Contributions of the bone marrow microenvironment to bone and skeletal metastasis

by

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DEDICATION

To my family for all their support and love: my husband Rubens Ribeiro, my son , Victor Soki Ribeiro and my parents, Kazunao and Fausta Soki

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PREFACE

This thesis includes significant work of Dr. Laurie K. McCauley who assisted in experimental design and thesis writing.

Description of my contribution to the work presented in this thesis:

All the chapters in this thesis were written by me and revised by Laurie McCauley.

I wrote Chapter 1 where I summarized the background information that was relevant to the thesis.

Chapter 2 has been published as a review paper (Soki, F. N., Park, S. I., & McCauley, L. K. (2012). The multifaceted actions of PTHrP in skeletal metastasis. Future Oncology (London, England), 8(7), 803–17. doi:10.2217/fon.12.76).I wrote the for publication with the assistance of Drs. Serk In Park and Laurie K McCauley.

Chapter 3 was an original article published in the Journal of Cellular Biochemistry (Soki, F. N., Li, X., Berry, J., Koh, A., Sinder, B. P., Qian, X., McCauley, L. K. (2013). The effects of zoledronic acid in the bone and vasculature support of hematopoietic stem cell niches. Journal of Cellular Biochemistry, 114(1), 67–78. doi:10.1002/jcb.24301). Xin Li and Laurie McCauley conceived the experiments and and I assisted with some of the *in vivo* experiments, wrote the manuscript, and prepared all the figures for publication. Dr. Li and I are co-first authors on the manuscript.

Chapter 4 is my original work in conjunction with Laurie McCauley. We conceived the experiments and I prepared the figures and wrote the chapter with Dr. McCauley's revisions.

Chapter 5 was an original article published in the Journal of Biological Chemistry (F. N. Soki, A. J. Koh, J. D. Jones, Y. W. Kim, J. Dai, E. T. Keller, K. J. Pienta, K. Atabai, H. Roca, and L. K. McCauley. Polarization of prostate cancer-associated macrophages is induced by milk fat globule-EGF factor 8 (MFG-E8)-mediated efferocytosis. J.Biol.Chem. 289 (35):24560-24572, 2014). Laurie McCauley and I conceived the experiments and I wrote the manuscript with Dr. McCauley's assistance.

In Chapter 6, I summarized the conclusions from each chapter of the thesis and discussed future directions.

All the figures and data from published papers that I am an author had permission for publication in this thesis.

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ABSTRACT

The skeleton, a favored organ for prostate cancer is organized by a mineralized connective tissue, and a rich marrow where hematopoiesis replenishes a variety of blood cells and gives rise to many cell populations. Thus, when tumors metastasize to bones they encounter many cells, once considered bystanders such as hematopoietic stem cells and macrophages, which play key roles in tumor growth progression and metastasis. Macrophages are implicated in both skeletal homeostasis and tumorigenesis; yet their role in skeletal metastasis is unclear. Macrophage phagocytosis of apoptotic cells is referred to as efferocytosis, and is an integral process by which harmful by-products of dead and dying cells are removed to create a pro-resolving environment. The purpose of this study was to determine the role of macrophages and efferocytosis in prostate cancer skeletal metastasis. *In vivo* experimental approaches resulting in macrophage ablation showed significant reduction in tumor growth in tibiae after intratibial tumor inoculations. Efferocytosis of apoptotic tumor cells increased MFG-E8 expression and promoted macrophage polarization into the M2 macrophage phenotype. Conversely, efferocytosis inhibition with neutralizing MFG-E8 antibody resulted in reduced M2 polarization, suggesting that efferocytosis is important for macrophage polarization into tumor promoting M2 cells. The involvement of the STAT3/SOCS3 activation pathway in macrophage polarization was observed. Increased MFG-E8 levels when bone marrow macrophages were co-cultured with apoptotic cells was accompanied by SOCS3 downregulation. Inhibition of STAT3 phosphorylation resulted in decreased efferocytosis and M2 macrophage polarization with an associated increase in SOCS3 protein expression. This suggests that SOCS3 and phospho-STAT3 act in an inversely dependent manner when stimulated by MFG-E8 and efferocytosis. Therefore we report a novel mechanism by which MFG-E8, by mediating efferocytosis of prostate cancer cells, can support tumor growth through facilitation of M2 macrophage polarization and regulation of SOCS3/STAT3 activation.

In conclusion, the bone microenvironment provides a dynamic and rich soil for tumors to thrive. Continued investigation on the role of bone marrow cells will provide a better understanding of the metastatic bone environment and aid in the advancement of new targets for the treatment and prevention of skeletal metastasis.

CHAPTER 1

INTRODUCTION

PROSTATE CANCER AND SKELETAL METASTASIS

Prostate cancer is the most common non-dermatologic cancer and the second leading cause of cancer death in American men. According to the American Cancer Society, it is estimated that about 30,000 men will die of prostate cancer and 233,000 new cases will be diagnosed in the United States for 2014 (Siegel et al., 2014). Advanced stage prostate carcinoma has a high tropism to bones, and an estimated 90% of patients with metastatic disease have bone lesions at postmortem analyses (Bubendorf et al., 2000). There is a high morbidity associated with skeletal metastasis with increased pain and decreased quality of life and survival (Coleman, 2006).

When tumors reach the bone they interact with the resident bone and bone marrow cells disrupting normal skeletal homeostasis. This interaction results in the release of many osteogenic and osteolytic factors that stimulate the uncontrolled production (osteoblastic) or resorption (osteolytic) of bone, driving its destruction (Mundy, 1997;Weilbaecher et al., 2011a). Tumor cells secrete factors such as PTHrP, tumor necrosis factor-α (TNF-α), interleukin-1, interleukin-6, interleukin-8, and interleukin-11 that stimulate bone cells (Chirgwin and Guise, 2007). Activated osteoblasts and osteoclasts in turn, secrete other factors that promote tumor growth, feeding a destructive cascade of metastatic growth (Chirgwin and Guise, 2007). Therefore, skeletal metastasis depends on both priming the seed (tumor cells) as well as nurturing the soil (bone). Bone metastasis can be characterized according to the radiographic and pathologic appearances as osteolytic, osteosclerotic or mixed

(Weilbaecher et al., 2011a). Prostate cancer metastatic lesions are predominantly osteoblastic. Tumor-derived factors such as Wnt family ligands, bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF) and endothelin-1 activate osteoblast bone formation contributing to skeletal metastasis (Weilbaecher et al., 2011b). Despite that prostate cancer skeletal lesions are predominantly osteoblastic, osteoclast activities are also increased and osteolytic lesions are commonly found in bone lesions (Keller and Brown, 2004;Coleman, 2006). Skeletal related events (SREs) are complications that afflicted patients endure when tumors metastasize to bones as consequences of the abnormal bone response (Weilbaecher et al., 2011a). Patients can present with ineffective hematopoiesis, hypercalcemia, pathologic fracture, and spinal cord or nerve root compressions (Weilbaecher et al., 2011a). Treatment utilizing osteoclast-target agents have shown to reduce the skeletal complications and ameliorate the morbidity associated with skeletal metastasis (Gartrell and Saad, 2014). Bisphosphonates are a commonly used therapy to improve patients' outcomes and reduce the morbidity associated with skeletal metastasis. They not only reduce bone resorption but also directly affect tumor cells and cells of the microenvironment including macrophages (Rogers and Holen, 2011). Zoledronic acid (ZA) is a potent third generation bisphosphonate utilized for treatment of metabolic bone diseases such as osteoporosis, Paget's disease and cancer-related bone diseases. The high affinity for hydroxyapatite results in accumulation in bone. Bisphosphonates are internalized by osteoclasts during bone resorption, inducing apoptosis through inhibition of the mevalonate pathway (Rodan and Fleisch, 1996;Winter and Coleman, 2009). Despite that randomized clinical trials showed that adjuvant ZA did not reduce the incidence of skeletal metastasis (Wirth et al., 2014), its effective role in the reduction of skeletal related events facilitated the approval of this drug for patients with prostate bone metastases (Gartrell and Saad, 2014).

PTHRP IN SKELETAL METASTASIS

Tumor derived parathyroid hormone-related protein (PTHrP) is a key osteolytic factor in bone metastasis. This peptide that was initially identified during the elucidation of mediators of malignancy-induced hypercalcemia plays various roles in normal physiology as well as pathological conditions. In cancers with high bone tropism such as prostate and breast tumors, PTHrP can act in priming the seed, participating in cell autonomous processes such as tumor cell proliferation, apoptosis, survival and anoikis, qualities that enhance the capability of tumor growth, dissemination and metastasis (Soki et al., 2012;McCauley and Martin, 2012b). PTHrP also has endocrine or paracrine roles in modulating bone responses and cellular aspects of the bone microenvironment, thus contributing to the formation of a conducive environment for cancer establishment in bone. When osteolytic tumors metastasize to bone, they promote a destructive cascade of events also known as the “vicious cycle” (Yin et al., 1999). Tumor derived PTHrP binds to and stimulates the PTH/PTHrP receptor present in osteoblasts and osteocytes to express receptor activator of nuclear factor kappa B ligand (RANKL) leading to osteoclast differentiation and bone loss. As a result of bone resorption, factors such as calcium, TGF-β, insulin-like growth factor 1 (IGF-1) and fibroblast growth factors (FGFs) are released to the environment, contributing to tumor proliferation and augmenting PTHrP production. In addition, PTHrP can also induce expression of chemokine (C-C motif) ligand 2 (CCL2 or MCP-1) thus contributing to tumor growth (Li et al., 2009). CCL2 directly stimulates tumor cell migration, proliferation and survival and indirectly establishes a niche for growth eliciting angiogenesis and macrophage recruitment and polarization to the M2 type (pro-tumorigenic macrophages) (Rozel et al., 2009;Mizutani et al., 2009). CCL2 also participates in skeletal metastasis, promoting increased osteoclastic numbers and activity, which are important for tumor growth in bone (Loberg et al., 2007a). A review of the role of PTHrP in skeletal metastasis and cancer will be discussed in Chapter 2.

THE BONE MICROENVIRONMENT: MORE THAN JUST BONE

Bone is a complex organ, organized by a mineralized connective tissue, which gives support and protection to various organs. Bone is a dynamic tissue under the control of local and systemic factors and is constantly remodeling itself (Mundy, 2002a). This tightly regulated balance between bone formation by osteoblasts, and bone resorption by osteoclasts controls this dynamic tissue. In addition to bone cells, the bone microenvironment is comprised of a rich marrow where hematopoiesis replenishes a variety of blood cells and gives rise to many cell populations. Thus, when tumors metastasize to bones they encounter many cell populations that can mediate critical roles in tumor growth and progression (Park et al., 2011b). Cells present in the bone marrow that were once considered only bystanders such as hematopoietic stem cells and macrophages play key roles in tumor growth progression and metastasis of different types of cancer.

Hematopoietic stem cells are found in close association with the endosteal interface of bone and bone marrow, and perivascular sinusoidal blood vessels, also known as stem cell niches that maintain and regulate these cells (Adams et al., 2006;Kiel et al., 2005). The hematopoietic niche is necessary to balance the expansion and mobilization of HSCs to the peripheral blood as well as the regulation of self-renewal capacity and hematopoietic commitment to differentiation. Cells that constitute this tightly regulated niche, including osteoblasts, osteoclasts, endothelial cells, adipocytes and stromal cells, participate in the regulation and maintenance of HSCs (Bianco, 2011). Hematopoietic stem cells and stromal cells actively participate in tumor growth mediated through effects in angiogenesis, competing with the metastatic tumor cells for the osteoblastic niches (Muller et al., 2001;Taichman et al., 2002;Jung et al., 2007;Shiozawa et al., 2008;Sun et al., 2010). Osteoclast targeted therapies with bisphosphonates in clinical settings are applied not only in osteoporosis, but also as adjuvant therapy for the skeletal related events in bone metastases. Modifications of the bone microenvironment may affect HSC niches that were shown to be important for tumor cell adhesion and colonization to the bones. Therefore, to determine the effects of the potent osteoclast inhibitor zoledronic acid in the HSC niche will bring a better

understanding of the mechanisms that osteoclasts orchestrate in the bone microenvironment and subsequently how this affects tumor metastasis to bone. The effects of zoledronic acid in the bone and vasculature support of hematopoietic stem cell niches will be discussed in chapter 3.

In addition to hematopoietic stem cells, emerging data on the role of other cell types including myeloid and bone marrow macrophages support their involvement in the growth, progression and metastasis of various tumors.

