2 supplied by epididymal fat resulting in increased tumor growth *in vivo*, enhanced incidence of lung and liver metastases, and induced tumor invasion *in vitro* [240]. Our studies presented in Chapter 4 showed that both chemokines are highly secreted by bone marrow adipocytes and they directly contribute to osteolysis in tumor progression. Studies have previously focused on CXCL1 and CXCL2 derived from tumor cells directly to aid in cancer-associated inflammation [241, 242]. Only recently have stromal-supplied CXCL1 and CXCL2 been investigated in tumorigenesis, particularly in prostate cancer [243]. Because CXCL1 and CXCL2 may have multiple effects on the bone marrow microenvironment, this current study investigated other sources of these chemokines and their roles in macrophage homing and tumor invasion.

Physical and paracrine interactions between tumor cells and their surrounding environment can have effects on macrophages in the bone as well as other sites, particularly in the context of macrophage phenotype. M1 macrophages are classically activated in response to bacteria and other foreign invaders and release highly pro-inflammatory factors to activate the

adaptive immune response [244]. M2 macrophages are either inactive or aid in wound healing after tissue injury [244]. These two types of macrophages are predominantly characterized by the cytokines and cell surface receptors they express. M1 macrophages express tumoricidal chemokines and factors, such as IL-1β, IL-6, nitric oxide synthase (NOS), and reactive oxygen species (ROS), while M2 macrophages produce arginase 1, CD163, IL-10 and the mannose receptor CD206 among others [244-246]. Interestingly, macrophage phenotype can be influenced by the factors secreted by the neighboring cells, including adipocytes. Resident visceral adipocyte macrophages of lean individuals have more of an M2 phenotype while obese individuals have an activated M1 phenotype, validating previous reports that obesity and organs with high fat content have characteristics of chronic inflammatory state [247]. This may prove to be a critical factor accounting for increased tumor aggressiveness in overweight and obese individuals because the accumulation of immune cells can be a strong stimulator of cancer progression by supporting angiogenesis and other changes to the tumor microenvironment [248], particularly in bone [42].

In this study, we investigated the effects of prostate tumor cells and adipocyte-derived factors on BMM-stimulated inflammation in bone. We have shown in Chapter 4 that that gene expression of host-derived CXCL1 and CXCL2 is increased in tumor-bearing mice and even more pronounced in HFD-fed mice. We also showed that adipocytes are a significant source of these chemokines in bone (Figure 4.7). Here, we demonstrate that CXCL1 and CXCL2 are also supplied by macrophages and that secretion and expression of these chemokines is increased in Transwell co-culture with prostate cancer cells in the presence of conditioned media from bone marrow adipocytes. We also show that BMMs are more invasive toward tumor- and adipocyte-

secreted factors, and these effects are inhibited in the presence of blocking antibodies to CXCL1 and CXCL2. In addition, we demonstrate that CXCL1 and CXCL2 induce prostate tumor invasion through reconstituted basement membrane. This present study also provides evidence that paracrine factors released by prostate cancer cells and bone marrow adipocytes induce expression of markers associated with a pro-tumorigenic M2 macrophage phenotype that may drive tumor progression in bone. Together, our results reveal and new role of macrophages in adipocyte- and tumor-driven inflammation in bone.

5.2 Results

5.2.1 Tumor and adipocyte-derived factors drive CXCL1 and CXCL2 expression in BMMs

Previous studies have shown that mesenchymal precursors [241, 249], the common progenitor cell type of adipocytes or osteoblasts, tend to differentiate into adipocytes with increased age resulting in more fatty marrow [250]. Interestingly, our studies demonstrated that BMMs isolated from aged mice are more invasive through collagen I than macrophages from younger mice, and these effects are more pronounced in the presence of adipocyte-derived factors, suggesting that bone marrow adipocytes may aid in inflammation in bone with age [42]. With age, stromal components of the prostate increase expression of CXCL1 and CXCL2 which contributes to chronic inflammation and benign prostatic hyperplasia [251]; however, the roles of these chemokines in tumor and macrophage invasion during adipocyte-driven inflammation in bone have not been fully investigated.

We previously showed that adipocytes express and secrete CXCL1 and CXCL2. To determine if CXCL1 and CXCL2 levels in BMMs cultured are affected by their interaction with

prostate tumor cells especially in the presence of bone marrow adipocyte-derived factors we utilized *in vitro* Transwell co-culture system of BMMs and PCa cells. As shown in **Figure 5.1 A**, gene expression of CXCL1 was increased in BMMs exposed to adipocyte-conditioned medium. The CXCL1 mRNA levels were also augmented by co-culture with PC3 cells with no additional effects from simultaneous exposure to Adipo CM. Interestingly, contrary to clear modulation of CXCL1 levels, expression of CXCL2 remained fairly unchanged.

CXCL1 and CXCL2 chemokines are rapidly secreted during inflammatory events, thus it is important to note that their gene expression may not necessarily reflect the secreted levels. To address this, we performed CXCL1 and CXCL2 ELISA analyses of media conditioned by BMMs cultured alone or in co-culture with PC3 cells. Our data revealed that upon co-culture with PC3 cells BMMs secreted 6 fold more CXLC1 (134.7 pg/mL) compared to BMMs cultured alone (22.77 pg/mL). BMMs secreted the highest levels of CXCL1 in the presence of Adipo CM (446 pg/mL) and the combined PC3-Adipo CM (1142 pg/mL) cultures **(Figure 5.1 B)**, suggesting BMMs secrete CXCL1 primarily in response to adipocyte-supplied factors. As shown in **Figure 5.1 C**, CXCL2 secretion was enhanced in BMMs treated with Adipo CM (36.96 pg/mL) and even more with PCa cells (282.3 pg/mL). CXCL2 levels remained elevated when BMMs were cultured with PC3 cells and Adipo CM (212.4 pg/mL), suggesting BMMs secrete CXCL2 in response to paracrine interactions with PCa cells. Together, these data suggest that BMMs-secreted inflammatory factors may differ depending on the externally supplied stimuli in the bone-tumor microenvironment.

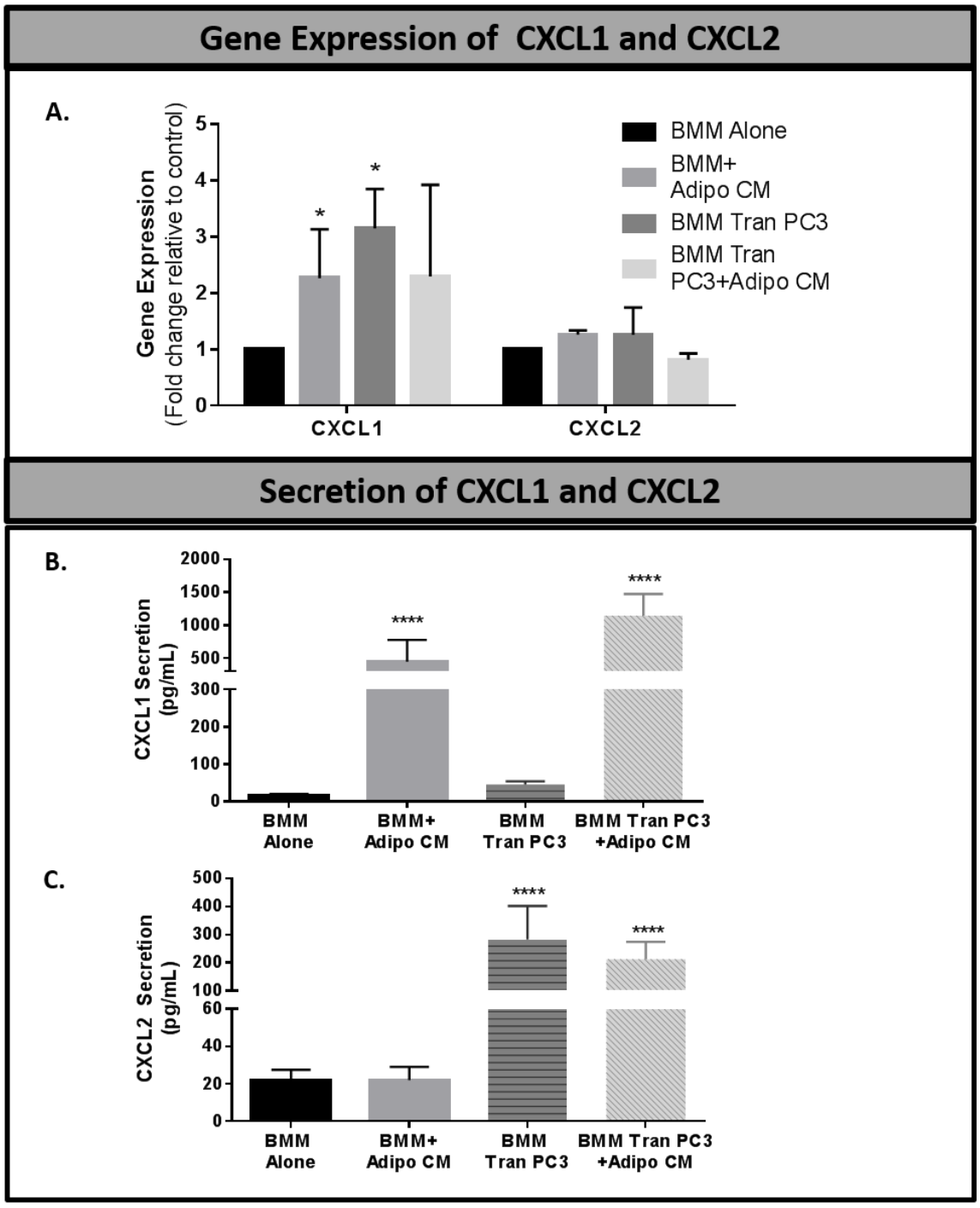


Figure 5.1: Interaction of BMMs with prostate tumor cells leads to upregulation of BMM-derived CXCL1 and CXCL2. BMMs were cultured alone or in a transwell system with PC3 in the absence or presence of Adipo CM. **(A)** Taqman RT-PCR analysis of CXCL1 and CXCL2 expression in BMMs. RT-PCR analysis was normalized to 18S and expressed as fold change relative to control. CXCL1 **(A)** and CXCL2 **(B)** ELISA assay results for BMMs grown in a transwell system with PC3 cells in the absence or presence of Adipo CM. Media samples from duplicate wells/condition were diluted based on DNA concentrations in cell lysates. (****p<0.0001 and *p<0.05 are statistically significant)

5.2.2 CXCL1 and CXCL2 signaling promotes BMM invasion

CXCL1 and CXCL2 are normally secreted by macrophages and serve as potent neutrophil chemoattractants during inflammation and injury [130]. Importantly, both tumor- and stromal supplied CXCL1 and CXCL2 are implicated in tumor progression [130, 252]. Previous studies have shown that astrocytes in the spinal cord secrete CXCL1 in response to increased bone destruction associated with femoral prostate tumor growth, resulting in increased bone pain and overexpression of CXCR2 in neurons [253]. Further evidence showed that ovarian cancer cells overexpressing CXCR2 activate epidermal growth factor receptor-Akt pathway and promote increased migration, invasion, growth, and colony formation in part by activation of CXCL1 and CXCL2-mediated signaling [254].

Therefore, we examined the effects of CXCL1 and CXCL2 signaling in invasion of BMMs towards tumor-derived factors. BMMs were cultured on collagen I and allowed to invade toward PC3 cells in the presence or absence of Adipo CM. BMMs were comparably invasive toward PC3 cells and Adipo CM and their invasive potential was further increased towards PC3 cells cultured in the presence of Adipo CM **(Figure 5.2)**. We next determined if CXCL1 and CXCL2 are directly involved in promoting BMM invasion toward PC3 and adipocyte-derived factors by blocking the activity of these ligands with neutralizing antibodies **(Figure 5.3)**. We observed that blocking either chemokine results in significant reduction of macrophage invasiveness toward either tumor- or adipocyte-supplied factors **(Figure 5.3, bottom left and middle panels)**. Surprisingly, BMM invasion was not significantly affected by CXCL1 and CXCL2 blocking antibodies when PC3 cells were cultured in the presence of Adipo CM **(Figure 5.3, right panel)**.

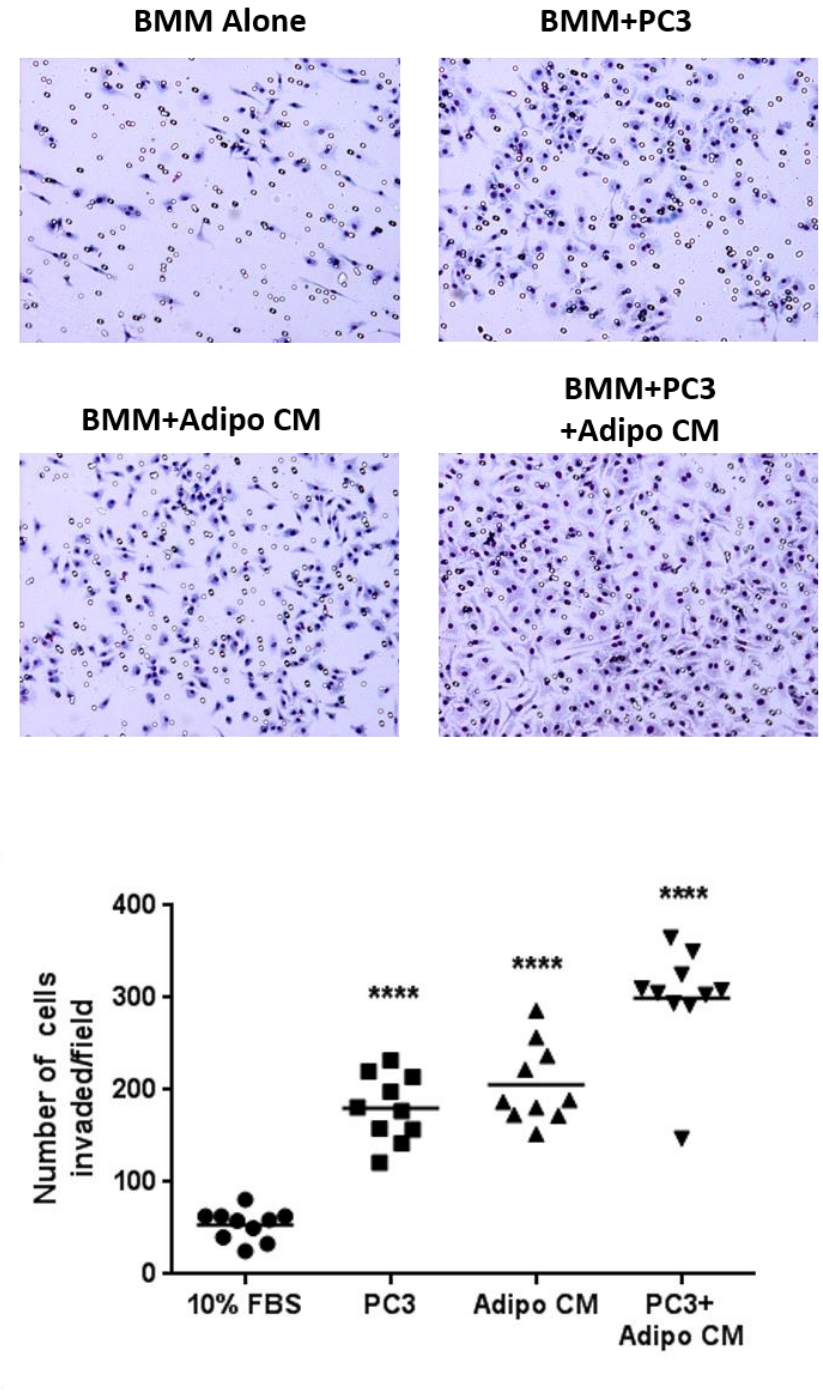
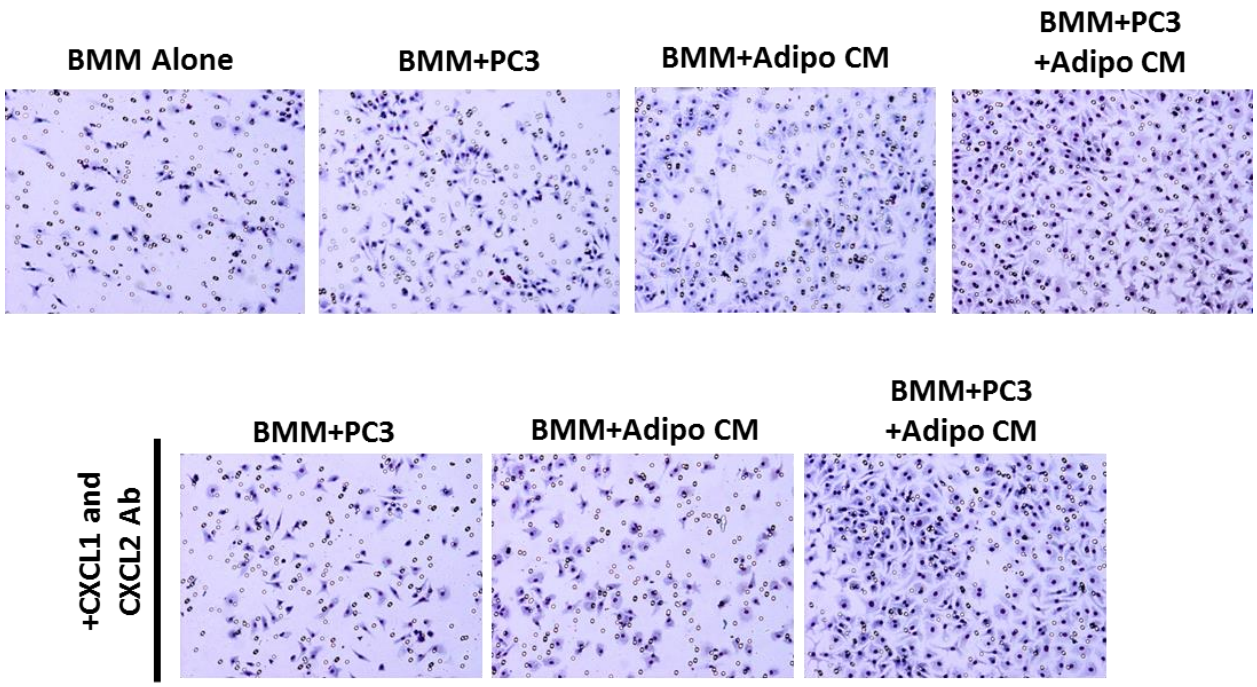


Figure 5.2: Adipocyte- and tumor-derived factors stimulate BMM invasion. BMMs were serum-starved for 4 hours and seeded on collagen I. Cells were allowed to invade toward DMEM containing 10% FBS (control), Adipo CM, PC3 cells, or PC3 cells cultured in Adipo CM for 24 hours. Cells were stained using Diff Kwik Staining kit and 5 representative images were taken under 10X magnification. Cells were counted using ImageJ software and expressed as average number of cells invaded per field are representative of at least 4 experiments. (****$p<0.0001$ considered statistically significant).