Macrophages are myeloid phagocytic cells recruited in response to infection, inflammation and tissue injury, therefore, having a role in the innate and adaptive immune response (Pollard, 2009). They also have important roles in tissue development such branching morphogenesis, neuronal patterning, angiogenesis, bone morphogenesis and adipogenesis (Pollard, 2009). In bone, osteoclasts are considered specialized resident macrophages. Indeed, osteoclast participation in tumor growth and progression of skeletal metastasis has been extensively studied and these cells identified as key contributors for the vicious and destructive cycle of bone metastasis (Mundy, 1997;Keller and Brown, 2004). Resident macrophages other than osteoclasts have been identified in close association to the endosteal bone and termed as 'osteomacs' or osteal macrophages. Osteal macrophages constitute one sixth of the total cells and differ from osteoclasts, by expressing specific surface markers: F4/80 and CD68 (Austyn and Gordon, 1981;Chang et al., 2008). Interestingly, resident macrophages are found in close association with osteoblasts and play key roles in bone remodeling, bone healing and hematopoietic niche maintenance, skeletal homeostasis and PTH anabolic actions on bone remodeling (Chang et al., 2008;Cho et al., 2014;Winkler et al., 2010a;Alexander et al., 2011a).

Macrophages are activated differentially according to the stimuli provided: M1 anti-tumorigenic macrophages are classically activated and M2 pro-tumorigenic macrophages (also known as tumor associated macrophages, TAMs) are alternatively activated (5,6). TAMs are prominently found and involved with cancer initiation, progression and metastasis, facilitating angiogenesis, matrix breakdown and tumor cell-motility (Pollard, 2004;Colotta et al., 2009). Modification of the myeloid cells in bone has demonstrated influence in tumor metastasis to bone. A single dose of

cyclophosphamide, a bone marrow–suppressive chemotherapeutic drug, administration prior to intracardiac tumor inoculation increased prostate cancer cell localization and growth in bone (Park et al., 2012a). This was associated with a transient expansion of myeloid cells and increased cytokines with myelogenic potential such as C-C chemokine ligand 2 (CCL2), interleukin-6 (IL-6), and VEGF-A that primed the environment for tumor growth. Moreover, the chemokine CCL2 known to attract and differentiate macrophages towards TAMS and osteoclasts has been shown to increase prostate cancer growth and bone metastasis (Mizutani et al., 2009;Loberg et al., 2007b;Loberg et al., 2007a;Roca et al., 2009;Rozel et al., 2009). Therefore, macrophages play important roles in cancer growth as well as in the bone niche. However, their role in bone pathologies such as bone metastasis remains elusive. Their involvement in the modulation of the bone microenvironment during skeletal metastasis was explored in Chapter 4.

MACROPHAGES AND EFFEROCYTOSIS

A key function that macrophages exert in normal physiology is their involvement in the later stages of inflammation. Macrophages are important phagocytic cells that actively participate in the cell clearance process, also known as efferocytosis, phagocytizing apoptotic cells to control and regulate the inflammatory response (Gregory and Pound, 2011a). Efferocytosis elicits changes in immune responses and can result in immunosuppression of anti-tumor defenses (Reiter et al., 1999a;Savill et al., 2002a), production of anti-inflammatory mediators including transforming growth factor–β (TGF-β) and interleukin-10 (IL-10), and inhibition of pro-inflammatory tumor necrosis factor (TNF) (Savill et al., 2002a;Michlewska et al., 2009). MFG-E8 mediated efferocytosis can also promote an anti-inflammatory status in LPS stimulated cells, by down-regulating nuclear factor–κB (NF-κB) and MAP kinase signaling pathways (Miksa et al., 2008). Remarkably, NF-κB inhibition is also linked with TAM polarization (Biswas et al., 2006;Saccani et al., 2006). Interestingly, even though tumors present high apoptosis rates concomitantly with their high proliferation rates, the presence of apoptotic cells is under appreciated due to rapid cell clearance performed by

macrophages and other phagocytic cells. Despite its important function in normal physiology, efferocytosis in cancer is an under explored field. Not much is known regarding the interactions of apoptotic tumor cells with macrophages, and subsequently, how this affects the remaining viable tumor cells in skeletal metastasis.

The phagocytosis process can be divided into 4 different stages (Savill et al., 2002b;Gregory and Pound, 2011a). The first stage consists of “find-me” signals that are released by apoptotic cells to facilitate the attraction and recruitment of the phagocytic cells within the damaged tissue. The second stage depends on “eat –me” signals that are externalized on the apoptotic cell surface for their recognition by phagocytic cells and subsequent internalization of the body. Sequentially, the internalized cargo undergoes degradation in the third stage, leading to the fourth stage of post-engulfment, when the phagocytic cells secrete anti-inflammatory cytokines and other factors, thus preventing further damage resolving the inflammatory response.

Milk fat globule-EGF factor 8 (MFG-E8) is a protein known as a phagocytosis “eat me” signal secreted by numerous cells including macrophages (Hanayama et al., 2002;Hanayama et al., 2004). MFG-E8 facilitates efferocytosis, functioning as a bridge between macrophage and apoptotic cells binding to both phosphatidylserine (PS) externalized on apoptotic cells and the $\alpha v\beta 3$/ $\alpha v\beta 5$ integrin expressed on macrophages (Hanayama et al., 2004). The role in efferocytosis was confirmed in MFG-E8 deficient mice that presented apoptotic cell accumulation. The deficiency also implicated in autoimmunity and inflammatory related diseases (Miksa et al., 2008;Asano et al., 2004;Thorp and Tabas, 2009).

Interestingly, phagocytic cell activation through MFG-E8 results in similar anti-inflammatory and tumor promoting properties, as seen in M2 TAMs (Lewis and Pollard, 2006;Gregory and Pound, 2011b;Pollard, 2009). Despite these suggestive links, efferocytosis via MFG-E8 and its role in tumor efferocytosis and M2 polarization has not been investigated in the context of prostate cancer or skeletal metastasis. The impact of efferocytosis mediated by MFG-E8 on macrophage polarization into M2 TAMs and the underlying mechanisms was investigated and discussed in Chapter 5.

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CHAPTER 2

THE MULTI-FACETED ACTIONS OF PARATHYROID HORMONE RELATED PROTEIN IN SKELETAL METASTASIS

ABSTRACT

Parathyroid hormone-related protein (PTHrP), identified during the elucidation of mediators of malignancy-induced hypercalcemia, plays numerous roles in normal physiology as well as pathological conditions. Recent data support direct functions of PTHrP in metastasis, particularly from tumors with strong bone-tropism. Bone provides a unique metastatic environment because of mineralization and diverse constituting cell populations in the bone marrow. PTHrP is a key regulator of tumor-bone interactions and regulates cells in the bone microenvironment, through proliferative and pro-survival activities that prime the “seed” and the “soil” of the metastatic lesion. This review highlights recent findings of the role of PTHrP in skeletal metastasis, including direct actions in tumor cells as well as alterations in the bone microenvironment and future perspectives involving potential roles of PTHrP in the premetastatic niche and tumor dormancy

INTRODUCTION

Parathyroid hormone related protein (PTHrP) was first discovered as an etiological factor of humoral hypercalcemia of malignancy (HHM), commonly found in

patients with certain types of advanced stage cancers such as breast, lung, renal, ovarian, pancreatic carcinomas, and myeloma (Mundy, 2002b;McCauley and Martin, 2012a). An association between hypercalcemia and malignancy was first postulated in 1920s with the development of the calcium assay (Kramer and Tisdall, 1921) which allowed calcium measurement in patients with cancer. In 1941, Albright raised a hypothesis that ectopic parathyroid hormone (PTH) could be the tumor-derived factor responsible for HHM (Albright F, 1941), due to its important role in calcium homeostasis, which is a tightly regulated process involving coordination of bone, kidney, gut and parathyroid glands (Clines and Guise, 2005). PTH, is an 84 amino acid hormone secreted by the parathyroid glands, and plays an essential role in regulating phosphate, vitamin D, extracellular fluids and calcium balance. Albright's hypothesis that PTH was the etiological factor in HHM was true from the functional standpoint but did not prove true clinically (Manring and Calhoun, 2011). It was only in 1987, that three independent groups identified a protein with similar biological activities and structure to PTH, hence named parathyroid hormone-related protein (PTHrP). Moseley *et al.* isolated an 18kDa protein from a human lung cancer cell line with biological activities and high homology to the amino terminal region of PTH in which 8 residues in positions 1-13 were identical to the human PTH (Suva et al., 1987). Interestingly, two other independent groups also isolated and purified the same PTH like factor from human renal carcinoma cells (Strewler et al., 1987) and from human breast cancer cells (Burtis et al., 1987;Stewart et al., 1987).

Subsequently, PTHrP was found to be expressed in numerous types of cancer and its role in HHM was elucidated, with activation of the PTH/PTHrP receptor (PPR) in the skeleton to evoke calcium release via bone resorption, and activation of the PPR in the kidney to decrease calcium excretion (Clines and Guise, 2005). Indeed, the main causes of hypercalcemia, primary hyperparathyroidism and HHM, show as-yet unexplained clinical differences, even though PTH and PTHrP have similar biological activities. For example HHM patients present lower levels of the active form of vitamin D (calcitriol), metabolic alkalosis and uncoupling responses of bone resorption and formation in contrast to what is observed with primary hyperthyroidism (Schilling et al.,

1993;Nakayama et al., 1996;Clines and Guise, 2005). Other potential mediators of HHM are tumor-associated factors with systemic or local actions. Systemic factors such as calcitriol 1,25-$(OH)_2D_3$ that are increased in lymphomas, acts on organs responsible for calcium homeostasis (kidney and intestine) resulting in elevated calcium levels (Seymour and Gagel, 1993). Tumor-secreted factors with local actions that stimulate bone resorption such as interleukin-1 (IL-1), interleukin-6 (IL-6), transforming growth factor alpha (TGF-α), tumor necrosis factor (TNF) and granulocyte colony-stimulating factor (G-CSF) also promote increased calcium (Clines and Guise, 2005) . In addition to its role in hypercalcemia, further investigation demonstrated that PTHrP also plays important roles in tumor progression and metastasis, which is the main topic of this review article.

PTHrP resembles PTH sharing 8 out of the 13 initial amino acids at the N-terminus and binds to the PTH receptor type 1 known as the PPR. The PTHrP gene *PTHLH*, located on chromosome 12, expands more than 15 kb including 9 exons and at least 3 promoters. Alternative splicing gives rise to three isoforms: 139, 141, and 173 amino acids (Martin et al., 1991). Furthermore, the PTHrP protein has several functional domains comprised of an N-terminal domain, a mid-region domain and a carboxyl-terminal domain. The N-terminal domain (amino acids 1-36) has the binding site to activate the PPR receptor, acting in autocrine, paracrine and endocrine manners and leading to different biological effects and cell autonomous functions (Figure 2.1). The mid-region (amino acids 37-106) includes a nuclear localization sequence (NLS) important for intracrine signaling of PTHrP in the nucleus and cytoplasm, regulating cell proliferation, survival and apoptosis. Lastly, the carboxyl-terminal domain (amino acids 107-139), also known as osteostatin, is associated with inhibition of osteoclastic bone resorption and anabolic effects in bone (Martin et al., 1991;de Castro et al., 2012).

Along with tumorigenic functions, PTHrP also participates in normal physiology, acting as a hormone in calcium transportation in the fetus, late pregnancy and lactation (McCauley and Martin, 2012a). PTHrP is also highly expressed in human tissues and plays an important role in the developmental stages of mammary glands, hair follicles and teeth (McCauley and Martin, 2012a). The biological function of PTHrP is very

important in development during endochrondral bone formation. Deletion of PTHrP in mice results in chondrodysplasia and early death and heterozygous *Pthlh* +/- mice have an early osteoporotic phenotype with reductions in trabecular volume (Karaplis et al., 1994;Amizuka et al., 1994;Amizuka et al., 1996). Altogether, these studies demonstrate the key role that PTHrP plays in normal physiology and developmental biology.

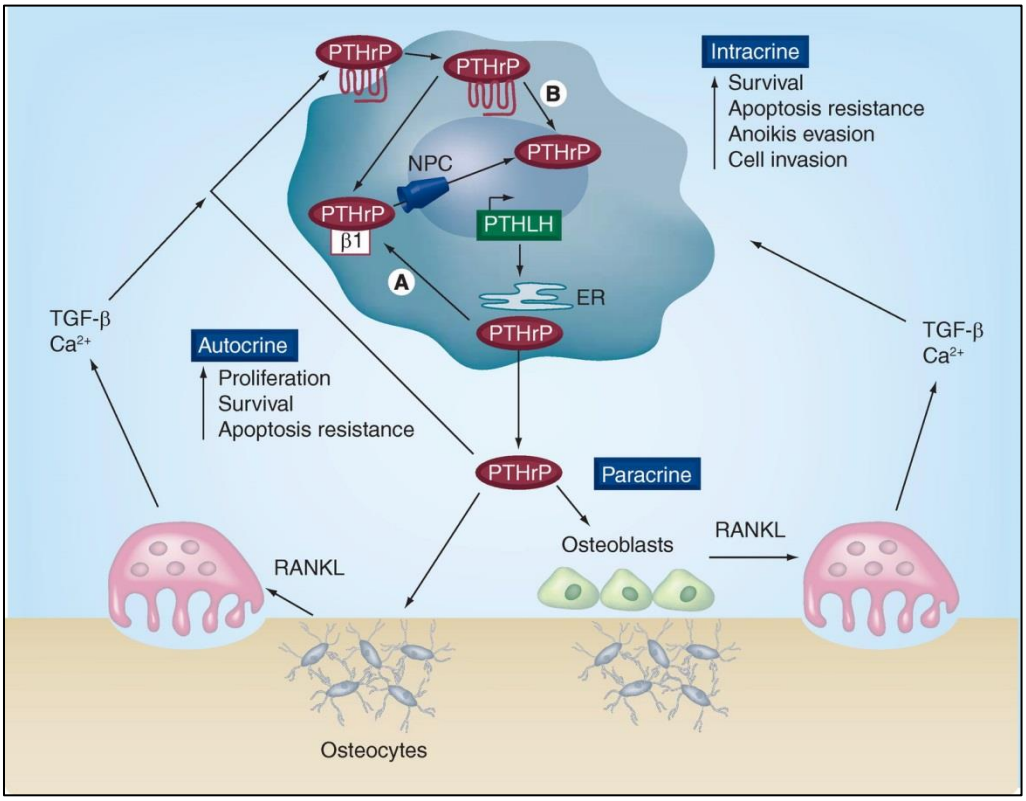

Figure 2.1. Multiple actions of PTHrP in tumor growth in bone.