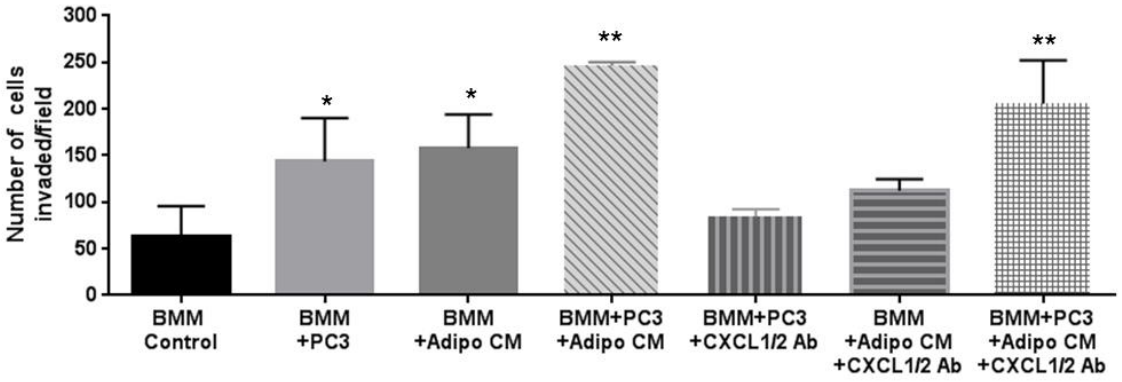


Figure 5.3: Blocking antibodies to CXCL1 and CXCL2 partially inhibits BMM invasion toward adipocyte-and tumor cell factors. BMMs were serum-starved for 4 hours and seeded on collagen I. Cells were allowed to invade toward DMEM containing 10% FBS (control), Adipo CM, PC3 cells, or PC3 cells cultured in Adipo CM in the absence or presence of blocking antibodies to CXCL1 (2µg/mL) and CXCL2 (0.075µg/mL) for 24 hours. Cells were stained using Diff Kwik Staining kit and 5 representative images were taken under 10X magnification. Cells were counted using ImageJ software and expressed as average number of cells invaded per field are representative of at least 3 experiments. (**p<0.01 and *p<0.05 are statistically significant)

This suggests that the combination of PC3 cells and Adipo CM may be inducing the levels of CXCL1 and CXCL2, thus reducing the sensitivity to blocking antibody treatment. Alternatively, prostate tumor cells themselves may be affected by Adipo CM and produce additional factors that induce macrophage invasion independent of CXCL1 and CXCL2 axis.

5.2.3 CXCL1 and CXCL2 promote invasion of prostate cancer cells in the absence of CXCR2

Our data presented thus far clearly indicate that gene expression of CXCL1 and CXCL2 are abundantly present in tibiae of HFD fed mice, especially under conditions of high marrow adiposity. To assess whether in addition to their involvement in osteoclast and macrophage function these chemokines may have any direct effects on tumor cells, we performed invasion assays in the absence or presence of CXCL1 and CXCL2 recombinant proteins. As shown in **Figure 5.4**, both chemokines stimulated invasion of PC3 cells through rBM. This suggests that these ligands may be playing novel roles in tumor progression in bone by regulating both tumor and macrophage invasion in the metastatic niche.

CXCR2, the common receptor shared by CXCL1 and CXCL2, is expressed in a variety of cell types including fibroblasts, smooth muscle cells, and neutrophils [129]. Since CXCL1 and CXCL2 are secreted by macrophages as well as bone marrow adipocytes *in vitro*, and both appear to have an effect on tumor cell invasiveness, we investigated if adipocyte-supplied CXCL1 and CXCL2 affect the expression of CXCR2 in prostate tumor cells. Surprisingly, despite previously reported expression of CXCR2 in prostate cancer cells [146, 255, 256], we observed almost undetectable levels of endogenous CXCR2 in PC3 cells cultured alone or with Adipo CM **(Figure 5.5)**.

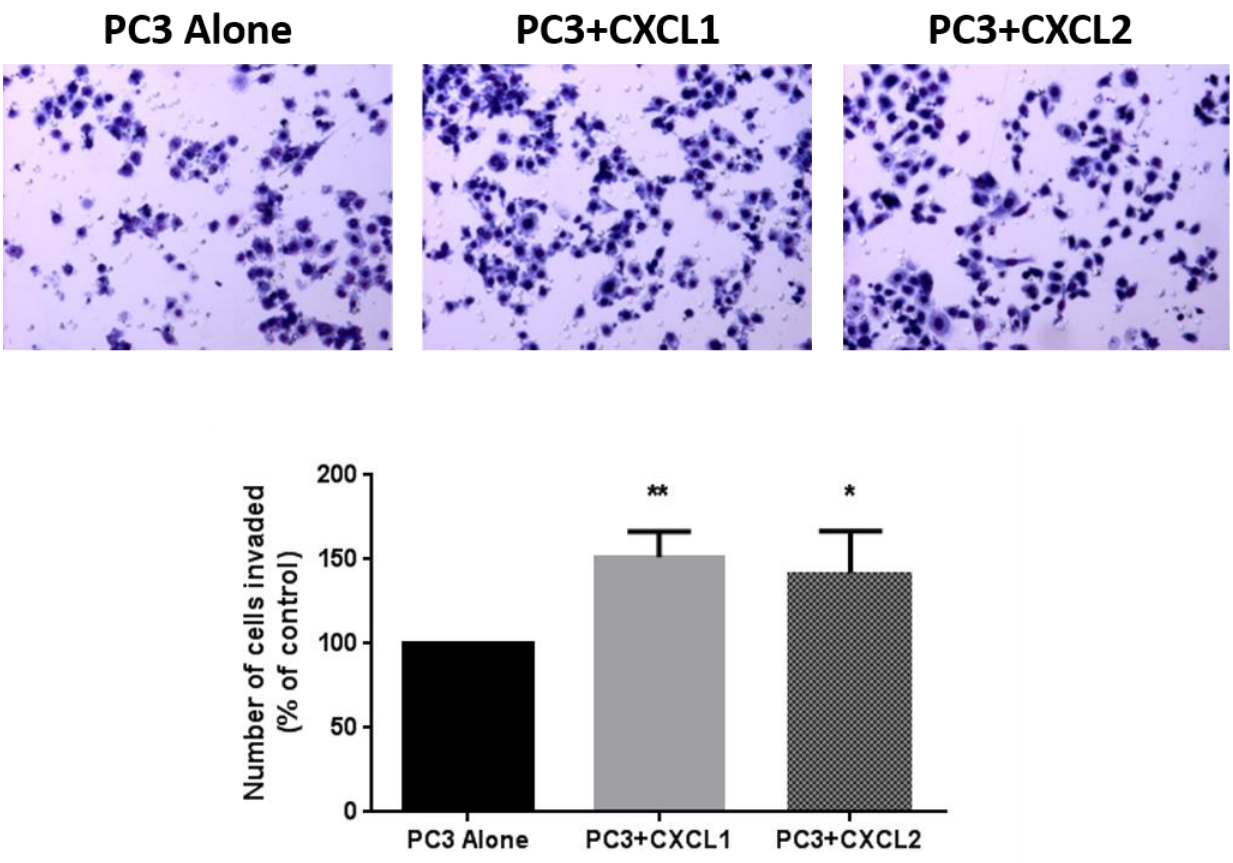


Figure 5.4: Recombinant CXCL1 and CXCL2 proteins stimulate prostate cell invasion through rBM. PC3 cells were serum-starved for 12-18 hours and seeded on reconstituted basement membrane (rBM) and treated with 50ng/mL CXCL1 or CXCL2. Cells were allowed to invade toward DMEM containing 10% FBS for 48 hours. Cells were stained using Diff Kwik Staining kit and 5 representative images were taken under 10X magnification. Cells were counted using ImageJ software and expressed as average number of cells invaded per field are representative of at least 3 experiments.(**$p<0.01$ and *$p<0.05$ are statistically significant)

mRNA Transcripts of CXCR2 in PC3 Cells Treated with Adipo CM

Ct Values	Ct Value 1	Ct Value 2	Ct Value 3	18s Average Ct Values
Sample				
PC3 Alone	**Undetermined**	**Undetermined**	**Undetermined**	10.66136667
PC3+ Adipo CM	**Undetermined**	**Undetermined**	**Undetermined**	10.75483333

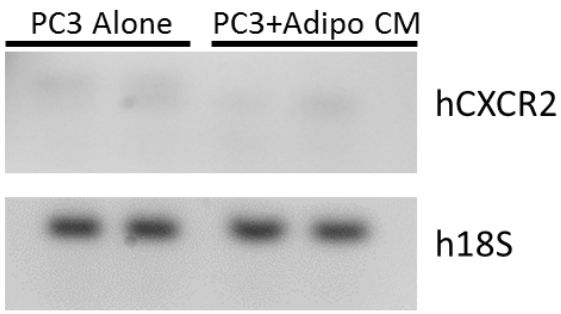


Figure 5.5: CXCR2 receptor is not expressed in PC3 cells. Taqman RT-PCR analysis of CXCR2 expression in PC3 cells treated with Adipo CM for 48 hours. **(Top panel)** C_T values of CXCR2 were undetectable. Data was normalized to 18S. **(Bottom panel)** CXCR2 PCR products were run on a 2% agarose gel. Faint bands were detected indicative of very low expression in PC3 cells in the absence or presence of Adipo CM. PCR data were normalized to 18S and analyzed using DataAssist Software.

Because we did not detect the receptor *in vitro*, we assessed whether PC3 cells may express CXCR2 *in vivo*. As shown in **Figure 5.6** PC3 tumors grown intratibially expressed very low or undetectable transcript levels of human CXCR2, result further confirming our *in vitro* findings that PC3 cells do not express CXCR2. In fact, CXCR2 transcripts were also absent in other prostate cell lines such as C4-2B and DU145 cells in the presence or absence of Adipo CM, in contrast to primary human neutrophils **(Table 5.1)**, which we used as a positive control [257]. These results suggest that CXCL1 and CXCL2 may bind other receptors on prostate tumor cells to promote this invasive behavior. Together, these data highlight the need for further investigation of the endogenous expression of CXCR2 in prostate malignancy in order to better understand how its ligands affect the bone-tumor microenvironment.

5.2.4 BMMs express markers of M2 phenotype in PC3-BMM co-culture

It is well documented that M2 macrophages promote tumor migration, invasion, angiogenesis, and tumor growth [258, 259]. Therefore, we investigated whether the interaction of BMMs with tumor cells *in vitro* and *in vivo* had any effect on their M1/M2 phenotype. Specifically, we assessed gene expression of the M1 marker nitric oxide synthase (NOS2) and M2 markers arginase-1 (ARG1), IL-10, and CD163. PC3 and ARCaP(M) tumor-bearing mice on LFD and HFD expressed high levels of M1 and M2 factors **(Figure 5.7 A, B)**. Most of these genes were further increased in HFD mice compared to LFD with the notable exception of CD163 **(Figure 5.7 B)**. To directly investigate tumor cell effects on phenotypic switch, we assessed the expression of these genes in BMM-PCa co-culture *in vitro* **(Figure 5.8).**

Taqman RT-PCR Analysis of CXCR2 in Various PCa Cells				
Ct Values	Ct Value 1	Ct Value 2	Ct Value 3	18s Average Ct Values
Sample				
LFD PC3 Tumor	37.7434	36.6728	Undetermined	9.02843
HFD PC3 Tumor	35.5911	37.0033	Undetermined	7.434

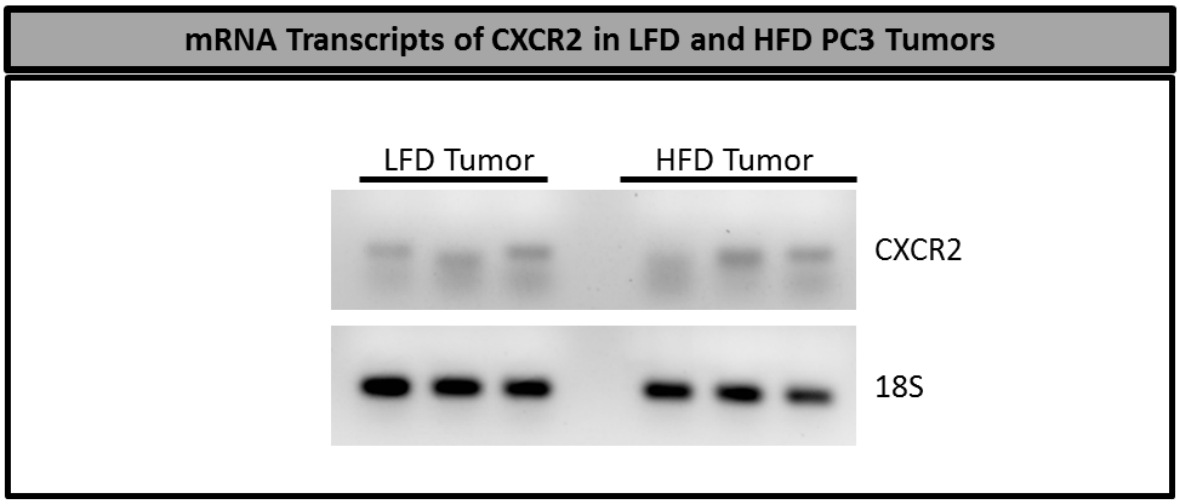


Figure 5.6: CXCR2 expression is very low in PC3 tumor-bearing mice. Taqman RT-PCR analysis of CXCR2 expression in PC3 tumors of tumor-bearing tibiae of HFD and LFD mice. **(Top panel)** C_T values of CXCR2 in PC3 tumors in LFD and HFD mice. Data was normalized to 18S. **(Bottom panel)** CXCR2 PCR products were run on a 2% agarose gel. Low mRNA amounts of products were detected indicative of very low expression in PC3 tumors *in vivo*.

Taqman RT-PCR Analysis of CXCR2 in Various PCa Cells				
Ct Values	**Ct Value 1**	**Ct Value 2**	**Ct Value 3**	**18s Average Ct Values**
Sample				
C42B Alone	**Undetermined**	36.6221	35.6081	11.49876667
C42B+Adipo CM	36.2022	37.0815	36.9907	12.23756667
DU145 Alone	**Undetermined**	**Undetermined**	36.2442	11.1798
DU145+Adipo CM	**Undetermined**	**Undetermined**	**Undetermined**	10.81893333
Human Neutrophils	27.1974	26.9467	26.5563	10.8367
Human Neutrophils	32.5911	32.4225	31.4764	11.09746667

Table 5.1: CXCR2 expression is very low or undetectable in metastatic prostate cancer cell lines. Taqman RT-PCR analysis of CXCR2 expression in C4-2B and DU145 grown in control conditions or in the presence of Adipo CM. cDNA isolated from primary human neutrophils was used as a positive control. All prostate tumor cell lines expressed very low or undetectable levels of CXCR2 mRNA transcripts. Unstimulated human neutrophils expressed CXCR2 confirming the specificity of the probes used. 18S was used as a loading control

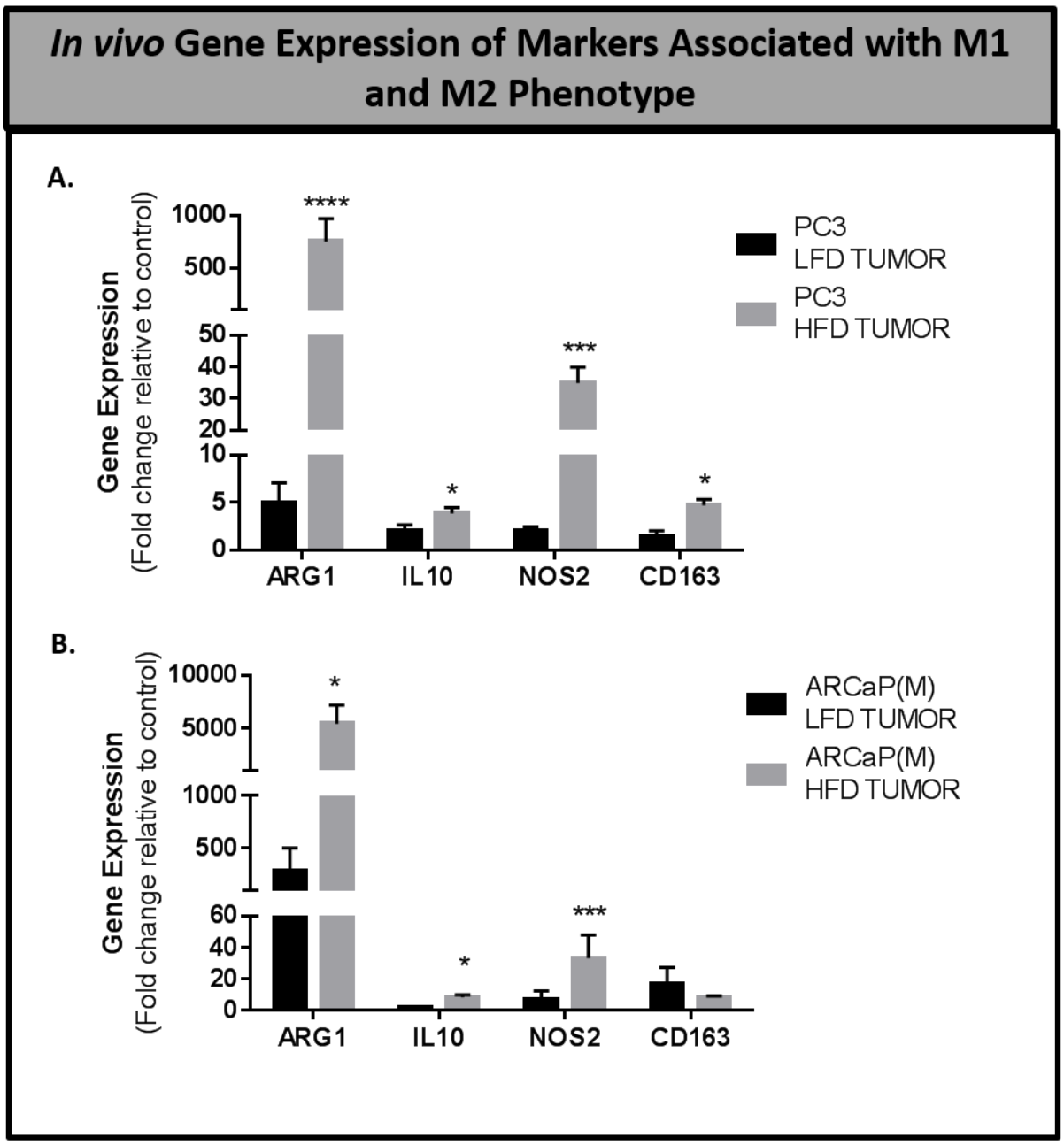


Figure 5.7: Host expression of markers associated with M1 and M2 phenotype are increased in tumor-bearing mice fed HFD. Taqman RT-PCR analyses of host-derived M1 marker nitric oxide synthase-2 (NOS2) and M2 markers IL-10, arginase-1 (ARG1), and CD163 in PC3 **(A)** and ARCaP(M) **(B)** tumor-bearing tibia of mice on low fat diet (LFD) and high fat diet (HFD). Both M1 and M2 markers are increased in PC3 and ARCaP(M) tumor tibiae and these effects are more pronounced in bone containing increased numbers of adipocytes. PCR data were normalized to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and expressed as fold change relative to PBS control bones. (****$p<0.0001$; ***$p<0.001$; and *$p<0.05$ are statistically significant)

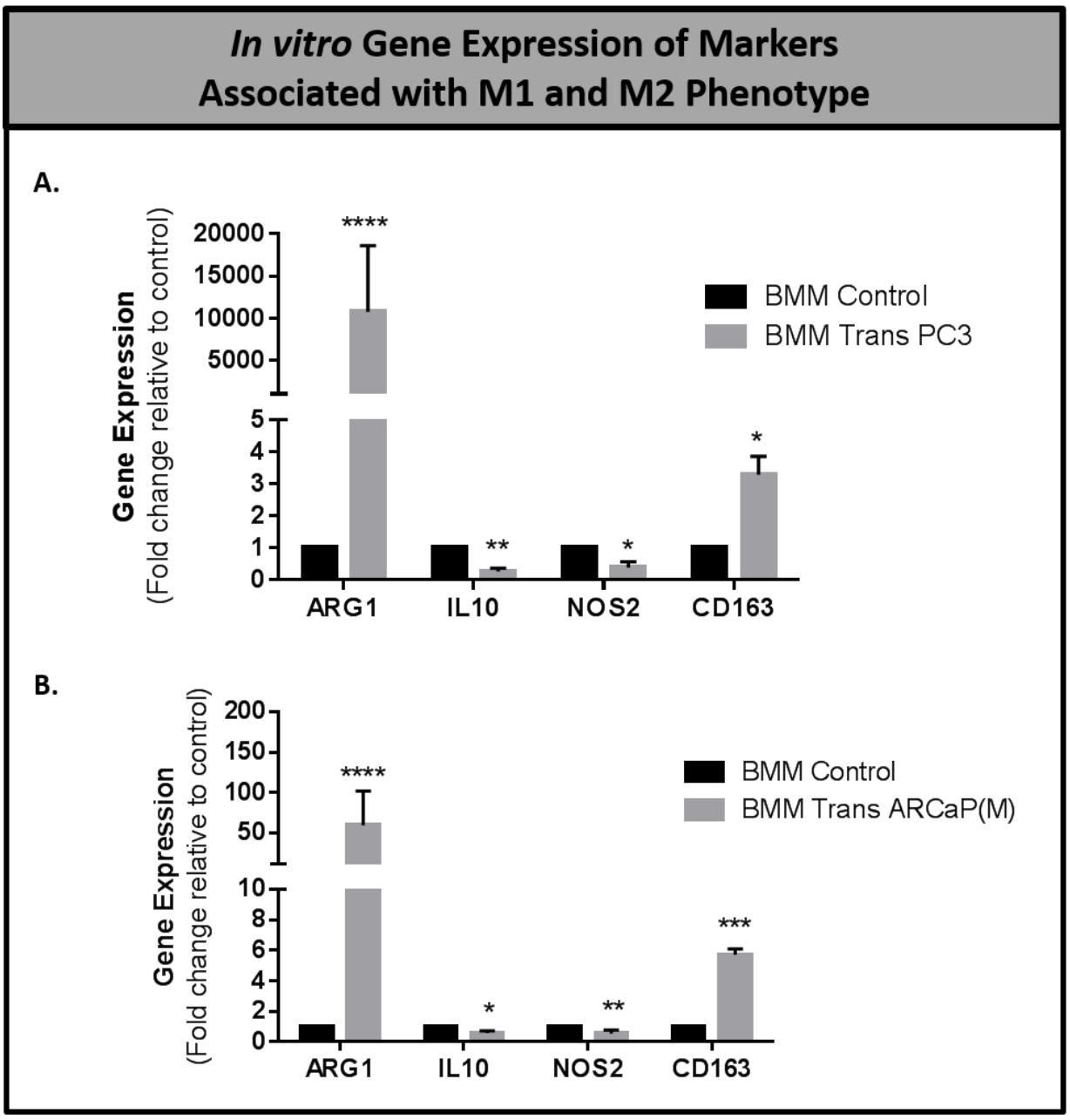


Figure 5.8: BMMs have increased expression of markers associated with M1 and M2 phenotype in PC3-BMM co-culture. Taqman RT-PCR analyses of BMM-derived M1 marker nitric oxide synthase-2 (NOS2) and M2 markers IL-10, arginase-1 (ARG1), and CD163 in co-culture with PC3 **(A)** and ARCaP(M) **(B)** cells. PCR data were normalized to control and are representative of at least three biological replicates. (****p<0.0001; ***p<0.001; **p<0.01 and *p<0.05 are statistically significant

Interestingly, BMMs cultured with PC3 and ARCaP(M) cells expressed significant amounts of ARG1, result in line with our *in vivo* findings. The M1 marker NOS2 was significantly reduced, suggesting that paracrine signals from tumor cells may stimulate BMMs to reduce pro-inflammatory stimuli. The expression of M2 marker CD163 was enhanced in BMMs interacting with tumor cells while surprisingly IL-10 cytokine expression was reduced. We further investigated why IL-10 expression may be reduced by assessing changes in expression of OPN, a regulator of macrophage behavior and phenotype as well as a potent negative regulator of IL-10 [151, 260]. As shown in **Figure 5.9**, OPN is increased both at the gene **(Figure 5.9 A)** and protein **(Figure 5.9 B)** levels in BMMs cultured with PCa cells and these levels are maintained in the presence of Adipo CM. Although we saw trends towards M2 macrophage phenotypic switch in our co-culture system, more directed studies are needed to verify these findings.

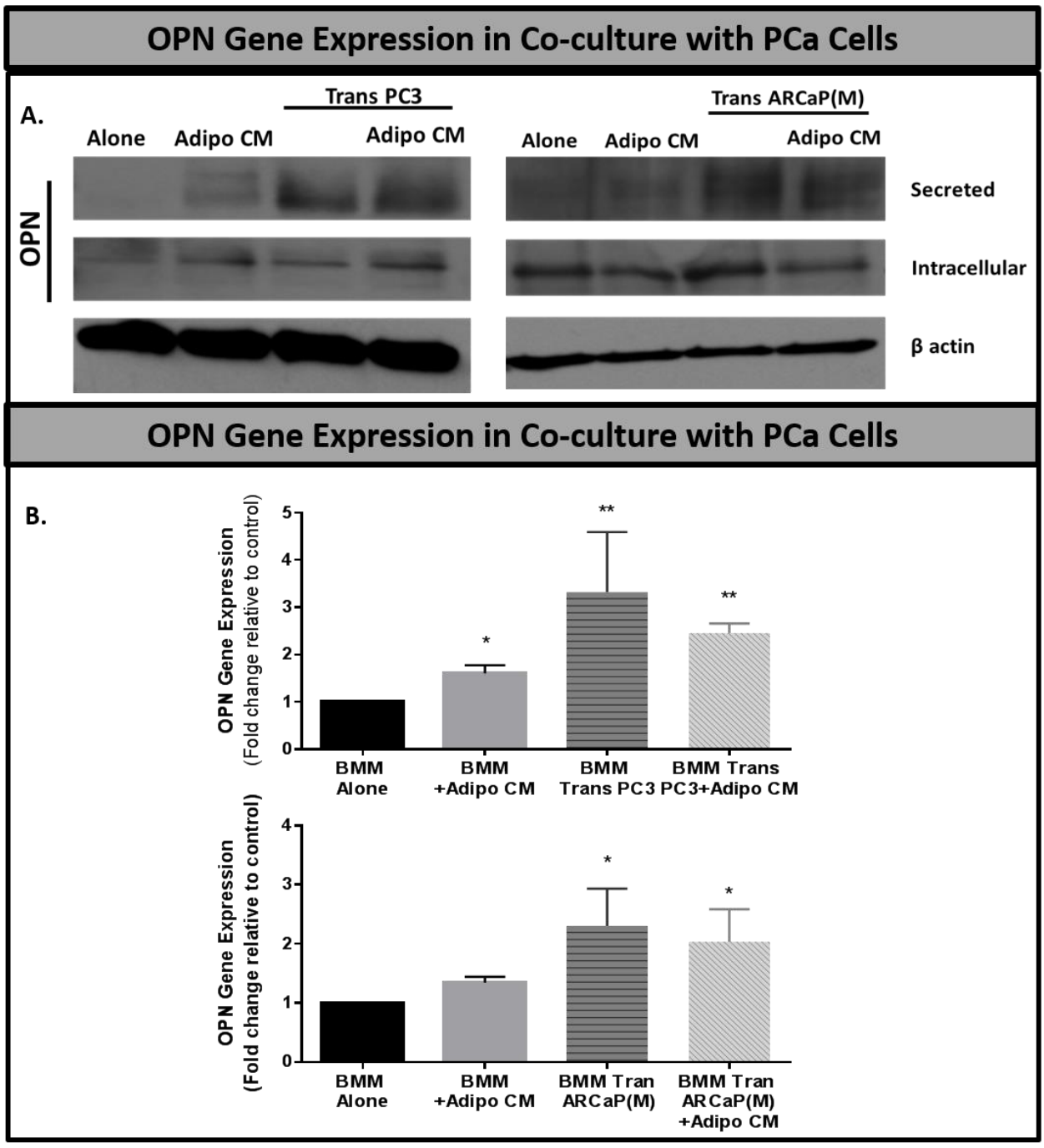


Figure 5.9: Adipocyte- and tumor-derived factors stimulate osteopontin expression and secretion in BMMs. (A) Western blot analyses were used to determine changes in intracellular and secreted levels of osteopontin in BMMs in Transwell with PC3 **(left panel)** and ARCaP(M) **(right panel)** prostate tumor cells. β actin was used as a loading control. **(B)** Taqman RT-PCR analysis (Life Technologies) of osteopontin in BMMs co-cultured with Adipo CM and PC3 cells **(top panel)** or ARCaP(M) cells **(bottom panel)** and normalized to 18S and represented as fold change relative to control. (**p<0.01 and *p<0.05 are statistically significant)

5.3 Discussion

Chronic inflammation is a driving force that promotes tumor progression and aggressiveness in several cancers and the various ways cancer cells hijack the immune system have been an area of intense study [196]. Sustained inflammation has been attributed to reduced metabolism resulting in delayed clearance of highly toxic chemotherapy agents and cytotoxicity in normal tissue [261]. We and others have shown that tumor-driven inflammation in bone is particularly detrimental, often leading to altered bone homeostasis, bone loss, and pain [262, 263]. Moreover, studies suggested that obesity perpetuates an inflammatory tumor microenvironment by enhancing nuclear factor kappa b (NFκB) activity in circulating mononuclear cells. Specifically, increased circulation of free fatty acids reduced the expression of IκB, an inhibitory protein of NFκB, in circulating mononuclear cells and this over activity resulted in high circulating levels of NFκB target factors, such as migration inhibitor factor (MIF), IL-6, TNF-α, and matrix metalloproteinase-9 [264]. Although the effects of systemic inflammation on bone remodelling have been well characterized, its roles in tumor growth in bone and the resulting changes in adipocyte-driven inflammation in bone have not been fully explored.

Macrophages are a major source of CXCL1 and CXCL2 [265], and limited reports have suggested that both chemokines are associated with tumorigenesis [239, 266]. Therefore, the present study investigated the role of macrophage-driven inflammation in tumor behavior and the effects of this interaction on macrophage phenotype. In chapter 4, we demonstrated that CXCL1 and CXCL2 were highly increased in tumor-bearing tibiae of mice fed a high fat diet compared to more modest changes in LFD mice. We also showed that bone marrow adipocytes

secrete CXCL1 and CXCL2 to accelerate bone remodeling. This is in line with our previous findings demonstrating that inflammation is clearly enhanced in the bone tumor microenvironment with increased marrow adiposity [43]. In the present study, we assessed the effects of adipocyte-derived factors on macrophage-driven inflammation in metastatic bone disease. Here we show specifically that BMMs are a source of CXCL1 and CXCL2 whose secretion is enhanced in the presence of adipocyte- and PCa-derived factors, and the origin of the stimuli determines how each chemokine is expressed. CXCL1 is highly secreted when BMMs are cultured with bone marrow adipocyte-supplied factors compared to CXCL2-stimulated expression primarily in BMM-PC3 co-culture conditions (Figure 5.1 B). This suggests that although CXCL1 and CXCL2 share similar amino acid sequences, they may have different functions in macrophage-driven inflammation in the bone tumor microenvironment.