Tumor-derived PTHrP acts by different modes in order to enhance tumor growth, progression and metastasis. PTHrP acts in an intracrine manner, increasing cell survival, apoptosis resistance and anoikis evasion. There are two potential pathways for nuclear localization of PTHrP: (A) translocation of PTHrP through the NPC in which the PTHrP nuclear localization sequence interacts with β1 and is then transported to the nucleus; and (B) internalization of the parathyroid hormone/PTHrP receptor–PTHrP complex by an endocytosis-dependent pathway to the cytosol and rapid transport into the nucleus. Moreover, tumor cells also express the parathyroid hormone/PTHrP receptor facilitating the autocrine actions of PTHrP and contributing to cell proliferation and apoptosis resistance and growth. When tumors metastasize to bone, PTHrP acts in a paracrine manner, secreting PTHrP in the bone microenvironment, activating osteoblasts and inducing a destructive cascade with release of numerous growth factors that contribute to tumor growth and enhanced PTHrP expression. Therefore, PTHrP participates in

all steps of the metastatic processes, from tumor growth, progression, invasion, migration and survival to bone modulation in order to support metastases. Permission for figure from Future Medicine

β1: Importin β1; ER: Endoplasmic reticulum; NPC: Nuclear pore complex.

The PPR is a class II G-protein coupled receptor comprised of seven transmembrane spanning domains. The gene that encodes the PPR is highly conserved and homologous in rat, mouse and human and the multiple exons that encode the gene are subjected to alternative splicing (Kong et al., 1994). PTH and PTHrP amino-terminal regions bind to and activate the PPR, which is expressed in the main target cells of PTH and PTHrP: osteoblasts in bone and renal tubular cells in the kidney. Remarkably, PPR was also found to be expressed in many tumor types such as prostate, breast, and many other cancers (Downey et al., 1997;Asadi et al., 1996) regulating tumor cell autonomous processes and contributing to tumor progression and growth. Consequently, PTHrP supports dual roles in skeletal metastasis: 1) modulating the bone and priming the metastatic microenvironment, and 2) promoting tumor cell autonomous function contributing to growth and progression. In bone, the PPR is primarily expressed in osteoblasts, osteocytes, and bone marrow stromal cells such as osteoblast precursor cells. Osteoclasts do not express the PPR evidenced by the lack of response to PTH (Fuller et al., 1998). The actions of PTH and PTHrP in osteoclasts are mediated by osteoblasts and osteocytes responsible for secretion of factors that activate osteoclasts. The PTH and PTHrP amino terminals interact with the J-domain functional portion of the PPR in osteoblasts stimulating multiple signaling cascades including adenylate cyclase-protein kinase A (AC/PKA), phospholipase C-protein kinase C (PC/PKC), and mitogen- activated protein kinases (MAPKs) pathways leading to different anabolic and catabolic responses in bone (Datta and Abou-Samra, 2009).

Tumor-derived PTHrP can act in different ways to modulate tumor growth, progression and metastasis. For example, in HHM, PTHrP is secreted from primary tumors and acts in an endocrine manner inducing bone resorption. When tumors metastasize to bone, PTHrP acts in a paracrine manner secreting PTHrP in the bone microenvironment, activating osteoblasts, and inducing bone remodeling. In addition,

tumor cells also express the PPR facilitating autocrine actions of PTHrP and contributing to cell proliferation and growth. Lastly, PTHrP also acts in an intracrine manner, increasing cell survival and apoptosis resistance (Fiaschi-Taesch and Stewart, 2003). Although PTHrP plays multifunctional roles in skeletal metastasis, most investigations have focused on PTHrP function as a tumor promoting factor. However, emerging evidence supports that PTHrP also alters the tumor microenvironment potentially contributing to metastasis development.

ROLES OF PTHrP IN SKELETAL METASTASIS OF CANCER

According to Stephen Paget's "seed and soil" hypothesis, disseminated tumor cells ("the seed") can produce metastases only when the tumor cells are seeded in the correct "soil" (Fidler, 2003;Paget, 1889). Therefore metastasis is a multistep process that requires coordination of 2 different subsets: tumor cells and the metastatic organ. The tumor cells must acquire the ability to invade the surrounding tissue, gain access to the circulation by the lymphatic or blood circulation, survive, and extravasate into a secondary site (Steeg, 2006). The second subset is the metastatic compartment that has to enable tumor invasion, colonization and growth. In other words, the metastatic organ is the fertile soil that favors tumor cell growth. PTHrP in skeletal metastases has the capacity to act on both parts of the story, nurturing the seed (tumor cells) and priming the soil (bone microenvironment).

PTHrP expression is commonly found in many types of cancer and increased expression is observed with tumor progression with the highest expression being found in metastatic lesions (Southby et al., 1990;Powell et al., 1991;Malakouti et al., 1996;Shen et al., 2007b;Guise et al., 1996;Iwamura et al., 1993). However, the use of tumor-produced PTHrP as a prognostic factor is still controversial. Clinical studies in breast and lung cancers have implicated PTHrP expression in primary tumors as a good prognostic factor (Montgrain et al., 2011;Henderson et al., 2006). In a recent clinical study in non-small-cell lung carcinoma, PTHrP expression was associated with

increased survival in females with both early or advanced stages of disease (Montgrain et al., 2011). Henderson *et al* demonstrated in a prospective study of 526 patients with breast cancer followed for a 10 year period that positive PTHrP expression in the primary tumors was correlated with improved survival and reduced development of bone metastases (Henderson et al., 2006). They concluded that PTHrP expression in the primary tumors conferred a less invasive phenotype that is distinct from its known osteolytic roles which support skeletal metastasis.

By contrast, clinical studies in invasive human breast tumors indicated that PTHrP was detected in 60% of the tumors but not in the normal breast tissues and expression was greater in bone metastases, with PTHrP expression detected in approximately 90% (Southby et al., 1990;Powell et al., 1991). A recent and exciting study identified the gene for PTHrP *(PTHLH)* as a breast cancer risk loci. In this large investigation that included two independent genome-wide associations (GWAS) from 41 case-control studies and 9 breast cancer GWAS, three new breast cancer loci were identified including *PTHLH* (p12p11). These data delineate the most convincing evidence implicating PTHrP in breast cancer pathogenesis. Still the genes identified must be proven to be the causal genes responsible for cancer pathogenesis and the mechanisms need to be explored (Ghoussaini et al., 2012). PTHrP is also expressed in more than 90% of colon cancer patients (Malakouti et al., 1996). Extensive investigation of PTHrP function in prostate tumors demonstrated that expression contributes to tumor growth and progression (Dougherty et al., 1999). Studies in human prostate cancer observed that PTHrP was differentially expressed depending on the cancer stage. PTHrP was expressed in 33% of benign prostate hyperplasia, 87% in well differentiated prostate cancer and 100% in poorly differentiated and metastatic tumors (Asadi et al., 1996;Iwamura et al., 1993).

The widespread thought is that PTHrP is a supportive factor for cancer growth and progression. Differences in its prognostic applicability may reflect temporal aspects and/or downstream events that have been difficult to elucidate in the context of cancer. Moreover, the fact that PTHrP is a polyhormone witth multiple biological active domains may explain the variability seen in cancer prognosis and the necessity to further

elucidate PTHrP actions in cancer. Alternative splicing and post translational proteolysis generate different PTHrP isoforms and fragments that can elicit various cellular responses. The variety of PTHrP fragments and different actions (autocrine, paracrine, endocrine and intracrine) may justify the complexity of PTHrP induced responses. The role of the different PTHrP fragments and the cell biological responses PTHrP generates are still not fully clarified.

PTHrP has been extensively investigated as an important bone factor in cancers that have significant bone tropism, especially in prostate and breast cancers. Indeed bone is a common site for tumor metastases and skeletal metastasis is the leading cause for mortality and morbidity among breast, prostate and lung cancer patients (Coleman, 2006). Postmortem examination demonstrated that around 70% of patients dying with breast cancer and approximately 90% of prostate cancer patients had evidence of bone metastases (Bubendorf et al., 2000;Coleman, 2006). Other cancers that also metastasize to the skeleton include renal tumors, melanoma and multiple myeloma (Chiang and Massague, 2008;Coleman, 2006).

Radiographic manifestation of bone metastases show different characteristics; osteoblastic lesions demonstrate exacerbated activity of osteoblasts evidenced by abnormal bone formation whereas osteolytic lesions show intensified osteoclast activity evidenced by abnormal bone resorption (Weilbaecher et al., 2011b;Loberg et al., 2005). However, most of the tumors present mixed lesions, with the presence of both osteolytic and osteoblastic aspects (Coleman, 2006). Osteolytic lesions are associated with bone fractures and HHM, a common finding with advanced bone destruction. They are most common in breast, multiple myeloma, melanoma, lung, thyroid, renal, and gastrointestinal malignancies. On the contrary, prostate cancer metastatic lesions are predominantly osteoblastic. Tumor-derived factors such as Wnt family ligands, bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF) and endothelin-1 activate osteoblast bone formation contributing to skeletal metastasis (Weilbaecher et al., 2011b).

The interplay of tumor cells with the bone microenvironment results in tumor growth and bone remodeling in skeletal metastasis (Loberg et al., 2005). Tumor cells

secrete factors such as PTHrP, tumor necrosis factor-α (TNF- α), interleukin-1, interleukin-6, interleukin-8, and interleukin-11 that stimulate bone cells (Chirgwin and Guise, 2007). Activated osteoblasts and osteoclasts in turn, secrete other factors that promote tumor growth, feeding a destructive cascade of metastatic growth (Chirgwin and Guise, 2007). Therefore, skeletal metastasis depends on both priming the seed (tumor cells) as well as nurturing the soil (bone). PTHrP is a pivotal tumor-derived factor playing a role in both steps. For instance, it can act in priming the seed, participating in cell autonomous processes such as tumor cell proliferation, apoptosis, survival and anoikis, qualities that enhance the capability of tumor growth, dissemination and metastasis. Importantly, PTHrP can act as an endocrine or paracrine factor modulating bone responses and cellular aspects of the bone microenvironment, thus contributing to the formation of a conducive environment for cancer establishment in bone.

TUMOR CELL AUTONOMOUS FUNCTIONS OF PTHRP

Tumor-derived PTHrP can act in different ways to modulate tumor growth and progression in a cell autonomous manner. PTHrP can act by paracrine, autocrine and intracrine modes to modulate diverse cell processes (Figure 2.1). The paracrine and autocrine actions of PTHrP derive from PPR activation through binding of amino terminal PTHrP but are not the only mechanism. Post translation protease cleavage generates biological active mid-region and carboxyl terminal PTHrP fragments that can act via paracrine and autocrine fashion through activation of presumably novel cell surface receptors. The C-terminal fragment also known as osteostatin has a role in osteoclast inhibition and bone anabolic actions suggesting an important role in skeletal metastasis although their function in cell autonomous processes in cancer cells are not well defined (de Castro et al., 2012). The intracrine action of PTHrP depends on its nuclear localization sequence (NLS) within the 87-107 amino acid region and regulates cell apoptosis, proliferation and cell cycle. The PTHrP NLS is required and interacts

with importin β1, independently of importin α (Λαμ ετ αλ., 1999) transporting the protein through the nuclear pore complex dependent of microtubule integrity (Lam et al., 2002). Another pathway is through internalization of PPR-PTHrP complex by an endocytosis-dependent pathway to the cytosol and rapid transport into the nucleus (Watson et al., 2000b;Watson et al., 2000a). PPR-PTHrP complexes were reported to be found in the nucleus of osteoblasts in bone and cells in other organs such as kidney, liver, gut and ovary, although the functional mechanisms of PPR-PTHrP complexes are still not fully understood (Watson et al., 2000a;Watson et al., 2000b). In addition, proteins smaller than 40 kDa may be translocated through the nuclear pore complex through mechanisms that are,as yet, unknown owing to the difficulty of visualizing and quantifying the transport (Nardozzi et al., 2010) The possibility of PTHrP peptides (<40 kDa) without the NLS interaction with importin proteins translocating directly through the nuclear pore complex cannot be ruled out, owing to the small size of the molecule, though this would likely be at much slower rates (Nardozzi et al., 2010). Nuclear PTHrP localization can then exert differential cellular responses than what is seen by paracrine and autocrine PTHrP highlighting the great diversity of PTHrP actions. Additional information on intracrine mechanisms of PTHrP can be found in detailed reviews (Nguyen and Karaplis, 1998;Fiaschi-Taesch and Stewart, 2003). Altogether, PTHrP differential actions can promote proliferation, evasion of apoptosis and survival, invasion and migration, and evasion of anoikis, contributing to tumor growth and progression.