The interactions between tumor cells and the surrounding stroma have received increased attention as possible prognostic indicators of reduced survival. Clinical and *in vitro* studies showed that macrophage infiltration into breast tumors results in increased disease recurrence, mammosphere formation, tumor migration, and invasion demonstrating that macrophages provide factors that aid in malignant progression [267, 268]. Further evidence suggests that tumor cells attract macrophages by secreting a number of chemotactic factors, namely monocyte chemotactic protein-1 (MCP-1), and monocyte colony stimulating factor (M-CSF) [68]. Interestingly, adipocytes further promote these effects when they directly interact with tumor cells and initiate homing of macrophages to adipocyte-rich areas of the surrounding stroma [269]. In the present study, we demonstrated that bone marrow macrophages are more invasive toward factors secreted by prostate tumor cells and adipocytes and this invasive

behavior is even more pronounced when tumor cells are treated with Adipo CM (Figure 5.2), effects that are blocked with neutralizing antibodies to CXCL1 and CXCL2 (Figure 5.3). Notably, we observed no significant reduction in BMM invasion toward PC3 cells and Adipo CM in the presence of CXCL1 and CXCL2 neutralizing antibodies, suggesting that other factors in addition to CXCL1 and CXCL2 may stimulate these effects. These results provide evidence that adipocytes, macrophages, and tumor cells work in concert to fuel tumor-associated inflammation.

As previously mentioned, CXCL1 and CXCL2 have inflammatory as well as pro-tumorigenic effects. CXCL1 is highly expressed in stromal fibroblasts surrounding breast tumors and its overexpression is associated with increased tumor grade and overall poor prognosis [270]. CXCL2 has been implicated as a survival factor secreted by primary bone marrow mononuclear cells that stimulates chronic inflammation and protects lymphocytic leukemia cells against apoptosis *in vitro* [271]. Interestingly, levels of CXCL1 and CXCL2 have been reported to be highly enhanced in prostate stromal cells when stimulated with factors secreted by newly transformed prostate epithelial cells, particularly Il-1β [243]. *In vitro* and *in vivo* models of prostate cancer reported that CXCL1 and CXCL2 are involved in prostate tumor migration and invasion [252, 272, 273]. However, contradictory to work previously published by others [146, 255], we were unable to detect any significant mRNA levels of CXCR2 in any of the tested prostate cancer cells both *in vitro* and *in vivo* (Figures 5.5-5.6 and Table 5.1). Notably, our results appear to be in line with findings from a breast cancer model demonstrating that breast cancer cells express and utilize CXCL1 and CXCL2 in tumor progression but have very low transcript levels of CXCR2 [131, 274]. Together, this suggests that CXCL1 and CXCL2 may

stimulate invasion by paracrine interactions with cell in the tumor microenvironment rather than direct effects on tumor cells.

It is noteworthy that cells in the bone tumor microenvironment are known to be versatile in response to changes in environmental stimuli, specifically macrophages [275, 276]. Phenotypic changes are a common occurrence when macrophages interact with tumor cells, as a downstream effect of altered expression of proteases, surface receptors, and cytokines [74]. It is reasonable to speculate that monocytes may be induced by CXCL1 and CXCL2 supplied by macrophages and adipocytes to differentiate into M2 macrophages that produce OPN to aid in tumor progression [277] and bone remodeling [278]. This suggests that the roles and M1 and M2 macrophage phenotypes of are more complex than previously thought.

Nonetheless, the traditionally recognized function of these macrophage subtypes in malignancy has remained fairly consistent. An *in vivo* model of glioma was used to demonstrate a disproportionate influx of M2 macrophages around and in the tumor and depletion of macrophages using the macrophage Fas-induced apoptosis (MAFIA) transgenic mouse model, showed greatly reduced tumor growth and angiogenesis [279]. This provides direct evidence for negative impact of M2 macrophages on tumor promotion. Others have shown that hyaluronan (HA), a matrix component found in most tissues such bone and breast, binds CD44 receptor on resting monocytes and drives their differentiation to an M2 phenotype while inducing apoptosis in M1 macrophages [280]. Clinical evidence has since followed, directly correlating M2 accumulation in breast tumors with levels of hyaluronan [281]. Many more studies have also linked M2 accumulation with poor disease outcome [282, 283]. In the present

study we investigated genes associated with M1 (NOS2) and M2 (CD163, ARG1, IL-10) and showed that most of these genes were upregulated in tumor-bearing mice, particularly those on HFD (Figure 5.7). Furthermore, we demonstrated that PC3 cells cultured with BMMs *in vitro* tended to shift the marker expression in BMMs to a more M2 phenotype with the exception of reduced IL-10 expression (Figure 5.8). We attributed this reduction in IL-10 levels to increased expression of OPN, a modulator of macrophage inflammation and phenotype [163, 284], and a negative regulator of IL-10 [151, 260] (Figure 5.11). Further studies are needed to determine if BMM-supplied osteopontin directly contributes to macrophage phenotypic switch.

With the growing attention on the detrimental roles of macrophages in tumorigenesis, numerous clinical trials are being conducted to investigate the effects of M1 versus M2 phenotype in disease prognosis and ways they can be targeted therapeutically. Notably, an anti-macrophage migration inhibitory factor (MIF) antibody is currently being tested in a phase I clinical trial in the treatment of solid tumors, particularly colon and rectal cancers, as the means of reducing macrophage recruitment and preventing tumor growth [270]. Results of another recently completed clinical trial examining the effects of administering recombinant GM-CSF to newly diagnosed prostate cancer patients before radical prostatectomy in an attempt to boost M1 macrophage activation and tumor-killing ability are currently pending [285]. If these therapies successfully promote M1 activation at the tumor site, it would be interesting to determine if levels of CXCL1 and CXCL2 are reduced with the presence of more M1 macrophages in the tumor milieu and how it may disrupt tumor growth, particularly in bone. Based on previous reports and evidence provided herein, therapies targeting inflammation may be a key element that slows tumor growth and aggressiveness in bone.

CHAPTER 6: BONE MARROW MACROPHAGE-DERIVED OPN PROMOTES PROSTATE TUMOR INVASION AND SURVIVAL IN BONE

6.1 Introduction

Metastatic bone disease is devastating and incurable, often causing bone pain, fracture, and reduced quality of life [211]. Patients with advanced prostate cancer commonly develop drug resistance and 85–100% of patients who die of prostate carcinoma have some evidence of bone metastases [286, 287]. Importantly, many complications associated with metastatic disease arise because of underlying inflammation, which is known to aid in altered bone remodeling, tumor colonization and invasion [66]. One cell type that mediates this inflammation in bone are marrow macrophages, which are capable of secreting both pro- and anti-inflammatory factors [288], recruiting tumor cells to the bone, and regulating the adaptive immune response [289]. Because macrophages play central roles in inflammation, it is not surprising that they may be a prime target of tumor cells to aid in progression, immune evasion, and tolerance [289, 290].

The interactions between macrophages and tumor cells are very widely studied, highlighting the importance of the stroma in malignancy [232, 237, 291]. For example, macrophage colony-stimulating factor 1 (M-CSF1) is a key factor that promotes monocyte recruitment and proliferation [292]. Previous studies have demonstrated that prostate cancer cells treated with androgen deprivation therapy express high levels of M-CSF1 leading to increased infiltration of macrophages into the tumor and eventual recurrence and these effects are reduced in the presence of the CSF1 receptor inhibitor PLX3397 [82]. Another study demonstrated that PC3 cells treated with docetaxel and co-cultured with macrophages showed

increased production of several host-derived chemokines including IL-4, IL-6, and macrophage inhibitory cytokine 1, all strongly correlated with progressive disease and reduced overall survival in prostate cancer patients [293]. However, although there have been numerous reports demonstrating the important role of macrophages in tumor recurrence and growth, few studies to date have investigated the roles of bone marrow macrophages in tumor progression in bone.

Several cell types in bone secrete osteopontin (OPN), a glycosylated bone protein belonging to the SIBLING (small integrin-binding ligand N-linked glycoproteins) family and involved in bone remodeling, insulin resistance, and inflammation (reviewed in [149]). Osteopontin is particularly important in tumor-driven inflammation because it promotes monocyte migration and regulates COX-2 and MCP-1 expression in tumor-associated macrophages [156, 294]. OPN is also expressed in tumor cells and studies have suggested that alternatively spliced isoforms OPNb and OPNc may have different functions in disease progression of the breast, glioma, lung cancer, and prostate cancers [159]. OPN binds to various receptors including αVβ1, 3, and 5, CD44 as well as its splice variants CD44v3 and v6 [295]. Recent attention has focused on the expression and roles of CD44v6 expression and signaling in tumor invasion. CD44v6 expression is upregulated in ovarian cancer and drives tumor adhesion, migration and invasion [296]. CD44 and CD44v6 expression were both correlated with reduced patient survival in head and neck cancers [297]. αVβ3 expression has also been linked to tumor progression because it plays a role in stimulating angiogenesis, invasion and metastasis in solid tumors [298, 299]. Studies have investigated osteopontin signaling in normal inflammatory and bone remodeling events [151]; however, the role of

osteopontin in metastatic prostate cancer growth and progression in bone is not fully understood.

We previously showed that osteopontin secretion is enhanced in macrophages cultured with PCa cells (Figure 5.11). Therefore, in this part of our studies we investigated the role of bone marrow macrophage-derived osteopontin, in tumor invasion and downstream signaling through its receptors CD44, CD44v6, and αVβ3. Utilizing previously reported meta-analyses of OPN gene transcript available in the Oncomine data bank, we show that OPN expression is increased in metastatic prostate cancer tissues compared to primary prostate tissues. Using an *in vivo* model of prostate tumor growth in bone, we show that OPN expression is increased in PC3 and ARCaP(M) tumor-bearing mice and these levels are further augmented in mice fed HFD. Utilizing *in vitro* Transwell co-culture system of PCa cells with BMMs, we also show that BMMs express and secrete OPN in response to tumor cells. We further demonstrate that BMM-derived factors, including osteopontin, promote prostate tumor invasion and blocking the receptors CD44v6 and αVβ3 effectively inhibits tumor invasion toward BMMs. We also provide evidence suggesting that invasive and proliferative effects observed are, in part, mediated by activation of Akt pathway, and upregulation of anti-apoptotic factors Bcl2, Bcl-xL, and survivin. We demonstrate that BMMs induce a hypoxic response in tumor cells as shown by increased expression of hypoxia markers carbonic anhydrase IX and VEGF as well as ER stress factors BiP and XBP-1, which are known mediators of stress response pathways associated with tumor cell survival and disease progression [300, 301]. Collectively, our results highlight the importance of tumor associated macrophages in metastatic disease and reveal a new mechanism of OPN involvement in tumor survival and ER stress.

6.2 Results

6.2.1 OPN is overexpressed in metastatic prostate cancer patients

Clinical evidence has suggested that gastric and lung cancer patients that express high levels of OPN have a higher rate of disease recurrence and reduced overall survival following chemotherapy or surgical resection, respectively [302, 303]. To determine if OPN expression is upregulated with disease progression in prostate cancer, we searched the Oncomine™ Gene Browser for changes in mRNA expression between patients with localized versus metastatic prostate cancer. Our analysis revealed that PCa patients with metastatic disease have over 70-fold increase in osteopontin levels based on the Ramaswamy *et al* gene expression dataset [304] **(Figure 6.1 A)** and over 12-fold increase based on Yu *et al* dataset [305] **(Figure 6.1 B)**.

6.2.2 Host bone OPN expression is increased *in vivo* in tumor-bearing mice

Inflammation is one of the driving stimuli that promote tumor invasion and growth. Previous studies have clearly demonstrated that tumor-associated macrophages secrete significant levels of OPN at the tumor-stromal interface to promote progression of various malignancies such as colon, pancreas, breast, lung, prostate, melanoma, and ovarian cancers [306]. Therefore, we sought to determine if OPN expression is altered during prostate tumor growth in bone. PC3 and ARCaP(M) tumors were established in bone by intratibial injection and host OPN was assessed by Taqman RT-PCR analyses. Gene transcripts of OPN were significantly elevated in both PC3- and ARCaP(M) tumor-bearing tibiae compared to PBS control (4.77 fold increase in PC3 tumors and 2.48 fold increase for ARCaP(M) tumors) **(Figure 6.2 A, B)**.

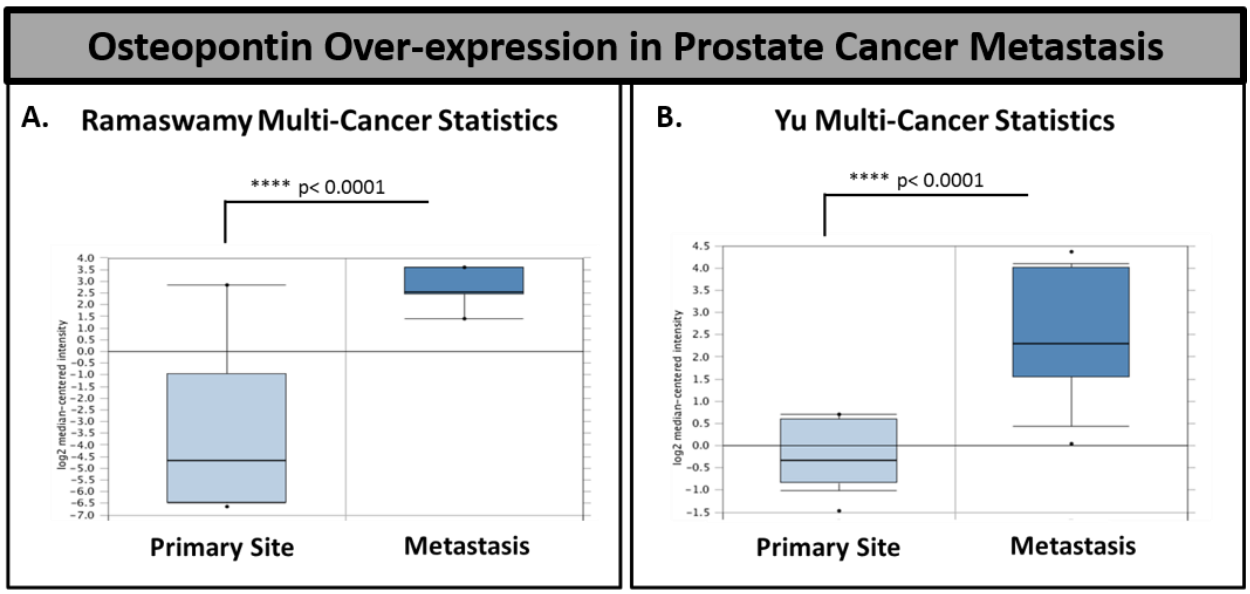


Figure 6.1: Osteopontin expression is increased in metastatic prostate cancer. (A) Box and Whisker plot of Oncomine data analysis of osteopontin mRNA transcript levels in primary versus metastatic prostate cancer in Ramaswamy prostate. There was a 75.095-fold increase in gene transcripts of OPN in metastatic tissues compared to localized prostate tissue. Primary tissue samples n=10 and metastatic tissues n=4. **(B)** Oncomine data analysis of osteopontin in Yu prostate. There was a 12.185-fold increase in gene transcripts of OPN in metastatic tissues compared to localized prostate tissue. mRNA transcript expression was expressed as $\log_2$ median-centered intensity. (**** p< 0.0001 considered statistically significant)

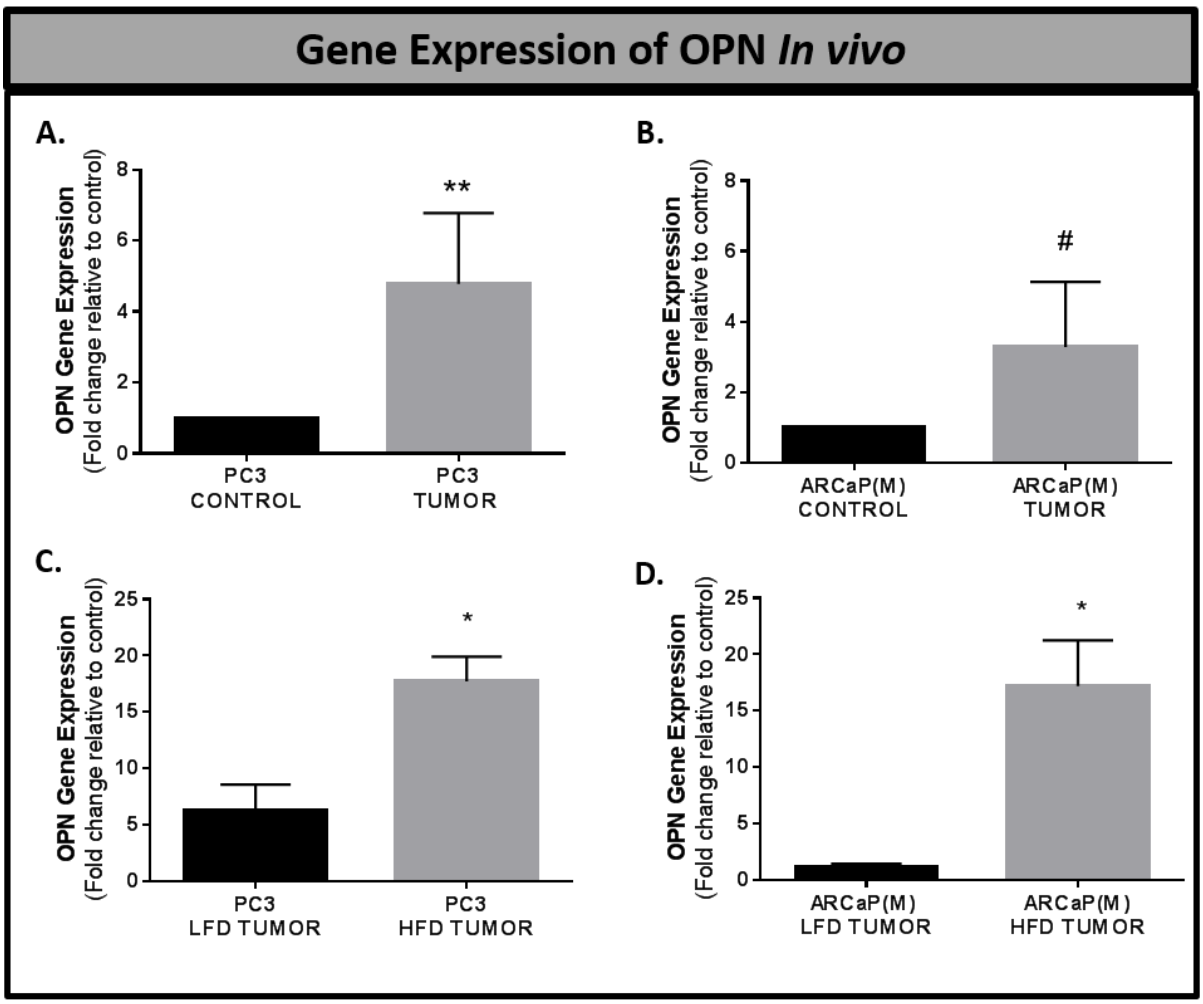


Figure 6.2: Host osteopontin expression is increased in prostate tumor-bearing tibiae. Taqman RT-PCR analyses of murine osteopontin expression in PC3 **(A, C)** and ARCaP(M) **(B, D)** tumor-bearing tibia of mice on low fat diet (LFD) and high fat diet (HFD). Osteopontin expression is increased in PC3 and ARCaP(M) tumor tibiae **(A, B)** and these effects are more pronounced in HFD fed mice **(C, D)**. PCR data were normalized to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and expressed as fold change relative to PBS control bones. (**p<0.01 and *p<0.05 are statistically significant #0.1971)

Interestingly, increased OPN expression in macrophages has been implicated in adipocyte-driven inflammation, promoting insulin resistance [156]. We observed that tumor-bearing mice fed a high fat diet expressed significantly more OPN than low fat diet-fed mice **(Figure 6.2 C, D)**.

6.2.3 OPN expression is increased in macrophages cultured with prostate cancer cells

Several cell types in bone express OPN in addition to macrophages and they include osteoclasts, osteoblasts, endothelial cells and natural killer cells [307]. To investigate the changes in tumor-stimulated secretion of macrophage osteopontin, PC3 and ARCaP(M) cells were co-cultured with BMMs. As shown in **Figure 6.3**, gene expression of OPN is enhanced in macrophages in response to paracrine interactions with PCa cells **(Figure 6.3 A, B)**. Since OPN is largely a secreted protein, we assessed the protein expression of this glycoprotein in lysates and media from BMM-PCa co-cultures. Mirroring RT-PCR analyses, osteopontin was robustly secreted by BMMs co-cultured with PC3 and ARCaP(M) while intracellular levels were largely unchanged **(Figure 6.3 C)**. These data suggest that prostate tumor cells promote the production of BMM-supplied osteopontin to drive inflammatory events in the bone marrow milieu.

6.2.4 CD44 expression is altered in prostate cancer cell lines

OPN can bind to several integrin receptors to mediate its downstream effects; however, its primary receptors are CD44, CD44v6, and αVβ3 [308]. Importantly, studies have suggested that increased CD44 expression in PC3 and DU145 cell lines is a marker of a stem-like

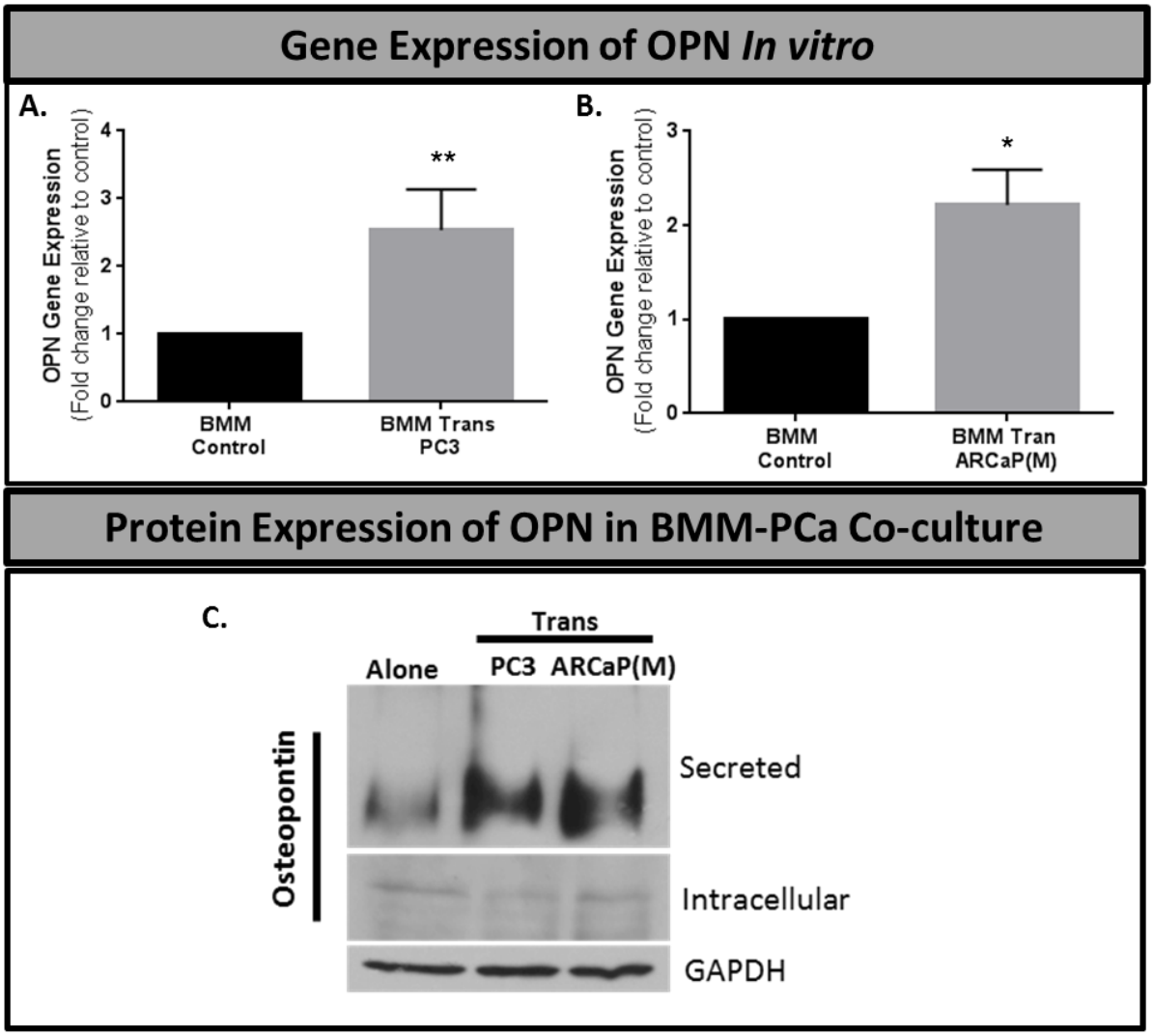


Figure 6.3: Tumor-derived factors stimulate osteopontin expression and secretion in BMMs. **(A)** Taqman RT-PCR analysis (Life Technologies) of osteopontin in BMMs co-cultured with PC3 cells **(A)** or ARCaP(M) cells **(B).** PCR data were normalized to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and expressed as fold change relative to control. **(C)** Western blot analyses were used to determine changes in intracellular and secreted levels of osteopontin in BMMs co-cultured with PC3 and ARCaP(M) prostate tumor cells. GAPDH was used as a loading control. (**p<0.01 and *p<0.05 are statistically significant)

phenotype *in vitro* [309, 310]. Therefore, we examined changes in these receptors in tumor cells co-cultured with BMMs **(Figure 6.4)**. Protein expression of CD44 was increased in both PC3 and DU145 cells cultured with BMMs while levels of this receptor were reduced in ARCaP(M) cells **(Figure 6.4 A)**. Notably, endogenous, baseline protein expression of CD44 was higher in ARCaP(M) cells compared to DU145 and PC3 cells, suggesting that the decline in expression upon ligand stimulation might be a result of saturation and receptor degradation. αVβ3 and CD44v6 protein expression remained unchanged; however, we observed that all three PCa cell lines expressed high levels of both receptors even in the absence of BMMs **(Figure 6.4 B, C)**. We also assessed levels of CD44v6 and αVβ3 expression in tumor cells treated with recombinant osteopontin protein by flow cytometry and we observed no significant differences **(Figure 6.5)**. Immunocytochemical analysis of CD44v6 also demonstrated that PC3 cells cultured with BMMs do not result in recycling or cytoplasmic accumulation of the receptor **(Figure 6.6)**.

6.2.5 Osteopontin promotes prostate tumor invasion

Tumor-derived OPN has been implicated as a driving factor of invasion and growth of cancers of the breast, liver, lung, stomach, prostate, and colon [311] and more recently oral squamous cell carcinoma [312]. Additional evidence has suggested that macrophage-secreted OPN may be a negative predictive factor of survival in non-small cell lung cancer [313].

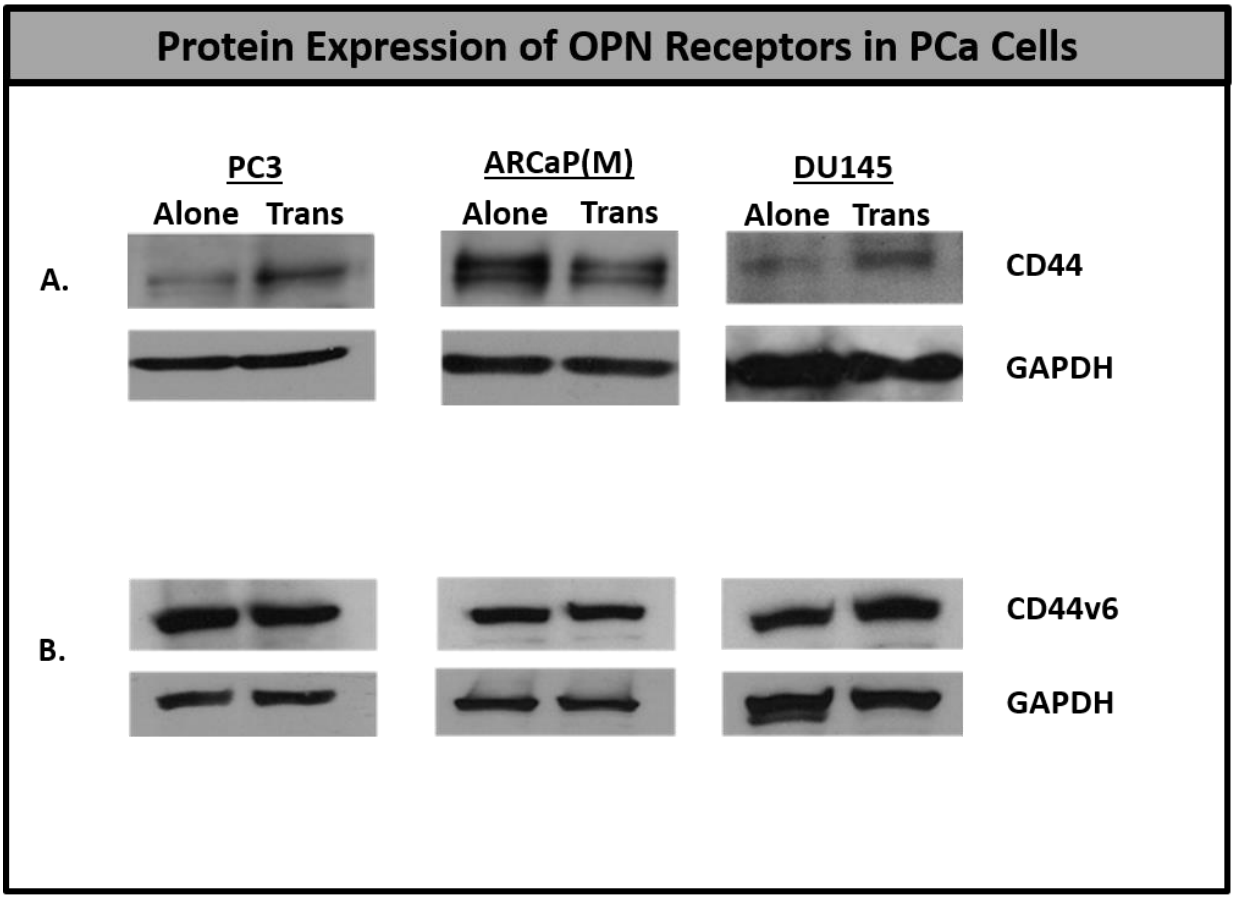
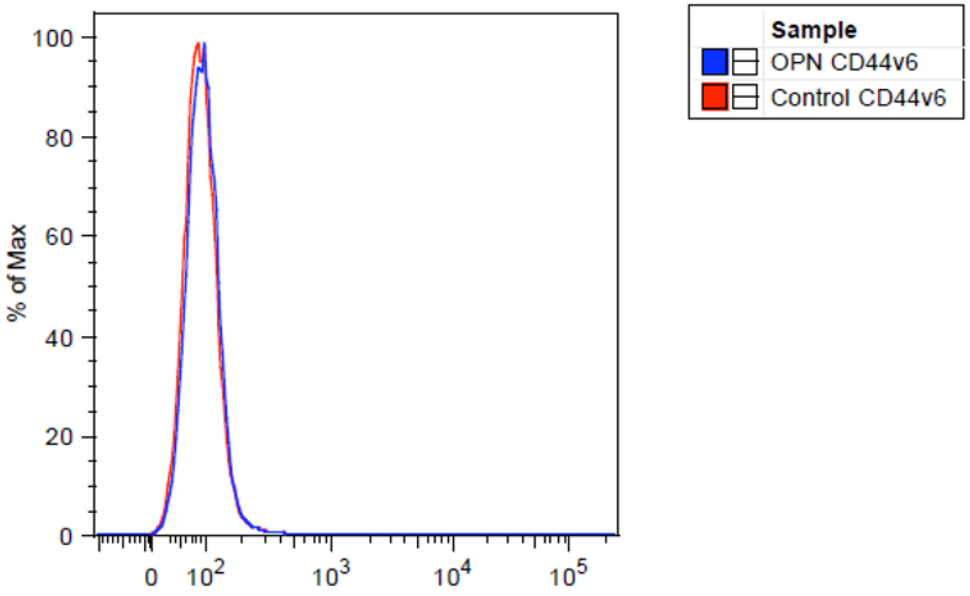


Figure 6.4: Protein expression of osteopontin receptors CD44, CD44v6, and in PC3, ARCaP(M), and DU145 cells. Immunoblot analyses of **(A)** CD44 and **(B)** CD44v6 protein expression in PC3, ARCaP(M), and DU145 prostate tumor cells co-cultured with BMMs. GAPDH was used as a loading control.

A.



B.

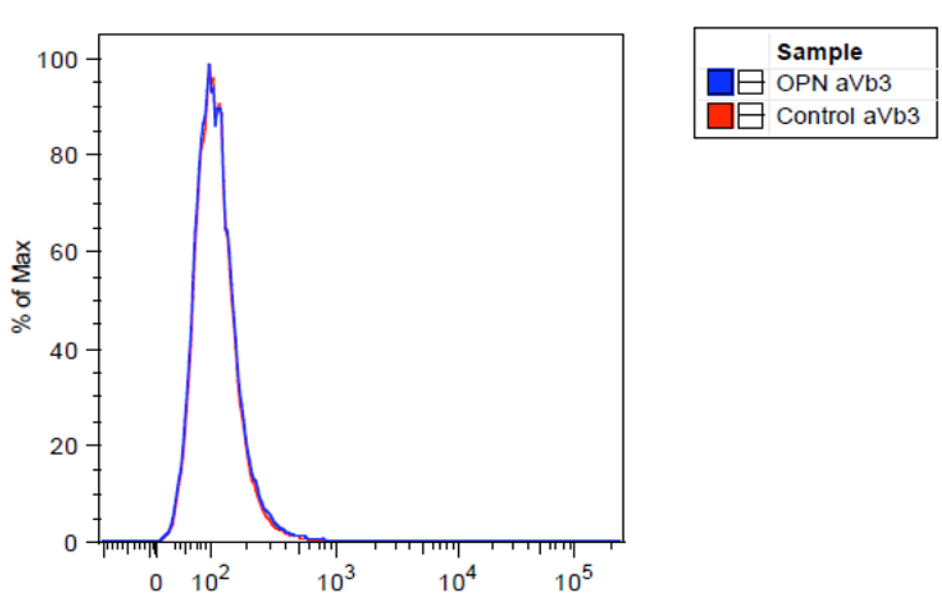


Figure 6.5: CD44v6 and αVβ3 expression is unchanged in PC3 cells treated with recombinant osteopontin. PC3 cells were cultured with 500ng/mL osteopontin for 48 hours. Antibodies against CD44v6 and αVβ3 were used for flow cytometry analysis of surface expression of both receptors.