Proliferation

PTHrP stimulates tumor cell proliferation in different types of cancer. Recently, a study in breast cancer demonstrated that PTHrP is involved with tumor initiation, growth and metastasis (Li et al., 2011a). In a spontaneous breast cancer model, *PTHLH* gene deletion significantly delayed tumor initiation and tumor growth. Reduced PTHrP expression resulted in reduced proliferation demonstrated by lower Ki67 and cyclin D1 staining as well as cell cycle arrest suggesting an important PTHrP role for breast tumor proliferation (Li et al., 2011a). In prostate cancer, PTHrP also promotes proliferation.

Prostate cancer cells that overexpressed PTHrP had enhanced tumor growth and tumor size in bone (Dougherty et al., 1999). Another study demonstrated that transfected cells that overexpressed PTHrP (1-87) stimulated cell proliferation and the intracrine production of IL-8, a known growth promoting and angiogenic factor (Gujral et al., 2001). The contribution of PTHrP to proliferation is also evident in renal carcinoma. Burton *et al.* demonstrated that autocrine PTHrP induced renal carcinoma cell proliferation and tumor growth, whereas antiserum and antagonists to PTHrP inhibited tumor growth *in vitro* (Burton et al., 1990). Therefore, PTHrP contributes to tumor cell proliferation promoting tumor growth, an important step for subsequent tumor progression and metastasis.

Evasion of Apoptosis and/or Promotion of Survival

PTHrP intracrine actions have been under investigation for their roles in intracellular biology, especially cell survival, growth and apoptosis. In prostate cancer, PTHrP and its nuclear localization sequence was found to be protective against cell apoptosis (Dougherty et al., 1999). Prostate cancer cells that overexpressed PTHrP had enhanced tumor growth and tumor size in bone. In addition, cells with deletion of the NLS were more susceptible to undergo apoptosis than full length PTHrP - transfected cells or controls. These findings indicated a role of PTHrP in prostate cancer cell survival via an intracrine manner. Similar results were also observed in a breast cancer cell line, demonstrating a critical role for nuclear targeting in the anti-apoptotic and cell cycle regulatory effects of PTHrP (Tovar Sepulveda et al., 2002). MCF-7 breast cancer cells that overexpressed PTHrP with the NLS sequence were protected from apoptosis induced by serum starvation and presented cells in G2-M stage of the cell cycle compared with cells overexpressing NLS-mutated PTHrP, indicating an intracrine role for PTHrP in cell apoptosis and cycle regulation. The role of PTHrP autocrine/paracrine actions in cell growth and cell death *in vivo* was demonstrated in renal carcinoma cells, where anti-PTHrP antibody treatment reduced tumor growth by inducing cell death (Massfelder et al., 2004). Neutralizing antibody for

PTHrP was also used against different renal carcinoma cell lines and strategies blocking both the PPR and PTHrP signaling decreased tumor growth by inducing cell apoptosis (Talon et al., 2006). These studies highlight PTHrP as an important growth factor and a survival signal that contributes to tumor growth. Moreover, acquiring apoptosis resistance is an important quality for the survival of cells that eventually enter the circulation and colonize different organs, therefore establishing a metastatic foci.

Invasion and Migration

Intracrine PTHrP signaling is also thought to influence tumor invasion and metastasis. In a prostate cancer study, PC-3 cells that overexpressed intact PTHrP upregulated the expression of the α1, α 5, α 6, and β4 integrin subunits (Shen and Falzon, 2003). The presence of NLS signaling was necessary for the increase in integrin expression known to facilitate cancer cell adhesion, migration and invasion, requirements necessary for cancer cell colonization in skeletal metastasis (Shen and Falzon, 2003). Interestingly, integrin α6 and β4 levels are also increased in colon cancer, suggesting a role of PTHrP in integrin expression in different types of cancers (Shen et al., 2007b). PTHrP also positively regulates LoVo (human colon cancer cells) proliferation, migration and invasion *in vitro* (Shen et al., 2007a). Overexpression of PTHrP augmented xenograft growth and expression of integrins α6 and β4, and phosphatidylinositol 3-kinase (PI3-K) pathway components. PTHrP mediates upregulation of integrin α6β4 expression, activating the PI3-K/AKT pathway (Shen et al., 2007a). A recent study investigated the link between PTHrP expression and Rac1, a GTPase. They demonstrated the PTHrP positive effect on Rac-1 activity was via guanine nucleotide exchange factor Tiam1. Interestingly, the effects of PTHrP expression were mediated by integrin α6β4 activation of PI3-K pathway that regulates both Rac1 and Tiam1 activity (Mula et al., 2010). Therefore, PTHrP expression in prostate and colon cancer is associated with tumor growth, migration and invasion. In addition PTHrP also influenced the expression of the chemokine receptor CXCR4, an adhesion factor expressed in breast cancer that binds to stromal cell-derived factor-1

(SDF-1 or CXCL12) present in bone (Li et al., 2011a). In this study, PTHrP was co-expressed with CXCR4 and was crucial for the metastatic spread. The role of PTHrP facilitating cell invasion and migration consequently contributes to the metastatic spread, by increasing cell motility, enabling cell invasion to the surrounding tissue and facilitating the access of tumor cells to the blood. Tumor cells can then intravasate into the bloodstream and disseminate into different organs where adhesion molecules would facilitate tumor cell adhesion and colonization into the metastatic organ.

Evasion of Anoikis

Anoikis is a phenomenon of cell apoptosis resulting from detachment with loss of cell-matrix interactions. Evasion of anoikis is an essential step in the metastatic process so that the cells can survive and colonize a distant organ (Sakamoto and Kyprianou, 2010). The PTHrP intracrine pathway plays an important role in tumor apoptosis evasion, however little is known regarding the role in anoikis. Recent studies, suggest that PTHrP could be important for anoikis. Bhatia *et al.* demonstrated in an in vitro study that the PTHrP intracrine pathway protected prostate cancer cell lines PC-3 and C4-2 from doxorubicin-induced apoptosis and promoted anchorage-independent cell growth (Bhatia et al., 2009). The intracrine effects of PTHrP were mediated via integrin α6β4-mediated activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, since knockdown of integrin α6β4 decreased the PTHrP-mediated activation of the PI3K/Akt pathway. PTHrP also increased nuclear factor-kappaB (NFkB) activity via a PI3K-dependent pathway. This study suggested a role of PTHrP in anoikis and activation of survival pathways.

Most recently, Park *et al*. investigated the participation of PTHrP and its NLS in the anoikis of prostate cancer (Park and McCauley, 2012) Here, downregulation of PTHrP in PC-3 cells conferred increased apoptosis of cells cultured in suspension. On the other hand, overexpression of the gene resulted in protection from anoikis. LNCap cells that expressed full length PTHrP or nuclear localization localization signal defective cells were generated and cultured under an anoikis challenge. Interestingly,

only full length PTHrP expression was able to rescue cells from anoikis. Investigation of an apoptosis-related gene array demonstrated that gene expression of tumor necrosis factor- alpha (TNF-α), a pro-apoptotic protein, is increased when PTHrP is downregulated and decreased with PTHrP overexpression, but not in NLS-defective PTHrP overexpressing cells. This suggests that the PTHrP-mediated reduction in pro-apoptotic TNF-α is dependent on full length PTHrP to confer anoikis resistance. Moreover, i*n vivo* low-PTHrP expressing cells resulted in fewer metastatic lesions compared with cells overexpressing PTHrP, suggesting an anoikis role due to loss of intracrine PTHrP activity. These findings suggest that PTHrP nuclear localization confers resistance to anoikis and delineates a new mechanism associated with prostate cancer metastasis (Park and McCauley, 2012). Tumor cells can survive after detachment from the primary tumor, and overcome the physical endurances of not having a protective matrix and neighboring cell interactions as well as surviving in the bloodstream, essential steps for metastasis onset.

PTHrP-dependent Expression of Growth Factors

When osteolytic tumors metastasize to bone, they promote a destructive cascade of events also known as “vicious cycle”. PTHrP secreted by tumor cells increases bone resorption and induces bone matrix release of calcium and numerous growth factors such as transforming growth factor- beta (TGF-β), promoting tumor growth in bone. TGF-β signaling is a very important aspect of PTHrP osteolytic actions in bone. Mutation of TGF-β type II receptor in MDA-MB-231 cells resulted in less bone destruction, decreased osteoclasts and prolonged survival in mice (Yin et al., 1999). Conversely, constitutively active TGF-β type II receptor breast cancer cells increased PTHrP production in tumors and enhanced osteolytic bone metastasis (Yin et al., 1999). In this context, a destructive cascade of tumor and bone interactions is established where PTHrP binds to and stimulates the PPR present in osteoblasts and osteocytes to express receptor activator of nuclear factor kappa B ligand (RANKL) leading to osteoclast differentiation and bone loss. Osteoclast-mediated bone resorption then

releases factors such as calcium, TGF-β, insulin-like growth factor 1 (IGF-1) and fibroblast growth factors (FGFs) that favor tumor proliferation and augment PTHrP production. In addition, PTHrP can also induce expression of chemokine (C-C motif) ligand 2 (CCL2 or MCP-1) thus contributing to tumor growth. Li *et al.* demonstrated *in vitro* and *in vivo* that prostate cancer–derived PTHrP induced osteoblastic secretion of CCL2 in bone and PTHrP antagonist treatment inhibited the secretion of CCL2 (Li et al., 2009). CCL2 then, supports tumor growth, progression and metastasis by different means. It can directly stimulate tumor cell migration, proliferation and survival or indirectly establish an appropriate niche for growth eliciting angiogenesis and macrophage recruitment and polarization to the M2 type (pro-tumorigenic macrophages) (Rozel et al., 2009;Mizutani et al., 2009). CCL2 also participates in skeletal metastasis, promoting increased osteoclastic numbers and activity, which are important for tumor growth in bone(Loberg et al., 2007a). These studies provided evidence that PTHrP in bone metastasis is an important modulator for the release and secretion of growth factors such as TGF-β and CCL2, which will further support tumor growth and skeletal metastasis progression.

Overall, PTHrP is a tumor promoting factor involved in each step of metastasis. First, PTHrP contributes to tumor growth in the primary tumor site, promoting cell proliferation, survival and evading apoptosis. Subsequently, PTHrP participates in cell invasion and migration required to penetrate the surrounding tissue and gain access to the circulation. Next, PTHrP participates in anoikis evasion, so that tumor cells can survive and extravasate into a secondary site where they can establish a metastatic growth. Finally, when tumors metastasize to bones, PTHrP still acts on the bone microenvironment to induce a destructive cascade with release of numerous growth factors that contribute to tumor growth and enhanced PTHrP expression. Hence, PTHrP participates in all steps of the metastatic processes, from tumor growth, progression, invasion, migration and survival to bone modulation to support tumor growth as summarized in figure 2.1.

ROLE OF PTHrP IN THE METASTATIC MICROENVIRONMENT

PTHrP actions in skeletal metastasis are not only restricted to the tumor cell autonomous functions but also in the modulation of the bone marrow microenvironment. Extensive evidence demonstrates that PTHrP is a tumor promoting factor. However, emerging evidence supports that PTHrP can also modulate the bone microenvironment, providing a congenial “soil” for tumor metastasis. Evidence is emerging that PTHrP nurtures the “soil” to house and subsequently “feed” the disseminated cells, leading to metastatic onset and growth. Evolving PTHrP participation in the modulation of the bone metastatic environment includes modulation of the cellular contents and promotion of angiogenesis, all of which are known to contribute to metastasis (Figure 2.2). In this section, recent findings in PTHrP actions in the bone microenvironment will be discussed.

PTHrP Actions in Bone: Direct and Indirect Effects

PTHrP binds to PPRs primarily expressed in osteoblasts, osteocytes, and bone marrow stromal cells such as osteoblast precursor cells. However, the net effects of PTH/PTHRP on bone (i.e. anabolic or catabolic) are dependent on the duration and exposure. For example, intermittent administration of PTH *in vivo* results in bone formation while continual infusion of PTH causes significant bone loss (Datta and Abou-Samra, 2009). In a recent study, Horwitz *et al.* investigated the effect of continuous infusion of human PTH (1-34) or human PTHrP (1-36) at low doses (2 and 4 pmol/kg/h, respectively) in healthy adult volunteers for 7 days (Horwitz et al., 2011). Continuous infusion induced hypercalcemia and hypercalciuria and rapidly increased bone resorption. Interestingly, bone formation was suppressed by 30 to 40% causing sustained arrest in the osteoblast maturation program. Indeed PTHrP has a direct effect on osteoblast cell cycle that is dependent on the developmental stage (Datta et al., 2005;Datta et al., 2007). Differentiated osteoblast treated with PTH/PTHrP had upregulated JunB expression with reduction in cyclin D1 and G1 cell cycle arrest (Datta

et al., 2005). Such findings suggest that PTHrP may influence the life span and activity of osteoblasts in bone.