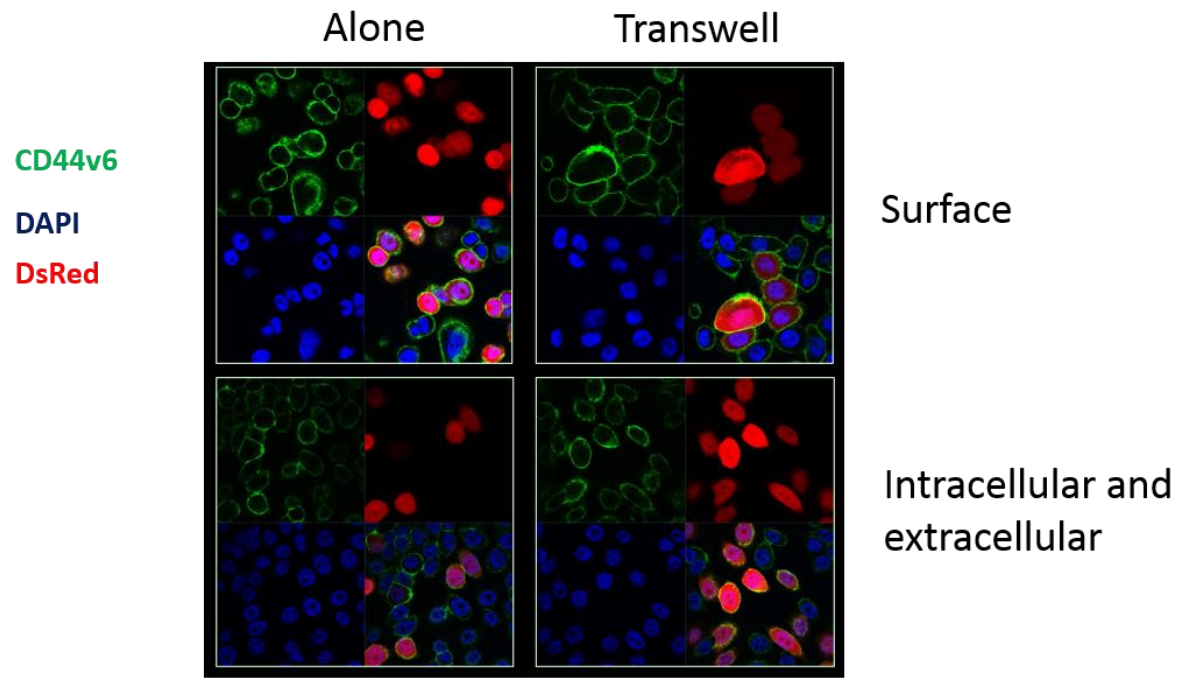


Figure 6.6: CD44v6 is localized on the cell the surface of PC3 cells cultured with BMM cells. PC3 cells were cultured in 6-well dishes on glass coverslips. BMMs were seeded on Transwell filters and stained after 48 hours. Both intracellular and surface expression of CD44v6 were assessed. Representative images were taken using 40X magnification.

To examine the effects of macrophage-supplied OPN in prostate tumor invasion, PC3 and ARCaP(M) cells were allowed to invade toward BMMs for 48 hours. Exposure to BMM-derived factors promoted increased invasion of PCa cells through reconstituted basement membrane-coated inserts **(Figure 6.7 A, B)**. Given that several macrophage-supplied factors other than OPN can promote invasiveness, we utilized blocking antibodies to the OPN receptor integrin αVβ3. Neutralization of the receptor effectively inhibited invasion of PC3 and ARCaP(M) cells. We also observed a similar reduction in tumor cell invasion toward BMMs when PC3 cells were treated with neutralizing antibodies to CD44v6 **(Figure 6.7 C)**. To investigate this more closely, we treated PC3 and ARCaP(M) cells with recombinant OPN protein to directly assess the effects of OPN on tumor cell invasiveness. PC3 cells were significantly more invasive in the presence of OPN **(Figure 6.8 A)**; however, we observed a less dramatic increase in invasiveness for ARCaP(M) cells **(Figure 6.8 B)**.

To examine why this may be the case, we performed Taqman RT-PCR analyses of tumor-derived OPN in both cell lines. Our results revealed that ARCaP(M) cells have much higher baseline OPN levels (C_t value, 19.6912) than PC3 cells (C_t value, 35.6002) **(Table 6.1)**. This suggests that PCa cells that express higher levels of endogenous OPN are not as responsive to externally supplied OPN and other factors secreted by BMMs may bind to the same receptor and promote the observed invasive behavior. Together, these data suggest that blocking the receptors for OPN partially inhibits invasion; however, other BMM-supplied ligands may promote these effects as well.

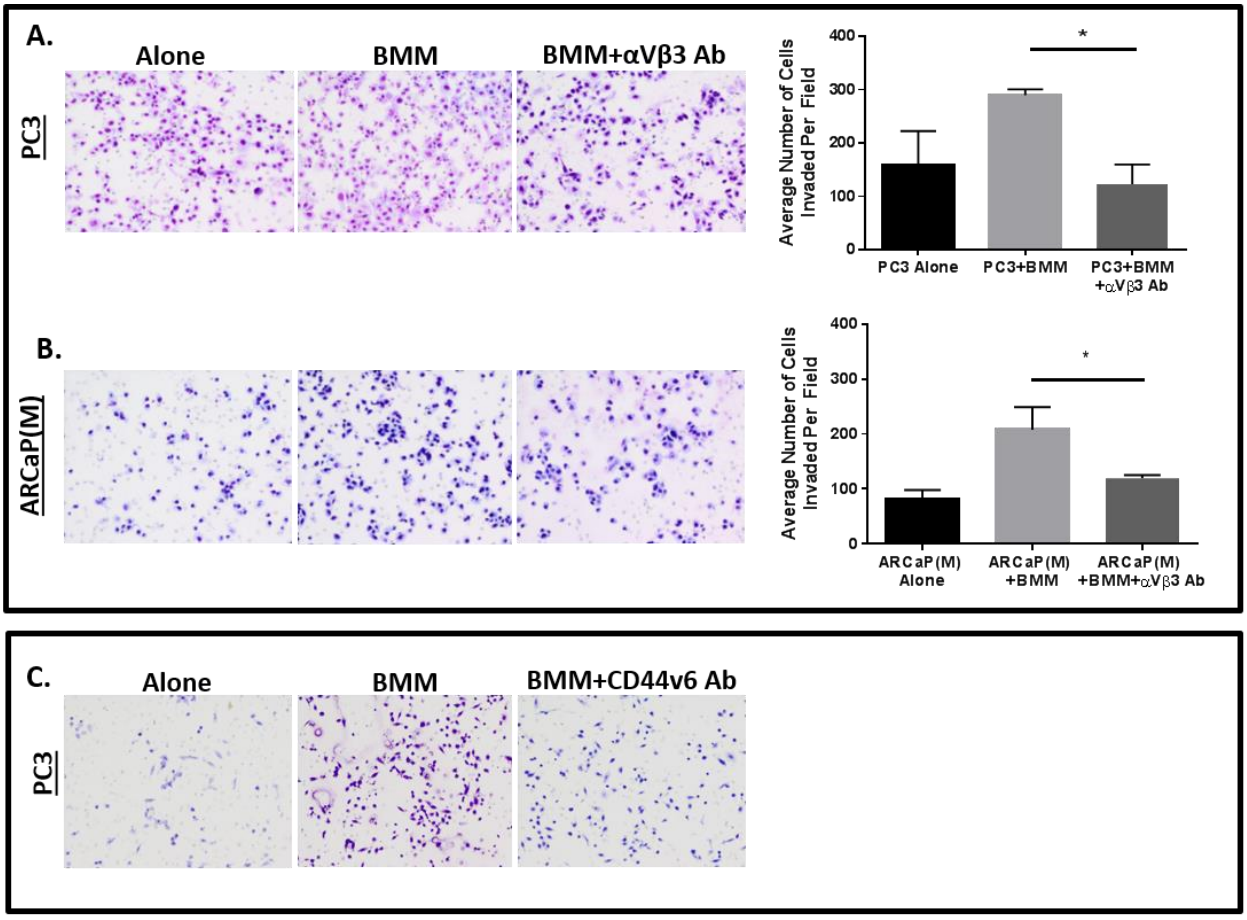


Figure 6.7: Prostate tumor invasion is inhibited with blocking antibodies to αVβ3 and CD44v6. (A) PC3 (top panel) and ARCaP(M) (bottom panel) cells were serum-starved overnight and seeded on reconstituted basement membrane (rBM). Cells invaded for 48 hours toward MEMα containing 10% FBS (control) or BMMs in the absence or presence of αVβ3 blocking antibodies. **(B)** PC3 cells invaded toward BMMs in the absence or presence of blocking antibodies to CD44v6. Cells were stained with Kwik Diff Staining Kit (Thermo Scientific) and images taken under 10X magnification. Cells were counted using ImageJ software and represented as average number of cells invaded per field. (*$p<0.05$ is statistically significant)

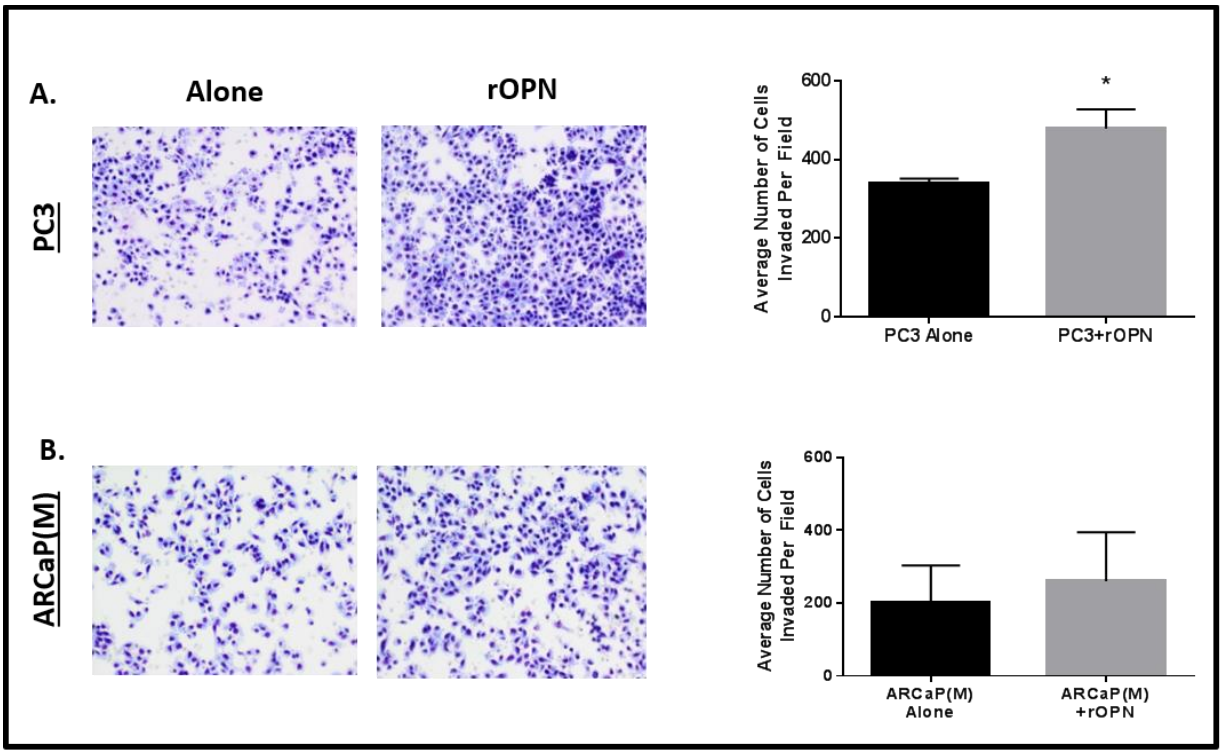


Figure 6.8: Osteopontin stimulates increased invasion of tumor cells. (A) PC3 and **(B)** ARCaP(M) cells were serum-starved overnight and seeded on reconstituted basement membrane (rBM) in the absence (control) or presence of 200ng/mL recombinant OPN. Cells invaded for 48 hours toward DMEM containing 10% FBS. Cells were stained with Kwik Diff Staining Kit (Thermo Scientific) and images taken under 10X magnification. Cells were counted using ImageJ software and represented as average number of cells invaded per field. (*$p<0.05$ is considered statistically significant)

Cell Type	PC3	ARCaP(M)
OPN (Average C_T) Values	35.6002 (s.d. 1.1922)	19.6912 (s.d. 0.1691)
18S (Average C_T) Values	10.3181 (s.d. 0.0496)	8.9431 (s.d. 0.0166)

Table 6.1: Osteopontin expression in PC3 and ARCaP(M) prostate tumor cells. Taqman RT-PCR analysis of endogenous levels of osteopontin in PC3 and ARCaP(M) prostate tumor cells. PCR data was normalized to 18S. PC3 cells less osteopontin than ARCaP(M) cells.

6.2.6 Akt and pro-survival factors are increased in prostate tumor cells cultured with BMMs

Akt activation has been well characterized as one of the key signaling mechanisms involved in tumor migration, invasion, growth, enhanced tumor metabolism, chemoresistance and survival [52, 314, 315]. Based on this evidence, we investigated the contribution of paracrine interactions between BMMs and PCa cells to tumor survival **(Figure 6.9)**. PC3 cells co-cultured with BMMs showed increased phosphorylation of Akt and protein expression of survival proteins Bcl-xL and survivin **(Figure 6.9 A-C)**. Moreover, gene transcripts of Bcl-2 were elevated while the pro-apoptotic marker Bax was moderately decreased **(Figure 6.9 D)**, results suggesting that BMMs secrete factors in the tumor microenvironment that act on prostate tumor cells to promote survival. To directly assess the effects of BMM factors on tumor survival, PC3 cells were pre-treated for 48 hours with docetaxel, a widely used chemotherapy agent that stabilizes microtubules resulting in disruption of mitosis and apoptosis [316]. Following pre-treatment, PC3 cells were co-cultured with BMMs. As shown in **Figure 6.9 E**, Akt phosphorylation was highly pronounced in PC3 cells that received docetaxel treatment and exposed to BMMs, suggesting a possible role of BMMs in tumor survival.

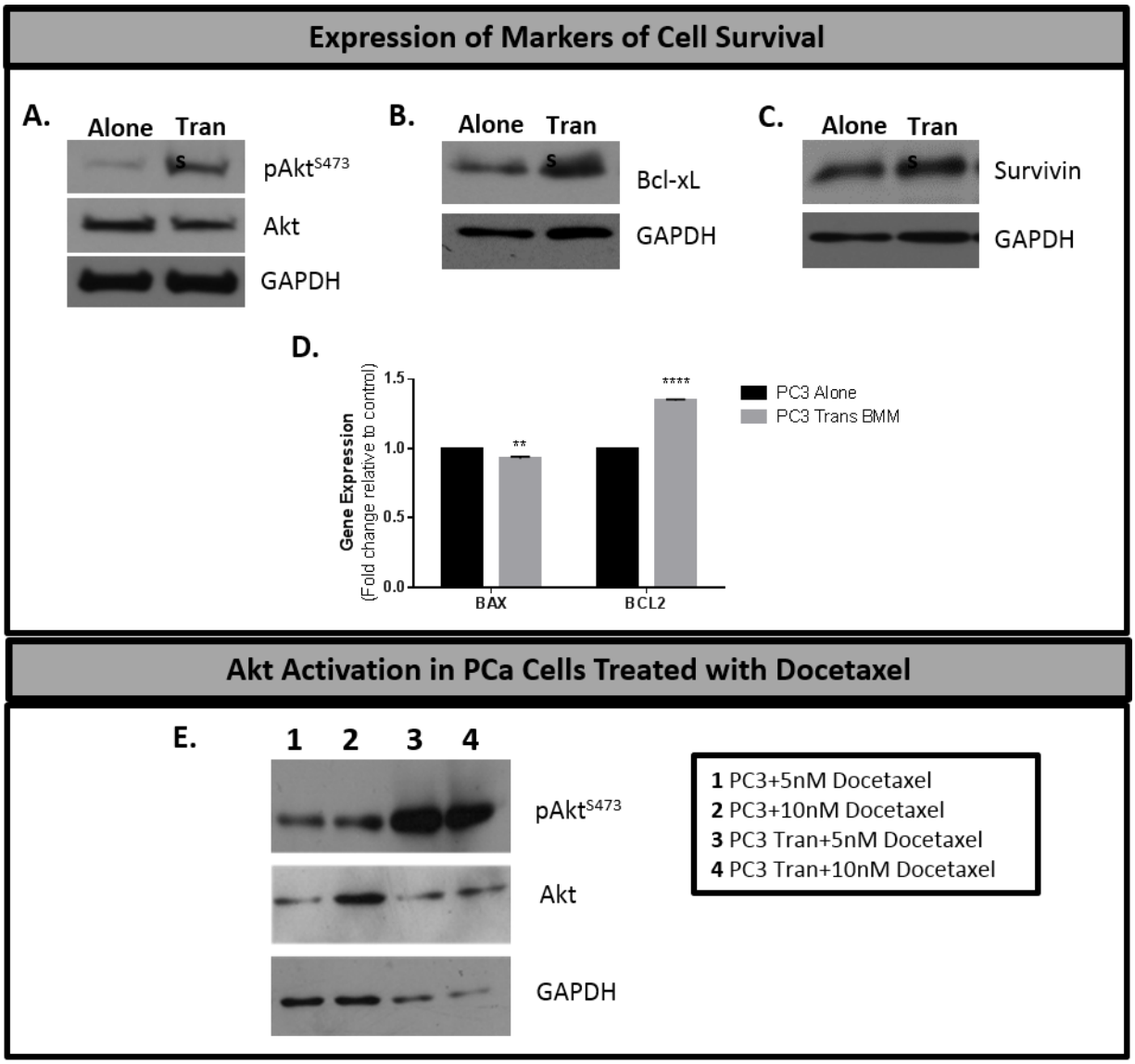


Figure 6.9: Akt and pro-survival genes are enhanced in tumor cells cultured with BMMs. PC3 cells were cultured with BMMs and protein expression of **(A)** pAkt and total Akt, **(B)** Bcl-xL, and **(C)** survivin were assessed by immunoblot analysis. **(D)** Taqman RT-PCR analysis of pro-survival Bcl2 and pro-apoptotic marker Bax were examined and was normalized to HPRT1. **(E)** PC3 cells were pre-treated with 5nM or 10nM docetaxel for 48 hours and allowed to recover under control conditions or in transwell co-culture with BMMs. pAkt and total Akt were assessed. GAPDH was used as a loading control. (***p<0.001; **p<0.01 are statistically significant).

6.2.7 BMMs induce hypoxic response in tumor cells resulting in ER Stress

In addition to enhanced expression of classic pro-survival factors, evidence has suggested that tumor cells utilize other mechanisms such as the hypoxia and ER stress response pathways to thrive in the metastatic niche [317, 318]. To determine the effects of BMMs on hypoxia and ER stress response, we assessed expression of hypoxia markers carbonic anhydrase IX (CA9) and vascular endothelial growth factor (VEGF) as well as ER stress factors binding immunoglobulin protein (BiP) and X-box binding protein 1 (XBP1). Notably, CA9 is a target gene of hypoxia inducible factor 1α that is overexpressed in malignant tumors resulting in metastasis, inflammation, and angiogenesis [319-321]. As shown in **Figure 6.10**, BMMs do indeed induce a hypoxic response in PC3 cells as demonstrated by increased gene expression of CA9 and VEGF **(Figure 6.10 A)**. To determine if hypoxia in tumor cells is in part driven by BMM-derived OPN, we cultured PC3 cells with BMMs isolated from OPN -/- mice **(Figure 6.10 B)**. Interestingly, CA9 was increased in tumor cells similarly to wild-type BMMs; however, VEGF remained unchanged in PC3 cells cultured with OPN -/- BMMs. It is important to note that OPN has been implicated as a potent positive regulator of VEGF, particularly in cancer progression [294, 322, 323].

Given a previously reported link between hypoxia and ER stress pathways, we investigated the effects of BMM-stimulated hypoxia in ER stress response in PC3 cells. **Figure 6.10 C** shows that BMM stimulate tumor cells to express robust amounts of intracellular BiP, a heat shock chaperone protein that binds misfolded proteins and shuttles them for proteasome degradation [324]. Although not as pronounced, BiP was also detected in the media,

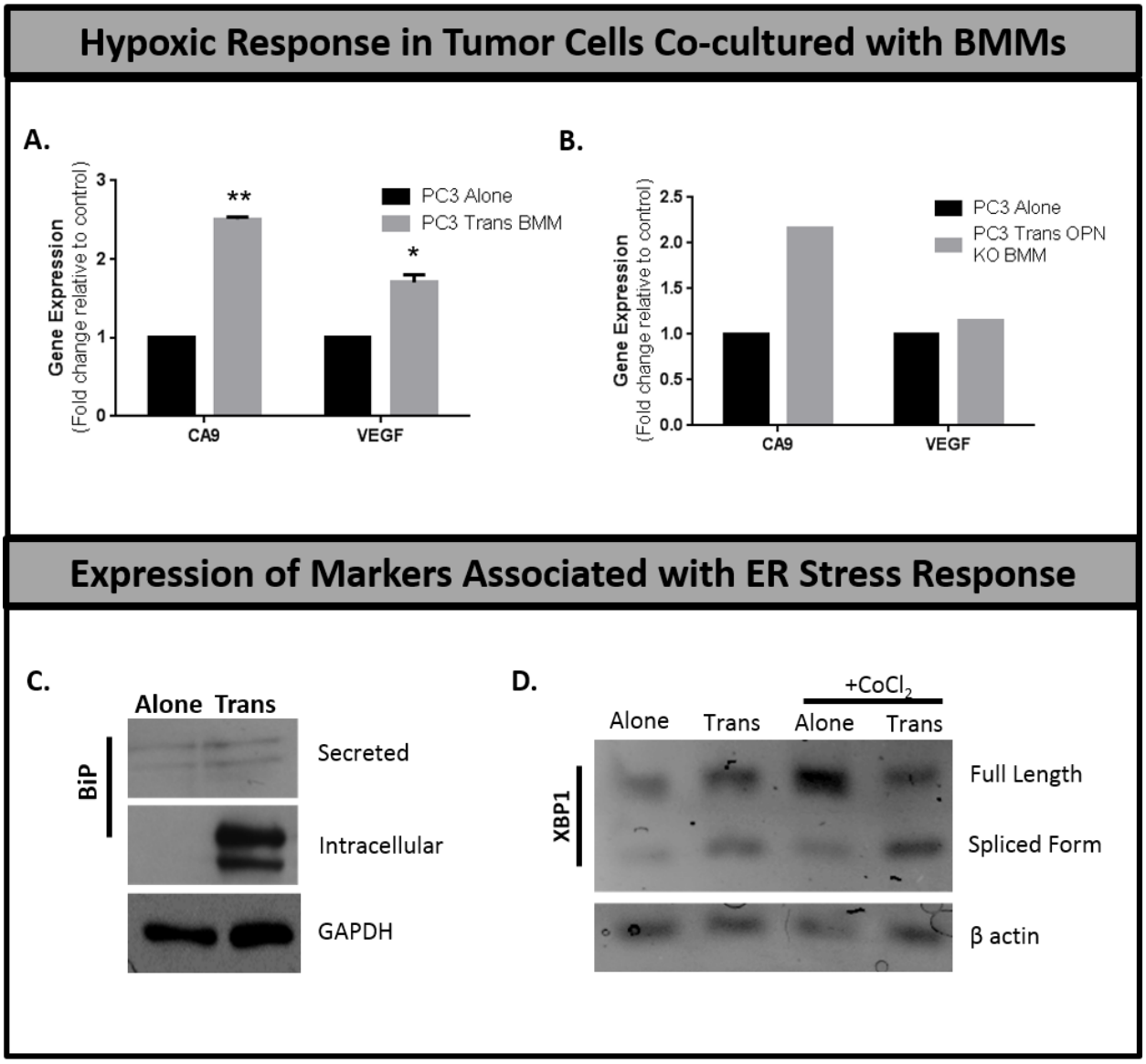


Figure 6.10: Markers of hypoxia and ER stress are induced in tumor cells co-cultured with BMMs. Taqman RT-PCR analysis of hypoxia markers carbonic anhydrase IX (CA9) and vascular endothelial growth factor (VEGF) in PC3 cells cultured with primary **(A)** WT or **(B)** OPN KO BMMs. PCR data was normalized to HPRT1 and represented as fold change relative to control. **(C)** Immunoblot analysis of binding immunoglobulin protein (BiP) protein expression in media and lysate of PC3 cells. GAPDH was used as a loading control. **(D)** PC3 cells were cultured with BMMs in the absence (normoxic) or presence of cobalt chloride ($CoCl_2$) to induce hypoxia. RNA was isolated and alternative splicing of X-box binding protein 1 (XBP1), indicative of induced ER stress, was evaluated. β actin was used as a loading control. (**p<0.01 and *p<0.05 are statistically significant).

suggesting that BiP can be secreted by tumor cells and signal to other cells or serve in autocrine signaling to further promote ER stress. A downstream factor of BiP is XBP1, a transcription factor that is activated by alternative splicing by the type I transmembrane protein IRE1-α expressed on the surface of the ER [325].Once activated, XBP1 can bind to promoter regions of target genes associated with tumor survival, proliferation, and cell fate in hypoxic or stressed conditions [320, 326-328]. Indeed, mRNA expression of both the full-length and spliced forms of XBP-1 was increased in PC3 cells cultured with BMMs and these effects were further enhanced under hypoxic conditions **(Figure 6.10 D)**. These data suggest that BMMs simulate hypoxia and ER stress in tumor cells and this is in part promoted by osteopontin.

6.2.8 Blocking αVβ3 and CD44v6 receptors on tumor cells inhibit pro-survival signaling

We next investigated the effects of blocking OPN signaling in prostate tumor cells using neutralizing antibodies against αVβ3 and CD44v6. As expected, Akt phosphorylation was increased in PC3 cells cultured with BMMs and a non-targeting IgG antibody had no effect on its activation **(Figure 6.11 A)**. Bcl-xL and BiP were also enhanced in co-culture, mirroring our previous findings. In the presence of blocking antibodies to αVβ3 and CD44v6, both pAkt, Bcl-xL, and BiP protein levels were reduced, suggesting that receptor-ligand interaction are important to downstream activation of pro-survival and ER stress signaling cascades. We also cultured PC3 cells with OPN -/- BMMs to determine if OPN has a direct role in PCa survival. We observed and increase in Akt phosphorylation and Bcl-xL protein expression in PC3 cultured with WT BMMs and levels were comparable in tumor cells cultured with OPN-/- BMMs **(Figure 6.11 B)**. Gene expression of BCL2 remained unchanged in PC3 cells culture with OPN -/- BMMs;

however, we did observe a moderate reduction in the pro-apoptotic gene Bax **(Figure 6.11 C)**. These data suggest that other ligands besides osteopontin may bind to CD44v6 and αVβ3 to activate downstream pro-survival mechanisms to promote tumor malignancy in bone.

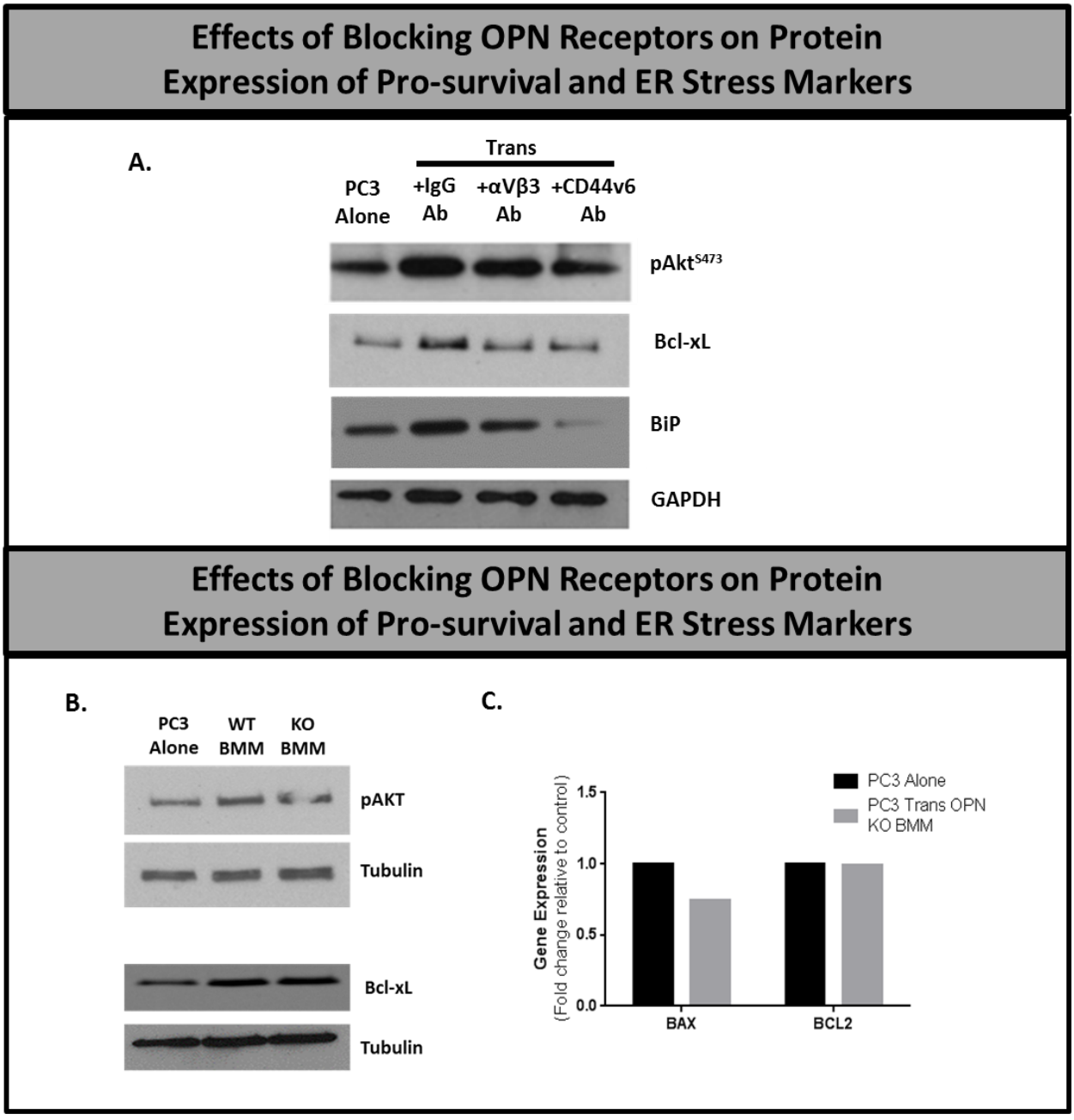


Figure 6.11: Markers of hypoxia and ER stress are reduced in tumor BMM co-cultures treated with blocking antibodies to αVβ3 or CD44v6, but are unchanged when cultured with OPN KO BMMs. (A) Immunoblot analysis of pAkt, Bcl-xL, and BiP in PC3 cells cultured with BMMs in the absence or presence of blocking antibodies to αVβ3 or CD44v6. **(B)** PC3 cells were cultured with OPN WT or KO BMMs and immunoblot analysis of pAkt and Bcl-xL,were assessed. **(C)** Taqman RT-PCR analysis of Bax and Bcl2 in PC3 cells cultured with OPN KO macrophages. HPRT1 was used as a loading control for PCR analyses. GAPDH was used as a loading control for protein expression.

Discussion

The rigid and hypoxic nature of bone requires that tumor cells find innovative ways to change and utilize their surroundings to promote survival and expansion. Tumor cells particularly thrive on certain cytokines produced by macrophages to stimulate pathways associated with survival, migration, and growth, and angiogenesis [329, 330]. Arising from these findings, the present study investigated the effects of BMM-driven inflammation, particularly macrophage-derived OPN, on tumor invasion, survival, and ER stress. We demonstrated that tumor growth in bone results in increased gene expression of host OPN levels (Figure 6.2 A, B). These results were in line with gene array meta-analysis data demonstrating OPN is enhanced in patients with metastatic disease compared to patients with localized disease (Figure 6.1). Strikingly, OPN expression was further elevated in tumor-bearing mice fed a high fat diet (Figure 6.2 C and D). These results are validated by other groups that reported OPN expression is correlated with chronic inflammation and insulin resistance in obesity [156, 331]. We also showed that OPN is robustly secreted into media from BMM-PCa co-cultures and these data mimicked gene expression analysis of BMMs cultured with tumor cells (Figure 6.3).