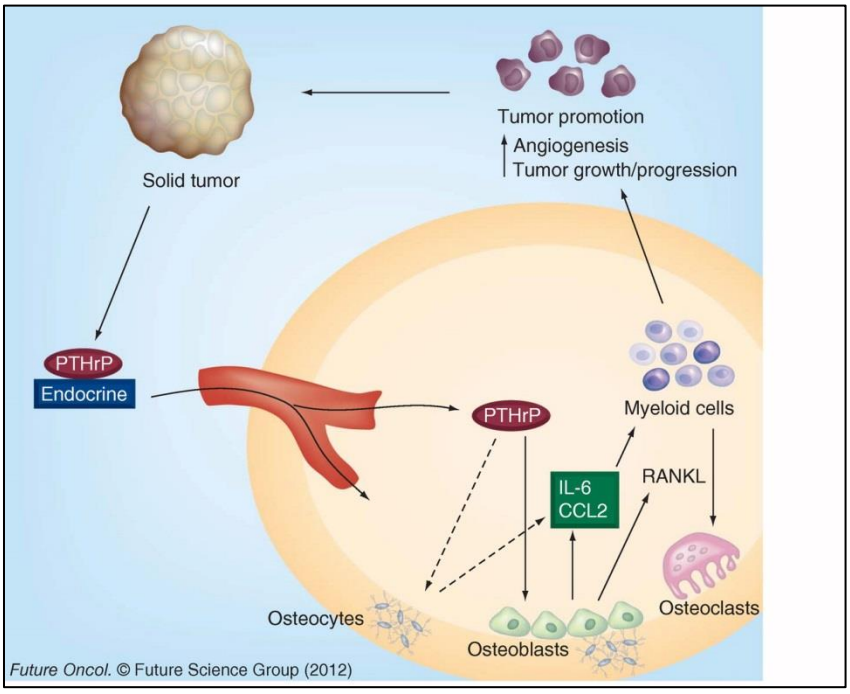

Figure 2.2. PTHrP actions in the modulation of bone microenvironment and a potential role for a premetastatic niche formation.

Tumor-derived PTHrP endocrine actions in bone are an inadequately studied area. An evolving model suggests PTHrP modulates the bone microenvironment by inducing osteoblast and potentially osteocyte secretion of CCL2 (MCP-1) and/or IL-6, which in turn mediates expansion of myeloid cells, such as macrophages and myeloid-derived suppressor cells, which are recruited to the tumor site, contributing to tumor growth, angiogenesis and progression. Moreover, growing evidence demonstrates that PTHrP is a potential candidate for premetastatic niche formation in bone with the expansion of these myeloid cells, forming a convivial niche for metastatic growth in bone. Permission for figure from Future Medicine (Soki et al., 2012)

Solid lines: Known pathways; Dashed lines: Potential pathways.

Osteoblasts are not the only cell responsible for RANKL production and bone remodeling. Osteocytes are cells located within bone matrix embedded and surrounded by mineral tissue and are the major cells present in bone, comprising 90-95% of all bone cells in adults. They are also the longest lived bone cells being able to survive up to decades (Bonewald, 2011). In the past 10 years much attention has been placed on osteocyte functions, shifting from only bystander cells into key players of the bone microenvironment. Recently, two independent groups investigated the role of osteocytes in vivo and found an important role in bone remodeling, being the main source of RANKL for osteoclastogenesis (Xiong et al., 2011;Nakashima et al., 2011). These studies challenged the dogma in bone biology, that osteoblasts are the key cells that modulate bone remodeling and bone coupling. For example, PTH and PTHrP actions were believed to be mediated in great part, if not exclusively, by osteoblast activation. However, osteocytes also express PPRs, therefore suggesting a significant role in the actions of both peptides. In a recent study, specific osteocyte deletion of PPR resulted in mild osteopenia, increased sclerostin expression and impaired homeostatic calcemic response demonstrating a significant role of PTH/PTHrP signaling in bone remodeling and homeostasis (Powell, Jr. et al., 2011). Since bone metastasis requires interactions of tumor cells and bone cells, the osteocytes as potential PTHrP responsive cells could be playing a role in modulation of the microenvironment with the secretion of different growth factors mediating not only the tumor growth but the bone microenvironment. These events would favor metastatic growth and progression, yet, such a role of PTHrP in osteocytes has not been delineated.

PTHrP actions are restricted not only to direct effects on bone cells such as osteoblasts and osteocytes; through the activation of these cells, PTHrP induces the release of a variety of growth factors and cytokines derived from activated cells as well as the bone matrix with the effect of modulating other cellular components, such as stromal cells and immune cells, which could be playing important roles in the metastatic ‘soil’ Emerging evidence suggests that PTHrP may also play a role in inflammatory responses associated with HHM. Studies demonstrated that PTHrP concomitant expression of inflammatory cytokines such as TNFα, interleukin-1 alpha (IL-1α) and IL-6

augment bone resorption activity (Sato et al., 1989;Uy et al., 1997;de la Mata et al., 1995). More evidence is necessary to delineate the regulation of PTHrP and cytokine expression in a cancer context. However, substantial advances have linked PTHrP actions in inflammatory responses and diseases (Funk, 2001) highlighting a possible role in cancer, since it is considered a "wound that never heals" and an inflammatory aspect has been strongly implied in its progression. Further studies are needed to explore PTHrP function in the cellular milieu of the bone microenvironment, the growth factors and cytokines expressed and how these may contribute in tumor growth and metastasis.

Angiogenesis

Angiogenesis is a well-studied process supporting tumor growth and progression. Growing evidence proposes that PTHrP can impact skeletal metastasis progression via stimulation of angiogenesis. Akino *et al.* first described a direct effect of tumor-derived PTHrP in angiogenesis, after observation that a metastatic pituitary tumor cell line (GH3) that expressed high levels of PTHrP had increased vascularity in xenografts. Using *in vitro* studies they demonstrated that PTHrP did not affect endothelial cell proliferation and migration but dose-dependently stimulated capillary tube formation (Akino et al., 2000). Although a contradictory study argued that PTHrP was an angiogenesis inhibitor functioning by activation of protein kinase A (PKA), little evidence exists to support this hypothesis (Bakre et al., 2002). In fact, a recent study in a spontaneous breast cancer mouse model with specific *PTHLH* gene deletion, demonstrated that PTHrP expression not only affected tumor initiation, progression and metastasis but also influenced tumor angiogenesis. PTHrP ablation resulted in reduced angiogenesis (Li et al., 2011a). In addition, Gujral *et al.* investigated the role of PTHrP in IL-8 production in prostate cancer cells, a known contributing factor for tumor angiogenesis and growth. Transfected cells that overexpressed PTHrP (1-87) and (1-173) stimulated cell proliferation and the production of IL-8, but not VEGF, suggesting a specific IL-8 response. Surprisingly, the PTHrP (65-87) region was required for PTHrP

1-87 to robustly stimulate IL-8 in prostate cancer cells. Since exogenous PTHrP (1-36 and 1-87) did not affect IL-8 expression they concluded that PTHrP (1-87) was required for intracrine IL-8 enhanced production by PTHrP (Gujral et al., 2001). A PTHrP paracrine effect in angiogenesis in bone metastasis has been also investigated. Liao *et al.* showed in *in vitro* studies that the PTHrP pro-angiogenic effect was dependent on the presence of bone marrow stromal cells (Liao et al., 2008). A potential mechanism could be through PTHrP mediated osteoblastic secretion of CCL2, a known angiogenic factor (Zhang et al., 2010;Roca et al., 2009;Li et al., 2009). Indeed recent data demonstrate that the PTHrP angiogenic effect is dependent on osteoclast activity and MMP9 production (Cackowski et al., 2010). Further studies are necessary to elucidate the PTHrP role in tumor angiogenesis especially in bone metastasis.

In summary PTHrP activates cells in the bone microenvironment promoting angiogenesis thus priming the bone microenvironment for a conducive metastatic onset and growth in bone. There is convincing evidence that PTHrP participates in angiogenesis in bone, yet the precise role of angiogenesis in skeletal metastasis needs further elucidation.

PTHrP as a therapeutic target.

Given the multiple roles PTHrP have in hypercalcemia of malignancy and in cancer autonomous and metastatic setting in bone, PTHrP is a potential therapeutic target. Strategies utilizing neutralizing antibody, small molecular inhibitors or targeting the signaling pathways PTHrP elicits are promising to inhibit PTHrP actions. Neutralizing antibodies demonstrated positive responses in animal models, reducing skeletal metastasis, bone lesions and also hypercalcemia (Guise et al., 2002;Onuma et al., 2005). However, human clinical evidence is lacking.

In addition to direct inhibition of PTHrP actions, chemotherapeutic drugs can also result in suppression of PTHrP production. Furugaki et al demonstrated that erlotinib, an EGF receptor tyrosine kinase inhibitor, reduced osteolytic bone resorption induced by lung cancer cells through its effect in the RANKL production by osteoblasts/stromal cells

(Furugaki et al., 2011). Interestingly, erlotinib also suppressed the production of osteolytic factors including PTHrP production. Lorch et al. also demonstrated the role of EGF receptor in two different squamous lung carcinoma xenografts that had reduced PTHrP expression after treatment with EGF receptor tyrosine kinase inhibitor PD153035 and gefitinib (Lorch et al., 2007). Moreover, targeting cells that release the growth factors and cytokines known to stimulate PTHrP production such as TGFβ may also contribute for the decrease expression of PTHrP (Yin et al., 1999).

Concurrently with the need to develop new strategies to inhibit PTHrP actions is the need to fully define PTHrP roles in the different stages of cancer. The controversial results seen on cancer prognosis depending on the cancer stage as protective to destructive raises concerns on the right time PTHrP therapy should be applied. Moreover, the variety of PTHrP isoforms and fragments generated by posttranslational proteolysis highlight the different potential roles PTHrP can have in multiple cell types. Still, many answers are needed to safely test potential therapies against PTHrP.

PTHrP, the premetastatic niche and tumor dormancy

PTHrP is a potent and efficient tumor promoting factor, acting in two different compartments: the tumor and the bone microenvironment. This dual role makes PTHrP an outstanding therapeutic target, especially for tumors with high bone tropism such as breast and prostate cancer. Although extensive studies investigated the actions of PTHrP in cellular autonomous tumor function, PTHrP involvement in modulating the metastatic microenvironment warrants extensive further investigation. For instance, PTHrP when functioning as an endocrine factor secreted by primary tumors, regulating the bone microenvironment at a distance could be an important premetastatic niche factor in skeletal metastasis.

This paradigm proposes the existence of a premetastatic niche that provides a permissive microenvironment for the recruitment of tumor cells leading to micrometastasis initiation and establishment (Kaplan et al., 2006). Studies in lung metastasis demonstrated participation of bone marrow derived cells in the formation of

the premetastatic niche before the arrival of tumor cells in the lungs (Kaplan et al., 2005;Hiratsuka et al., 2008;Hiratsuka et al., 2006). Hematopoietic progenitor cells expressing VEGFR1 and fibronectin clustered in tumor-specific metastatic sites, suggesting the formation of a premetastatic niche that contributes to disseminating tumor cell engraftment in the lungs (Kaplan et al., 2005). In addition, conditioned media from distinct tumor types with different patterns of metastatic dissemination redirected the formation of these clusters and transformed the metastatic profile, therefore dictating organ-specific tumor metastasis. In addition to hematopoietic progenitor cells, macrophage recruitment has also been implicated in premetastatic niche formation (Hiratsuka et al., 2006;Hiratsuka et al., 2008). In 2006 Hiratsuka *et al* demonstrated, in a lung premetastatic and metastatic phase, that factors released by subcutaneous tumors induced expression of inflammatory proteins S100A8 and S100A9 in lungs which triggered macrophage recruitment to the site (Hiratsuka et al., 2006). Antibodies targeting S100A8 and S100A9 resulted in 80-90% reduction of colonized tumor cells to lungs. Later they demonstrated that Serum amyloid A3(SAA3) by acting through Toll-Like receptor 4 (TLR4) on macrophages and tumor cells , mediated S100A8 and S100A9 expression specifically in the lung (Hiratsuka et al., 2008).

In bone, the formation of a premetastatic niche is not well defined. The lack of spontaneous skeletal metastasis models challenges advances in this area. However, most evidence is focused mainly in the context of endocrine-like actions that modulate the bone microenvironment. Factors other than PTHrP that are secreted by tumors and that can modulate the bone microenvironment from a distance provide evidence of potential premetastatic niche formation in skeletal metastasis. For example, heparanase is an enzyme produced by breast cancer cells that cleaves heparan sulfate to produce syndecan-1. Tumor-derived syndecan-1 shed in the primary tumor acts in bone, increasing osteoclastogenesis and contributing to osteolysis (Kelly et al., 2005;Kelly et al., 2010). Other factors such as, osteopontin (OPN) and matrix metalloproteinase (MMP) may also play a role in promoting tumor growth and skeletal metastasis (Anborgh et al., 2010;Lynch et al., 2005).

Indeed, PTHrP is also an attractive potential factor for the premetastatic niche formation in bone. For instance, PTHrP can modulate the production of CCL2 in bone by osteoblasts, inducing macrophage recruitment, activation into M2 tumor-promoting cell and also stimulating osteoclastogenesis that will altogether enhance tumor growth and progression (Mizutani et al., 2009;Li et al., 2009;Rozel et al., 2009;Loberg et al., 2007a). This suggests a potential mechanism for a premetastatic niche formation in the bones, where tumor derived–PTHrP induces CCL2 expression in osteoblasts contributing to modulation of the bone microenvironment into a conducive niche. In conclusion, although the premetastatic niche formation is not yet defined in bone metastasis, PTHrP is a potential candidate for the endocrine actions in bone modulation and premetastatic niche formation (Figure 2.2)

Bone consists of an assorted cellular profile and PTHrP actions in this context are under explored. For example, myeloid cells such as macrophages have been associated with tumor progression and metastasis of different types of cancer as well as contributing to premetastatic niche formation (Pollard, 2004;Hiratsuka et al., 2008;Hiratsuka et al., 2006). Since macrophages share the same precursors as osteoclasts, PTHrP may indirectly regulate the myeloid population in bone and skeletal metastasis. A possible mechanism would be by PTHrP mediated osteoblastic secretion of CCL2 (Li et al., 2009). Another population likely involved in tumor progression and metastasis is myeloid derived suppressor cells (MDSCs) that are immature myeloid cells involved in immune suppression and tumor escape from host control, as well as angiogenesis and tumor growth (Younos et al., 2011). MDSCs are identified by the expression of myeloid cell (CD11b) and granulocyte (Gr-1) markers and are increased in bone marrow, spleen and peripheral blood in tumor-bearing hosts (Younos et al., 2011). Unfortunately, their role in skeletal metastasis is not yet defined, but possible roles have been suggested as a potential source for angiogenesis and osteoclastogenesis, contributing to the development of osteolytic lesions and the progression of metastasis (Yang et al., 2010). Tumor-derived PTHrP is involved in the expansion and potentiation of MDSCs in the bone marrow that are recruited to the tumor tissue contributing to tumor angiogenesis and growth (Park et al., 2011a).

Myeloid derived cells such as macrophages and MDSCs have been implicated in tumor growth, angiogenesis, immunosuppression, as well as mechanistic aspects for the formation of a premetastatic niche. PTHrP modulation of these cells in bone and how they can contribute to skeletal metastasis is an understudied area with future potential.

Another evolving area of study is tumor dormancy. Tumor cell dormancy is one of the biggest problems in skeletal metastasis and believed to be associated with tumor relapse (Gao et al., 1999). Metastasis is a very inefficient process where less than 0.01% of the tumor cells that achieve the circulation form metastatic foci (Fidler, 2003;Langley and Fidler, 2011). The skeleton is a very complex environment with constant bone remodeling, hematopoiesis and a very rich milieu with growth factors and mineral components being continually released. Therefore, the fact the bone is one of the common site of metastasis is not surprising. It is thought that tumor dissemination is a process that occurs earlier, but in this process most of the cells fail to overcome the challenges imposed by entering the circulation (Fidler, 2003). However, it has also been proposed that when cells circumvent this challenge and find a distant organ to colonize, they can stay dormant for decades (Nguyen et al., 2009). This may be the reason why patients with solid tumors that were completely excised present with bone metastasis decades later, even if the primary tumors were not present any longer. Another problem of cell dormancy is that their detection is not conceivable. Most of skeletal metastases diagnoses are made only when tumors are visible and in advanced stages. Cell quiescence is also a big challenge in cell dormancy since most anti-cancer therapies target the high and fast proliferative cells. Detection of circulating tumor cells in the bone marrow has raised the awareness that bone could also be a potential tumor cell housing place (Gao et al., 1999). In this case, bone could offer a shelter where tumor cells attach and stay quiescent until they can be recruited to the other sites or even go back to their place of origin. PTHrP makes an interesting candidate for abetting cell dormancy because it not only acts via intracrine and autocrine modes to modulate gene expression and cellular responses in tumors, but also has paracrine modulation of the bone microenvironment. The action of the PTHrP in the regulation of cell cycle and expression of integrins, could be useful for the tumors to attach to the

bone and acquire quiescence, until they get activated to proliferate and form detectable metastasis. As little is known about PTHrP in cell dormancy, this is an area of potential promise for elucidation and therapeutic targeting.

CONCLUSION

PTHrP is an important protein not only for normal physiological processes such as in bone and mammary development, but also as an important player in different cancers and skeletal metastasis. PTHrP is a key regulator of tumor-bone interactions and regulates cells in the bone microenvironment, through proliferative and pro-survival activities that prime the “seed” and the “soil” of the metastatic lesion. In summary, PTHrP multi-faceted actions as an endocrine, paracrine, autocrine and intracrine peptide display the variety of biological functions it can regulate at the cell level.

FUTURE PERSPECTIVES

Advances in the area of bone biology, such as the identification of osteocytes as potential key players in bone regulation bring novel concepts and expand the influence that PTHrP may cause in bone. In fact, understanding of PTHrP actions in bone is a crucial step to dissect the mechanisms for tumor cell growth and bone metastasis. Moreover, novel concepts in cancer research need to be applied and tested for PTHrP functions. For example, the fact that PTHrP exerts an endocrine function in bone in the case of hypercalcemia of malignancy suggests that PTHrP could also modulate different organs via an endocrine mode. Therefore, in bone, PTHrP is a potential premetastatic niche factor and further investigations in this area are needed to dissect the earlier steps of cancer metastasis. Another area that is under investigated is tumor cell dormancy and how this affects the onset of metastasis. Improved animal models and

specific molecular markers are needed to investigate these novel theories and concepts. Understanding the earlier steps of tumor progression and metastasis is necessary for the development of new therapeutic targets to overcome cancer.

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CHAPTER 3

THE EFFECTS OF ZOLEDRONIC ACID IN THE BONE AND VASCULATURE SUPPORT OF HEMATOPOIETIC STEM CELL NICHES

ABSTRACT

Hematopoietic stem cells (HSC) are maintained in a tightly regulated bone microenvironment constituted by a rich milieu of cells. Bone cells such as osteoblasts are associated with niche maintenance as regulators of the endosteal microenvironment. Bone remodeling also plays a role in HSC mobilization although it is poorly defined. The effects of zoledronic acid (ZA), a potent bisphosphonate that inhibits bone resorption, were investigated on bone marrow cell populations focusing on HSCs, and the endosteal and vascular niches in bone. ZA treatment significantly increased bone volume and HSCs in both young and adult mice (4 week and 4 month old, respectively). ZA increased vessel numbers with no overall change in vascular volume in bones of young and had no effect on vasculature in adult mice. Since both young and adult mice had increased HSCs and bone mass with differing vasculature responses , this suggests that ZA indirectly supports HSCs via the osteoblastic niche and not the vascular niche. Additionally, gene expression in Lin- cells demonstrated increased expression of self-renewal-related genes Bmi1 and Ink4a suggesting a role of ZA in the modulation of cell commitment and differentiation toward a long-term self-renewing cell. Genes that support the osteoblastic niche, BMP2 and BMP6 were also augmented in ZA treated mice. In conclusion, ZA-induced HSC expansion occurs independent of the vascular niche via indirect modulation of the osteoblastic niche.

INTRODUCTION

Bone is an important hematopoietic organ tightly regulated by the diverse cellular components that constitute its microenvironment. This unique microenvironment, also known as the stem cell niche, maintains and regulates hematopoietic stem cells (HSCs). HSCs are found in close association with the endosteal interface of bone and bone marrow, and perivascular sinusoidal blood vessels (Adams et al., 2006;Kiel et al., 2005). Osteoblasts, endothelial cells, adipocytes and stromal cells are key cells that constitute the niche and participate in the regulation and maintenance of HSCs (Bianco, 2011). The hematopoietic niche is necessary to balance the expansion and mobilization of HSCs to the peripheral blood as well as the regulation of self-renewal capacity and hematopoietic commitment to differentiation. Moreover, the bone microenvironment plays an important role in this regulation. For instance, studies have demonstrated that increased osteoblast number augments HSC numbers in bone marrow (Zhang et al., 2003;Calvi et al., 2003) whereas, osteoblast depletion leads to a reduction in HSC numbers (Visnjic et al., 2004). It not well defined whether enrichment of bone structures such as vasculature or the endosteum can alter the HSC pool in bone marrow. In this context, therapies that involve modulation of bone formation or remodeling may affect HSC expansion and mobilization.

Osteoclasts are believed to play a role in the bone microenvironment and consequently affect the HSC microenvironment. For instance, osteoclast- induced bone resorption is responsible for the release of various growth factors, proteins and bone mineral such as calcium, all of which are known to influence in the hematopoietic cells and niche maintenance and mobilization (Adams et al., 2006;Kollet et al., 2006). Still, the precise role of osteoclasts in HSC niche maintenance and mobilization is ill defined and controversial. Although augmented osteoclast activation results in increased stress-induced mobilization of HSCs (Cho et al., 2010), osteopetrotic animal models and pharmacological strategies of osteoclast inhibition resulted in increased mobilization of HSCs in response to G-CSF stimulation (Miyamoto et al., 2011).

Pharmacologic strategies such as bisphosphonates inhibit osteoclast-mediated bone resorption. Zoledronic acid (ZA) is a potent third generation bisphosphonate

utilized for treatment of metabolic bone diseases such as osteoporosis, Paget's disease and cancer-related bone diseases. The high affinity for hydroxyapatite results in accumulation in bone that when it is resorbed by osteoclasts inhibition of the mevalonate pathway and apoptotic cell death occurs (Rodan and Fleisch, 1996;Winter and Coleman, 2009). However, the mechanisms of which osteoclast inhibition affects the HSC niche and progenitor cells expansion are yet unclear.

It was hypothesized that osteoclasts may play an important role in hematopoiesis in the bone marrow. In this study, mice were treated with ZA, and HSCs in bone analyzed. Altogether, the treatment differential response in the different hematopoietic progenitor and stem cells suggests that osteoclast inhibition and changes in the bone microstructure may play a role in the increased hematopoietic stem cells in the bone marrow.

MATERIALS AND METHODS

Treatment with zoledronic acid (ZA)

All animals were maintained in accordance with institutional animal care and use guidelines and experimental protocols approved by the Institutional Animal Care and Use Committee of the University of Michigan. Male C57BL/6J mice at 4-or 16-wks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). Intraperitoneal injections were performed twice/week with 200 μg/kg of ZA (Zometa, Novartis, Stein, Switzerland) or vehicle (saline) for up to 4 weeks. At sacrifice, blood and hind limbs were collected for analyses.

Flow cytometry analysis (FACS)

One femur and one tibia were collected and flushed with FACS buffer (PBS, 1%FBS, 2nM EDTA). The enriched central bone marrow cells were filtered through a 100μm cell strainer and cells were enumerated and resuspended to a total of 5 million cells/ 500 μL. Lin cocktail (APC), Sca-1 (E13-161.7, PE), c-kit (2B8, FITC) antibodies were used to quantify the Lin- $Sca1^+$ $c\text{-}Kit^+$ cells. To evaluate the SLAM population

(CD48$^-$ CD41$^-$ CD150$^+$ Sca1$^+$ c-kit$^+$) cells were incubated with the antibodies Sca-1 (E13-161.7,biotin), c-kit (2B8, APC), CD48 (HM48-1,FITC) and CD41 (MWReg30, FITC), CD150(TC15-12F12.2, PE-Biolegend). For the long term reconstitution assay analysis, peripheral blood cells were stained with CD3, CD5, B220 (Ra3-6B2, PE). Endothelial progenitor cells (EPC) were identified as CD11b$^-$ (M1/70, APC) CD34$^+$(RAM34, FITC-eBioscience) and flt1$^+$(Avas12a1, PE-eBioscience) cells. After antibody incubation cells were washed twice with PBS and evaluated with a FACs Calibur (BD Bioscience, San Jose, CA, USA). All other antibodies used for flow cytometry were purchased from BD Bioscience.