The effects of OPN signaling via CD44, CD44v6, and αVβ3 and its effects on tumor progression have been investigated in various cancer types; however, many of these studies focused specifically on the role of tumor-derived OPN and less on the contribution of this inflammatory regulator by stromal components to promote malignancy. Previous evidence has shown that breast tumor cells that no longer express CD44 are less able to migrate, invade, and proliferate, and receptor signaling to key migratory and proliferative pathways such as MAPK,

focal adhesion kinase, c-Src, Akt, and JNK are disrupted [332]. Less is known about the effects of targeting CD44v6 and αVβ3 in metastatic bone disease aside from limited reports linking protein expression of these receptors with angiogenesis [333], invasion [334], and overall poor prognosis [335, 336]. Our study demonstrated that protein expression of CD44 was increased in PC3 and DU145, but reduced in ARCaP(M) cells cultured with BMMs (Figure 6.4 A), while there were no changes in expression and localization of CD44v6 and αVβ3 (Figure 6.4-6.6). As previously mentioned, CD44, CD44v6, and αVβ3 are associated with increased tumor invasion. We showed that PCa cells were more invasive toward BMM-derived factors and blocking αVβ3 and CD44v6 reduced these effects (Figure 6.7).

Increased OPN expression has been implicated in tumor invasion. Recent studies have shown that extracellular OPN can act on breast cancer cells to promote tumor invasion in part by altering the density of actin cytoskeleton and reducing adhesion [337]. OPN reportedly has similar effects on migration and invasiveness in gliomas [338] and hepatocellular carcinoma [339]. Unfortunately, there are very few studies that have investigated the effects of exogenous OPN in prostate cancer. Although one study demonstrated that overexpression of alternatively spliced OPN promotes invasion in prostate [340], studies have not been conducted looking at the role of macrophage-derived OPN in the context of bone metastasis and progression. Our study is the first to show that PC3 and ARCaP(M) cells treated with recombinant OPN are more invasive through reconstituted basement membrane, although we observed that PC3 cells are more invasive than ARCaP(M) cells (Figure 6.8). Upon further investigation, our results showed that ARCaP(M) cells express more endogenous OPN than PC3 cells (Table 6.1) which may account for the differences in tumor response to extracellularly-supplied OPN. Moreover,

these data suggest surrounding metastatic niche may alter the gene expression profile of invading tumor cells to allow them to adapt to an environment that may or may not supply the factors needed for tumor growth.

Tumor cells utilize external factors to activate signaling mechanisms such as Akt to drive expression of survival factors. A comprehensive study conducted by Dai et al. demonstrated that OPN signaling directly promotes phosphorylation of Akt and PI3K to promote tumor migration and also upregulate downstream VEGF to promote angiogenesis and tube formation of endothelial cells [155]. Importantly, they demonstrated that blocking OPN signaling using neutralizing antibodies significantly abrogated vasculature formation, VEGF expression, and reduced cell viability resulting from inactivation Akt and PI3K [155]. Blocking osteopontin signaling also reduced expression of anti-apoptotic markers Bcl-xL and Bcl-2, and increased expression of the pro-apoptotic marker Bax, events resulting in tumor cell death [341]. In line with previously published findings, our results demonstrated that BMM-secreted factors promote Akt phosphorylation, and increased expression of survival genes (Figure 6.9), suggesting that secreted factors from macrophages promote survival of prostate tumor cells. We specifically tested this theory by culturing BMMs with PC3 cells pre-treated with docetaxel and observed that tumor cells expressed higher levels of pAKT in co-culture conditions compared to cells grown alone (Figure 6.9 E). We also showed that pAkt and Bcl-XL were reduced in the presence of neutralizing antibodies to CD44v6 and αVβ3 (Figure 6.11 A). Together, these data add to the testament that tumor-associated macrophages can promote tumor survival and possible chemoresistance.

One of the key components of tumor growth in primary and metastatic sites alike, is the ability to survive under harsh conditions including hypoxia, chemical onslaught, over-activated immune response, and limited access to nutrients and growth factors [342]. An elegant study from Chen and colleagues demonstrated that the ER stress marker XBP1 may directly interact with HIF1α in the same transcriptional complex, even more so under hypoxic conditions [300]. Furthermore, depletion of XBP1 in triple-negative breast cancer cells resulted in reduced levels of CD44 and VEGF expression as well as increase rate of disease recurrence following chemotherapy [300]. Similar links between hypoxia-induced ER stress and VEGF have also be reported by others [343]; however, this link has not been investigated in prostate cancer, especially at the level of metastatic bone disease. In the present study, we provide evidence to suggest that BMMs promote both hypoxic and ER stress response in PCa cells and this is evident by increased XBP1 splicing and protein expression of BiP in tumor cells cultured with BMMs (Figure 6.10 C and D). To our knowledge, this is the first study to demonstrate that BMMs can induce hypoxia and ER stress in prostate cancer.

Future studies are currently underway to further investigate the role of BMM-supplied OPN in tumor invasion and survival. However, we must also consider the compensatory mechanisms used by tumor cells in the absence of external sources of OPN. This glycoprotein is expressed in prostate cancer cell lines [165, 344], so it is possible that endogenous levels in tumor cells could be enhanced, particularly under hypoxic conditions or in response to chemical induction. Moreover, BMMs lacking OPN may compensate for this loss and enhance other factors that may serve a similar purpose of supporting malignant progression. Additional studies are also needed to determine if BMMs lacking OPN have impaired ability to switch to

M2 phenotype in the presence of tumor-derived factors and if this phenotype can be rescued with exogenous OPN from the tumor. More in depth characterization of OPN null macrophages is needed to determine the role of this protein in inflammation and tumor invasion.

This work features the multiple roles of bone marrow-derived macrophages in tumor progression, survival, and invasion. Particularly, we highlight the effects of macrophage-supplied OPN in downstream signaling pathways in tumor cells via ligand bind on CD44v6 and αVβ3 to promote tumor invasion in bone. To date, inhibitors or antibodies targeting OPN, CD44 or its variants, and integrin receptor αVβ3 have not been evaluated. Targeting them may prove difficult because OPN and its receptors play roles in normal bone remodeling, metabolism, and inflammation [149, 345]. However, based on our previous reports on macrophage involvement in tumor metastasis [238], it may be feasible to design new therapies that specifically target tumor-associated macrophages. Several clinical trials have been designed to determine the clinical ramifications of macrophage infiltration to the tumor site and find feasible drug targets that may slow prostate cancer progression [346, 347]. It remains to be determined if these will be effective and further studies are needed to understand how the bone marrow niche contributes to malignant progression in an effort to reduce or eliminate the incidence of metastatic disease.

CHAPTER 7: DISCUSSION AND FUTURE DIRECTIONS

Obesity contributes to several chronic diseases including diabetes, hypertension, and atherosclerosis [348]. Obesity and the inflammatory state have also been directly correlated with cancer initiation, progression, and metastasis [349]. Inflammatory cells and mediators are present in the microenvironment of most tumors regardless of the site of origin [233]. Inflammation is also relevant in prostate cancer due to its tendency to metastasize to the bone and promote the over activation of genes associated with wound healing response that is never resolved [350]. Obesity-driven inflammation is closely associated with reduced overall patient survival and increased tumor aggressiveness [351]. Most studies to date have focused on linking BMI and general obesity with systemic effects on cancer risk and progression, but little is known about local effects of bone marrow adiposity in metastatic prostate cancer.

The studies presented here sought to uncover molecular mechanisms involved in interactions between prostate tumor cells, bone marrow macrophages, and adipocyte-supplied factors in promoting the inflammatory environment and driving tumor progression in bone. We demonstrate that macrophages are very invasive toward factors released by bone marrow adipocytes and tumor cells and these same factors promote BMMs to secrete inflammatory mediators that can in turn affect the tumor cells. Our studies specifically focused on CXCL1 and CXCL2, chemokines with previously reported overlapping functions in tumor invasion and survival, especially when endogenously expressed and secreted by tumor cells, such as in ovarian, endometrial, oral squamous cell, and prostate cancers [207, 219, 256]. We provided new evidence that stroma-supplied CXCL1 and CXCL2 are abundantly produced in bone and are

just as important in metastatic progression as tumor-derived CXCL1 and CXCL2.

Fibroblasts and macrophages have been reported to be sources of CXCL1 and CXCL2 and infiltrating tumor cells have been shown to stimulate their secretion to aid in tumor cell seeding during early stages of cancer development, maintenance of a chronic inflammatory state, and angiogenesis [8, 243, 352, 353]. In line with these previous findings, we show that bone marrow macrophages are more invasive and secrete CXCL1 and CXCL2 in response to adipocyte- versus tumor-derived factors. Additional studies may be necessary to determine the specific factors secreted by prostate cancer cells and adipocytes to drive the abundant release of CXCL1 and CXCL2 from macrophages. This may offer clues concerning the specific need tumor cells may have for each chemokine since the CXCR2 receptor is not detectable in prostate cancer cell lines *in vitro*.

It was previously thought that CXCL1 and CXCL2 serve solely as chemotactic factors for neutrophils and tumor cells. Here we highlight a new function of these chemokines as key factors involved in marrow adiposity-directed bone remodeling. We show that adipocyte-supplied factors drive RANKL/M-CSF stimulated osteoclastogenesis, and the CXCL1/CXCL2/CXCR2 signaling axis is directly involved in osteoclast maturation of precursor cells. This is in line with recent findings that pre-osteoclasts treated with lipopolysaccharide produce CXCL1 and CXCL2 and both chemokines directly promote osteoclastogenesis [208, 243]. The data herein reveal a potential pathway utilized by bone marrow adipocytes to alter bone remodeling events in the skeleton that are commonly associated with osteoporosis and tumor metastasis.

Notably, CXCL1 and CXCL2 are not the only ligands capable of binding CXCR2. CXCL3, 5, 6, 7, and 8 all have ELR motifs and bind CXCR2 with varying affinities [354]. It is currently unknown if these factors act similarly to CXCL1 and CXCL2 and if bone marrow adipocytes secrete these ligands. Notably, interleukin-8 (CXCL8), a human homolog of murine CXCL1 and CXCL2, is secreted by cancer cells and also stimulates osteoclastogenesis [355, 356]. Thus, it is probable that tumor-secreted factors in bone act in concert with adipocytes and macrophages to accelerate bone remodeling, which may explain why we observed extensive bone destruction in tumor-bearing mice fed HFD. CXCL1 and CXCL2 knock-out mice may be needed to specifically elucidate the roles of each chemokine in normal bone remodeling to determine the mechanisms used by bone-trophic tumors to further enhance osteoclast differentiation via these factors. Although there have been no reports investigating these chemokines in bone abnormalities *in vivo*, studies in CXCR2 knock-out mice have provided some clues concerning the role of these chemokines in bone. Mice lacking CXCR2 are smaller and weigh less than wild type mice [357]. Interestingly, femoral, tibial, and lumbar bone mineral density, and blood vascularization in bone are reduced in CXCR2 null mice [357]. The observed skeletal differences in CXCR2 null mice suggest important functions for this receptor in normal bone remodeling and prompt further investigation of this signaling mechanism in bone-related disease.

Inflammation in the tumor environment is often a double-edged sword because macrophages and other inflammatory cells can have both tumoricidal and tumorigenic capacities. In some cases, tumor-associated macrophages have been shown to be highly pro-inflammatory and to secrete factors that induce homing of T cells to the tumor site [358]. This was shown to result in apoptosis and reduced proliferation of colorectal tumors [358]. Similar

protective effects have also been reported in stomach cancers and melanomas [359]. However, majority of literature evidence links tumor-associated macrophages with overall poor prognosis in several cancers [359]. The present study demonstrated that resident macrophages and adipocytes supply CXCL1 and CXCL2 to promote inflammation, bone remodeling, and tumor invasion. In addition to CXCL1 and CXCL2, tumor-associated macrophages produce other inflammatory mediators, such as osteopontin, to aid in tumor progression.

In addition to its roles in macrophage function and differentiation [149, 360], osteopontin prevents apoptosis and promotes anchorage-independent growth in tumor cells [311]. We show that osteopontin is highly expressed *in vivo* and particularly secreted in PCa-BMM co-cultures *in vitro*. Notably, we also show that osteopontin and other macrophage-supplied factors enhance tumor invasion and seem to have effects on tumor survival by mediating hypoxia, ER stress, and Akt-driven expression of pro-survival factors. In fact, it has been demonstrated that osteopontin activates downstream targets of αVβ3 such as HIF1α to promote hypoxia in tumors [361]. These findings may be clinically relevant because hypoxic tumors are much less responsive to classic chemotherapies and have overall poor prognosis [318]. Bone marrow macrophages may prove to be an important mediator of tumor survival and progression as they may be 'priming' tumor cells via osteopontin signaling to turn on ER stress and hypoxia response and thrive in bone. This is evident by the reported *in vitro* investigations demonstrating that tumor cells treated with osteopontin demonstrate increased survival under hypoxic conditions and are protected against phagocytosis by activated tumoricidal macrophages [362]. Combined with current reports that hypoxia stimulates tumor cell survival [363], M2 phenotype [355], and osteopontin overexpression [278], these events

may all cooperate to drive more aggressive tumors in bone.

Because some cancers have a more positive outcome when macrophages are present, it begs the question: how can macrophages in the bone tumor microenvironment be re-trained to target cancer cells, especially when tumor expresses the same antigens as the host? This complex question represents a central focus of the field of immunotherapy. Pre-clinical and clinical trials have attempted to train the innate immune system to target tumor cells directly and promote a strong immune response in dormant M2 macrophages [364-366]. Using *in vitro* and *in vivo* bone xenograft models of multiple myeloma, studies have demonstrated that bone marrow macrophages effectively phagocytose tumor cells treated with blocking antibodies to CD47, an integrin surface receptor overexpressed in this tumor type [367]. However, the data provided in these published studies do not address the possible contributions of hypoxia, adipokines, and cytokines that may counteract these therapeutic strategies and prevent macrophage reprogramming.

Our current work features the involvement of CXCL1/2/CXCR2 and osteopontin signaling axes in bone remodeling, inflammation, and tumor invasion. This suggests that a single drug may not be able to effectively reduce the tumor-promoting effects of the supporting stroma. Activating an anti-tumor inflammatory response in addition to chemotherapy targeting the tumor cells may be a more effective option in severing the ties that tumors have literally grown to rely on.

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ABSTRACT

ADIPOCYTE-INDUCED INFLAMMATION IN PROSTATE TUMOR PROGRESSION IN BONE: ROLE OF CXCR2 AND OSTEOPONTIN SIGNALING AXES

by

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Prostate cancer (PCa) is the second leading cause of cancer-related deaths among men. Evidence suggests that age and obesity, conditions associated with adipocyte accumulation in the bone marrow, are linked to increased risk of developing PCa and progressing to metastatic disease. Studies presented in this dissertation were based on the hypothesis that metastatic progression in bone is a result of a cooperative effort between bone marrow adipocytes, macrophages, osteoclasts, and PCa cells. We specifically focused on two adipocyte-supplied chemokines, CXCL1 and CXCL2, and bone marrow macrophage-secreted osteopontin as key drivers of pro-inflammatory environment in the bone marrow and important regulators of tumor growth and survival in bone.

Our results revealed that adipocyte-supplied CXCL1 and CXCL2 are significant contributors to tumor-driven osteolysis in metastatic disease. We showed that interaction of CXCL1 and CXCL2 with their receptor CXCR2 on osteoclast precursor cells drives osteoclastogenesis *in vitro* and bone degradation *in vivo*. Our studies also demonstrated that in addition to its effects on

osteoclasts, CXCR2 signaling axis is important for macrophage phenotype and function. Specifically, blocking the CXCR2 interaction with its ligands reduces macrophage invasiveness. We also showed that interaction of PCa cells with bone marrow macrophages *in vitro* and *in vivo* promotes phenotypic switch towards more pro-tumorigenic phenotype. Importantly, we were able to show that upon exposure to tumor cells macrophages secrete significant levels of osteopontin, powerful pro-inflammatory protein that contributes to tumor growth and survival in the bone metastatic niche. Collectively, our studies unravelled new mechanisms behind metastatic PCa progression in bone. This work will serve as basis for future studies towards discovering novel therapeutic targets for treatment of this incurable disease.

AUTOBIOGRAPHICAL STATEMENT

I received a Bachelor of Science degree in Biological Sciences from Wayne State University in 2004. It was during my undergraduate years that I was first exposed to laboratory research under the guidance of Dr. James Marsh where I studied the effects of high glucose on NFκB expression in cardiomyocytes. I returned to WSU In 2009 and joined Cancer Biology Graduate Program at the WSU School of Medicine/Karmanos Cancer Institute to pursue dissertation studies in prostate cancer. I joined the laboratory of Dr. Izabela Podgorski in 2010, and as part of my thesis work I investigated the effects of bone marrow adiposity and inflammation on prostate tumor growth, invasion, and aggressiveness in bone. During my graduate studies, I was fortunate to be awarded a spot as a pre-doctoral trainee on NRSA T32 grant and to receive the NIH/NCI F31 Pre-doctoral Fellowship Award. I also published two first-author review articles, and authored three original articles, including one as a first author. I have one additional original manuscript currently in preparation. After graduation, I will be joining the lab of Dr. Nima Sharifi at the Cleveland Clinic as a post-doctoral fellow.