Long- Term Competitive Reconstitution Assays

For *in vivo* enumeration of HSCs, 300,000 CD45.2 bone marrow cells were isolated from ZA-treated and untreated C57BL/6 mice and were mixed with 300,000 CD45.1 cells from CD45.1 congenic mice. Cells were then intravenously injected into CD 45.2/CD45.1 recipient mice that had been lethally irradiated with 850 cGy of irradiation. Four weeks after transplantation, the frequency of the different cell populations were determined in the peripheral blood (PB) by fluorescence-activated cell sorting (FACS) using PE-CD45.1 and FITC-CD45.2 antibodies (BD Pharmingen) over a 3 month period.

Histology and immunohistochemistry

After 4 weeks of treatment, mice were sacrificed and tibiae were collected and fixed in 10% formalin. Decalcification was performed in 10% EDTA for 14 days prior to paraffin embedding. Paraffin-embedded specimens were sectioned at 5 μm and stained with hematoxylin and eosin (H&E), or tartrate-resistant acid phosphatase (TRAP) to detect osteoclasts (Acid Phosphatase, Leukocyte Kit, Sigma, St. Louis, MO) or with Von Willebrand factor (vWF) to identify vascular spaces. For microvessel density (MVD) analysis, after vWF staining, four random areas were selected. Any single or cluster of positively stained endothelial cells that was clearly separated from adjacent micro-vessels was considered as one countable microvessel. The average MVD was

determined for each specimen. Mayer's hematoxylin (Sigma) was used for counterstaining.

RNA isolation and Quantitative PCR assay

One tibia and femur were flushed with PBS and cells were sorted into Lin $^{-}$, Lin^{+} using magnetic cell sorting microbeads (MACS). After sorting, cells were pelleted and RNA was isolated with Tri reagent (Sigma) following the manufacturer's protocols. One microgram of total RNA was reverse transcribed in a 20-μL reaction volume containing random hexamers using a reverse-transcription assay system (Applied Biosystems, Foster City, CA, USA). Quantitative reverse transcription-PCR (RT-PCR) was performed using the ABI PRISM 7700 with a ready-to-use mix of primers and FAM-labeled probe assay system (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference and the delta delta CT method were used to calculate the data.

Serum TRAP5b and calcium measurement

Serum TRAP5b activity was measured by ELISA (Immunodiagnostic Systems, Inc.) following the manufacturer's instructions. A quantitative colorimetric assay with Calcium Reagent Set (Pointe Scientific, Inc., Canton, MI) was used to measure the total calcium levels in serum and bone marrow supernatants.

Mirofil and Micro CT analyses

A perfusion technique using the radiopaque silicone rubber injection agent Microfil ™ followed by microCT analysis was utilized as previously described (Park et al., 2012b;Guldberg et al., 2008). Briefly, mice treated with vehicle or zoledronic acid for 4 weeks were anesthetized and perfused with lactated Ringer's solution containing heparin, followed by 10% neutral buffered formalin, and then with a Microfil™ compound of 1.04 specific gravity mixed 4:1 with a 0.92 specific gravity diluent. Femora, tibiae and spleens were dissected, and bones were fixed and decalcified. Samples were scanned in air at a 7um voxel size by microcomputed tomography (eXplore Locus SP (GE Healthcare Pre-Clinical Imaging, London, ON, Canada).

Regions of interest were defined for both central bone vascularity and vascular regions near the growth plate, and quantitative differences in vessel numbers and sizes were determined using the stereology package of commercially available software (MicroView v2.2, GE Healthcare Pre-Clinical Imaging, London, ON, Canada)..

Statistical analysis

The GraphPad Instat 3 software program (GraphPad Software, San Diego, CA) was used to analyze the differences by one-way ANOVA or Student's t-test for independent analysis. The value $p<0.05$ was considered statistically significant. All assays were repeated at least twice with similar results.

RESULTS

ZA treatment increased bone area and osteoclast activity in young mice

To elucidate the role of osteoclasts in the bone microenvironment, 4 week old mice were treated with vehicle (saline) or zoledronic acid (ZA) twice a week for 4 weeks. As expected, ZA treated mice had increased bone volume, increased trabecular thickness and number and decreased trabecular spacing (Fig.3.1 A-D). Serum tartrate-resistant acid phosphatase 5b (TRACP 5b) serum levels were decreased (Fig3.1E) as well as osteoclast numbers in marrow following ZA treatment (Fig. 3.1F). ZA treatment decreased the calcium levels in the bone marrow (Fig. 3.1G) but did not alter serum calcium levels (Fig. 3.1H) as previously reported (Li et al., 2011b). Altogether, the inhibition of osteoclasts by ZA resulted in increased bone mass.

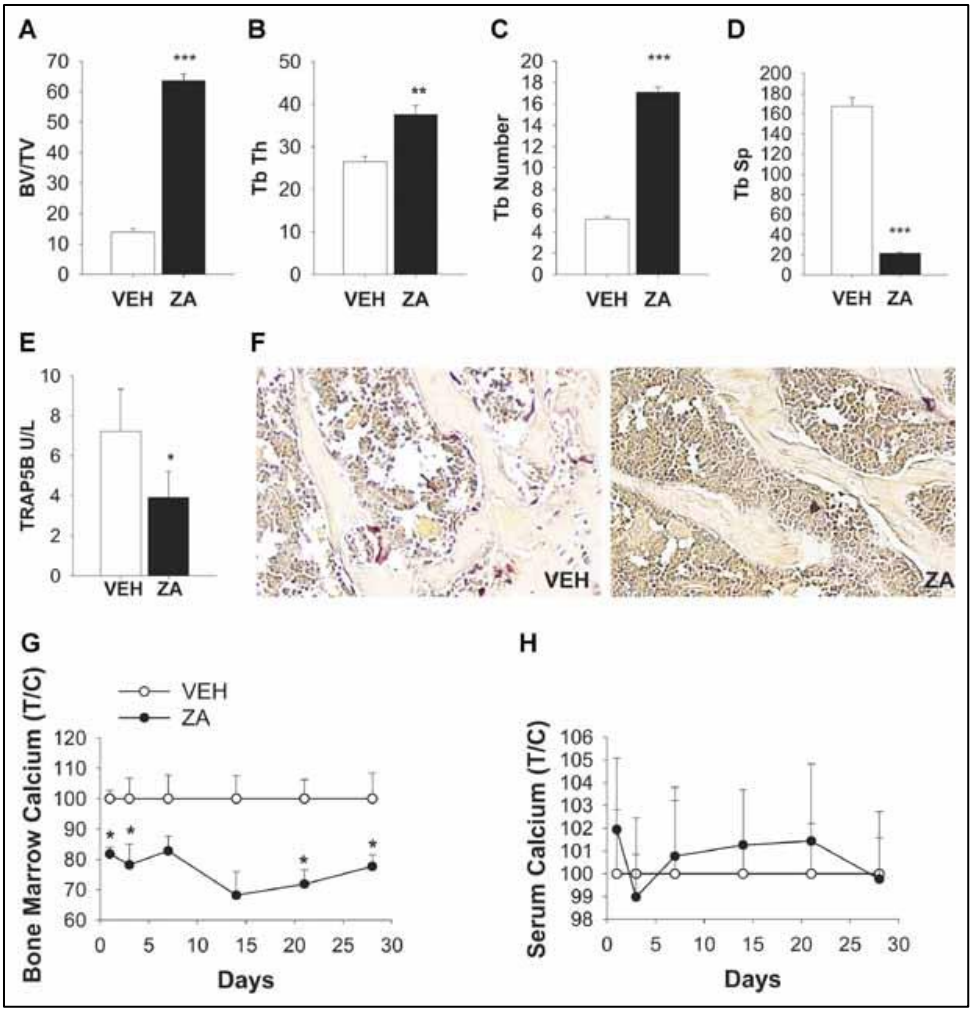

Figure 3.1. ZA treatment effects on bone area and osteoclast activity.

Four-week-old C57BL/6J male mice were treated with 200 µg/kg of ZA twice/week for 4 weeks. A–D: ZA treatment increased bone area. (A–D) Histomorphometric analysis of tibiae shows an increase of bone volume (BV/TV %), trabecular thickness (Tb Th, mm) and number(Tb number mm^{-1}), and decreased trabecular spacing (Tb Sp,) in tibiae from ZA treated mice versus vehicle (VEH); E: blood serum TRAP5b levels were reduced with ZA treatment; F: representative TRAP staining of tibia; G–H: bone marrow calcium levels were decreased and serum calcium levels were not changed; Calcium levels were measured in the bone marrow (G) and serum (H) of mice treated with ZA or vehicle for up to 4 weeks; (T/C) Treatment/control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$ versus vehicle [VEH]). Data are presented as mean ± SEM, n = 8/gp (A–E, G–H). Permission for figure from J Cell Biochem (Soki et al., 2013).

Hematopoietic progenitor cells were increased after ZA treatment.

To investigate the effects of osteoclast inhibition on hematopoiesis, mice were treated with ZA or vehicle for 4 weeks and the bone marrow cells were analyzed by FACS at different time points. Hematopoietic progenitor cells ($Lin^-Sca\text{-}1^+c\text{-}kit^+$ referred to as LSK) were significantly increased when compared to the vehicle treated mice (Fig. 3.2A). Since hematopoietic progenitor cells were increased the long term reconstitution hematopoietic stem cells were also analyzed using the SLAM markers ($CD48^-CD41^-CD150^+Sca1^+c\text{-}Kit^+$) as previously described (Kiel et al., 2005). As was observed with the number of LSK cells following ZA treatment, a trend of increased SLAM cells was observed with ZA treatment although it did not reach statistical significance (P=0.056) (Fig. 3.2B).

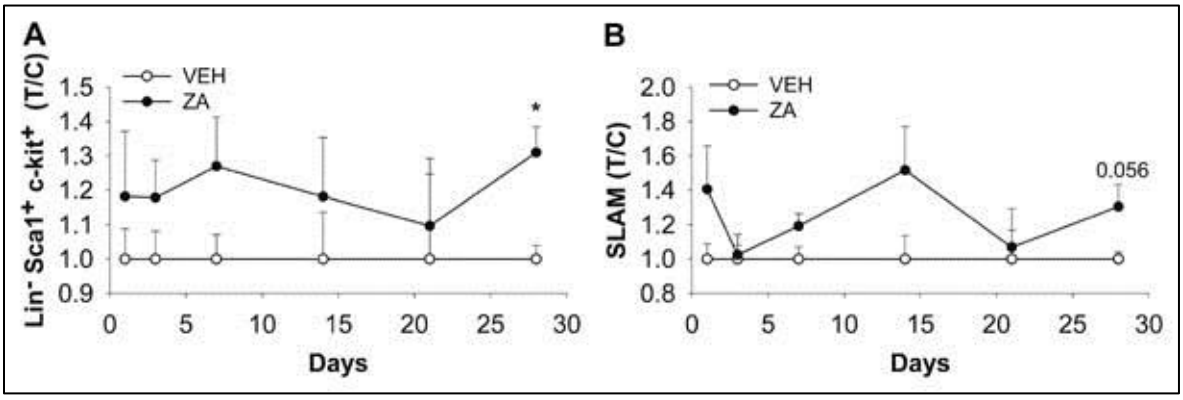

Figure 3.2. ZA treatment increased LSK population but not long-term hematopoietic stem cells.

Mice were treated with 200 µg/kg of ZA twice/week for 4 weeks and bone marrow cells were analyzed by flow cytometry. A: ZA treatment increased bone marrow $Lin^-Sca1^+c\text{-}Kit^+$ hematopoietic progenitor cells after 4 weeks of treatment versus vehicle (VEH) control. B: ZA treatment did not affect the long-term hematopoietic stem populations as identified using SLAM markers ($CD48^-CD41^-CD150^+Sca1^+c\text{-}Kit^+$; $^*P < 0.005$) T/C = treatment/control. Data are presented as mean ± SEM, n = 5/gp. Permission for figure from J Cell Biochem (Soki et al., 2013)

Long term reconstitution of lymphoid cells was higher in ZA treated mice

Since hematopoietic cells bearing the LSK and SLAM phenotype were increased in mice treated with ZA, their capacity for long term reconstitution was analyzed. Bone marrow cells were collected from isogenic CD45.1 mice treated with ZA or vehicle and mixed with CD45.2 donor cells at equal cell numbers (Fig 3.3A). Cells were transplanted into recipient mice (CD45.2) that had received lethal irradiation and engraftment of the CD45.1 donor cells was monitored in blood over a 3 month period. Lymphoid cells were increased in mice treated with ZA with higher B and T lymphocytes (Fig. 3.3 B,C). There were no differences in the myeloid cell populations (Fig. 3.3D).

Collectively the data show that HSCs are increased in the marrow following ZA treatment. To explore the mechanisms which could account for these data three potential pathways which could lead to increased HSCs were explored : 1) ZA reduced hematopoietic stem cell egress or mobilization from the marrow and subsequent retention of the cells in the bone marrow, 2) ZA increased endosteal or vascular niches allowing for greater localization of HPCs/ HSCs or 3) ZA altered stem cell composition and differentiation.

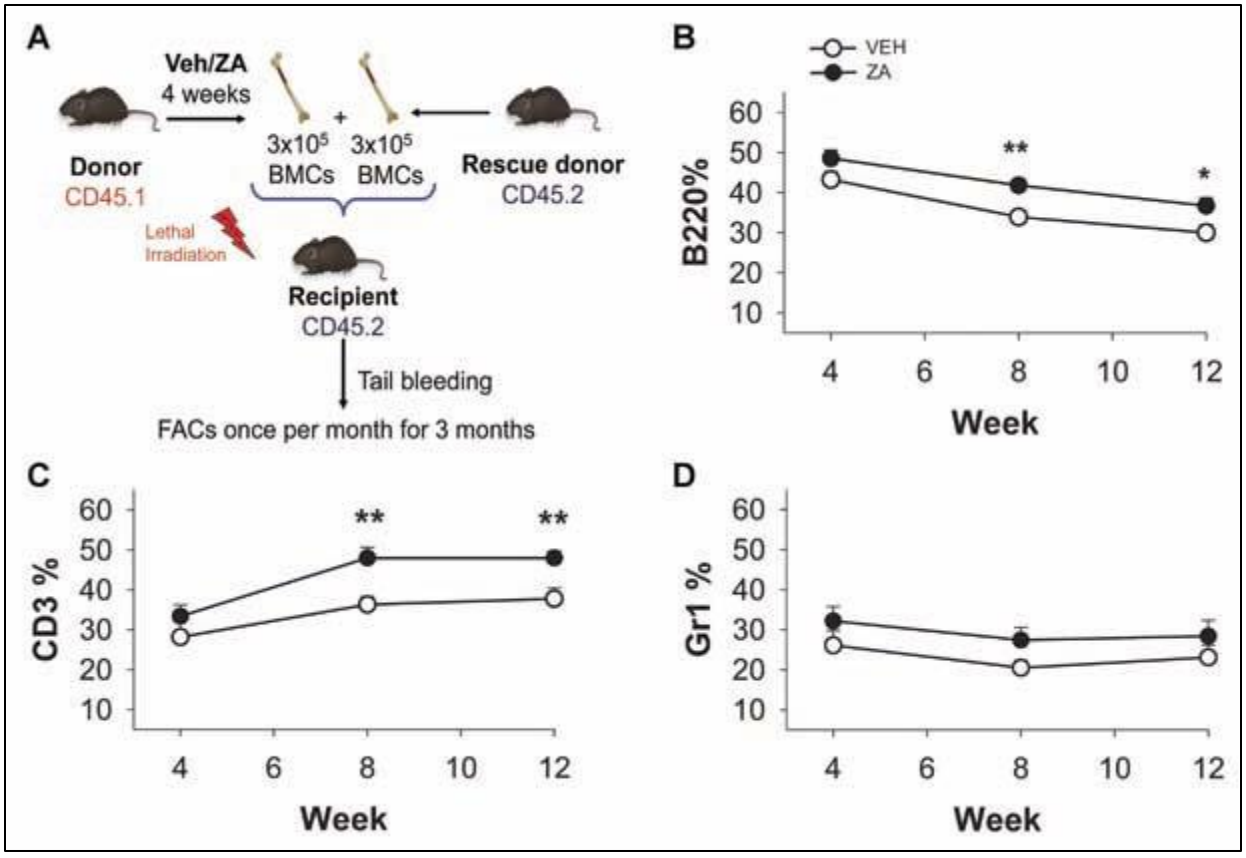

Figure 3.3. Long-term HSC reconstitution was increased in ZA treated bone marrow cells.

Donor CD45.1 mice were treated for 4 weeks with ZA or vehicle (VEH). Bone marrow cells were collected and mixed with rescue donor CD45.2 bone marrow cells. Mixed cells were injected intravenously in irradiated recipient mice, and blood cells were collected and analyzed by flow cytometry as indicated for CD45.1 positive and (A) B cells (B220), (B) T-cells (CD3), and (C) myeloid cells (Gr-1). Zoledronic acid treated mice had increased reconstitution of the B and T cell populations but no altered reconstitution of the myeloid Gr-1 population (**$P < 0.01$, *$P < 0.05$). Data are presented as mean ± SEM, n = 10. Permission for figure from J Cell Biochem (Soki et al., 2013)

Hematopoietic stem cell mobilization

To determine the extent to which ZA alters HSC egress or mobilization from the marrow, peripheral blood and spleens were analyzed by FACS in mice treated with ZA or vehicle. As a result of the ZA treatment, LSK numbers in the peripheral blood were not altered (Fig. 3.4A). No correlation was seen between LSK numbers in bone marrow and peripheral blood (data not shown). To investigate whether ZA has an effect on HSC mobilization or extramedullary hematopoiesis, the spleens of vehicle or ZA treated animals were analyzed. Spleen weight/ body weight and LSK numbers in mice with ZA or vehicle were not significantly different (Fig. 3.4B,C). Altogether this data suggests that increased LSKs in the bone marrow were not due to mobilization effects in mice treated with ZA.

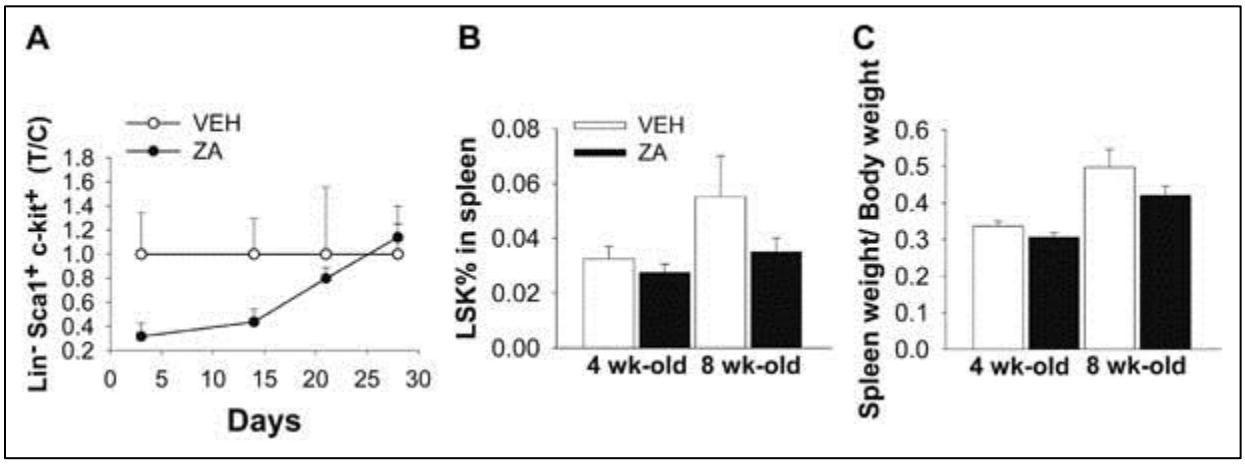

Figure3.4. Bone marrow HSC mobilization.

ZA did not alter the LSK population in the blood or spleen. Four-week-old C57BL/6J male mice were treated with 200 µg/kg of ZA twice/week for 4 weeks, and peripheral blood and spleens were analyzed. A: Flow cytometry analyses of LSK population in the peripheral blood; B,C: spleen cells were collected and LSK population was analyzed by flow cytometry (B); spleen weight/body weight were analyzed and no changes were observed (C). Data are presented as mean ± SEM, n = 10/gp (A), n = 8/gp (B,C). (T/C) Treatment/control. Permission for figure from J Cell Biochem (Soki et al., 2013)

Endosteal and vascular niches

The hematopoietic niche is formed in the bone marrow by hematopoietic and non-hematopoietic cells and localized in the endosteum (area between bone marrow and bone) and sinusoids. ZA increases bone mass and thus provides an increase in niches to support HSCs. Interestingly we observed in the bone sections of mice that augmented trabecular bone was also followed by increased small vessels number in 4 week old mice (Fig. 3.5A). Therefore changes in the vasculature of bone in mice treated with ZA or vehicle for 4 weeks were examined. Radiopaque silicone rubber agent Microfil™ was perfused intravenously and micro CT analysis of vascular spaces was performed (Fig. 3.5 B-F). Although the overall vessel volume fraction was not affected (Fig. 3.5C), ZA treated mice had reduced vessel thickness (Fig. 3.5D) and increased vessel numbers (Fig. 3.5E). In addition, bone sections were stained for Von Willebrand factor (vWF) and vessel numbers were quantified (Fig. 3.5G). Consistent with the Micro CT analyses, vessel number was increased with ZA treatment.

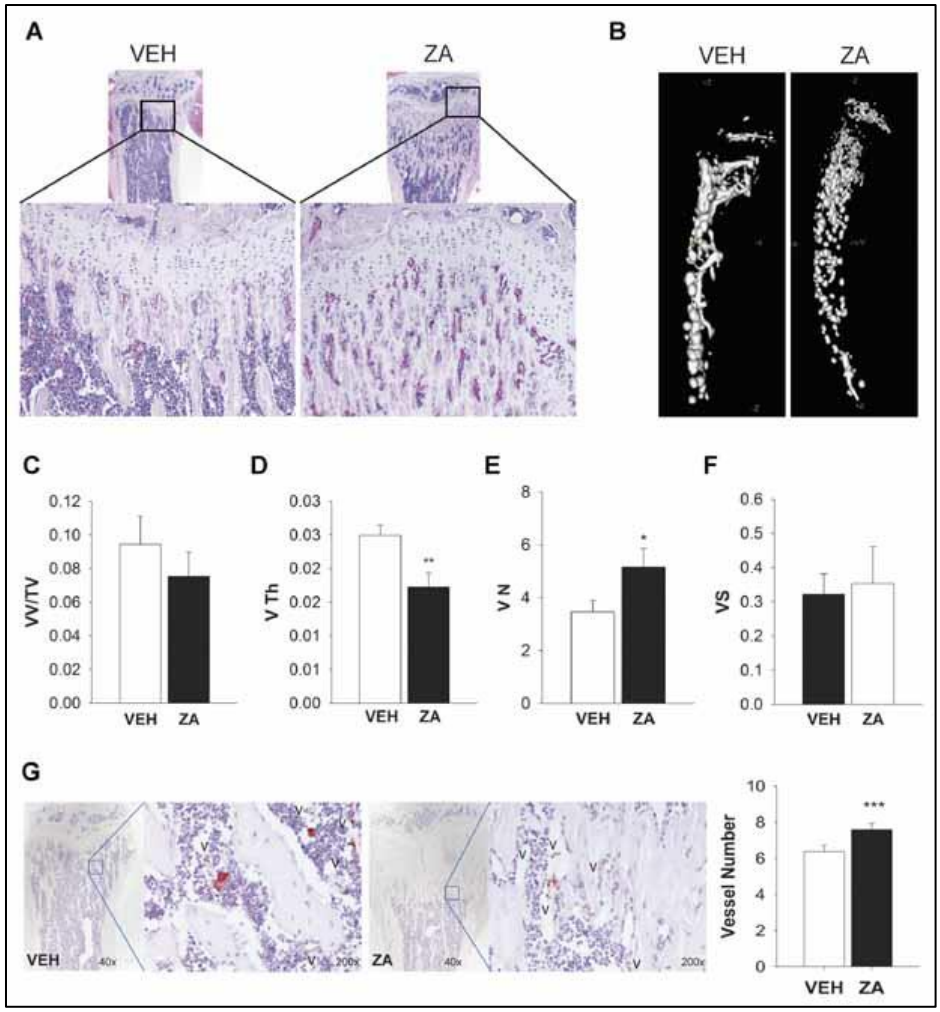

Figure 3.5. Effects of ZA on bone and vasculature.

ZA did not alter vessel volume but increased vessel numbers in 4-week-old mice treated with ZA or vehicle (VEH) for 4 weeks. A: H&E staining of mice treated with vehicle or ZA shows an increase of vessels in the trabecular area of mice tibiae; B–F: mice were perfused with radiopaque silicone rubber microfil and tibiae were analyzed by micro CT; (B) Representative micro CT image of bones with microfil perfusion; C–F: MicroCT analysis of microfil for vessel volume/tissue volume (VV/TV%), thickness (V Th, mm), numbers (VN, mm^{-1}), and spacing (VS, mm). ZA treatment decreased vessel thickness and increased vessel numbers; G: tibiae sections were stained for Von Willebrand factor (V) and vessel numbers were quantified (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$). Data are presented as mean ± SEM, n = 10/gp.Permission for figure from J Cell Biochem (Soki et al., 2013)

Hematopoietic cell composition

The changes seen in the hematopoietic progenitor cell populations could also be due to changes in the hematopoietic composition of the bone marrow. To assess whether ZA affected the mature hematopoietic composition, bone marrow was analyzed for $CD41^+$ cells by FACS and no significant changes were observed (Fig. 3.6A). In addition, peripheral blood cell count was performed and there was no alteration in the white and red blood cells or platelets (Fig. 3.6B).

The other possibility could be that zoledronic acid induced changes in the bone could be affecting the hematopoietic stem cell differentiation into progenitor cells. Thus, we decided to further investigate the constitution of the HSC population. Stem cells are classified according to their repopulation capacity into long-term HSCs (LT-HSC) and short-term HSCs (ST-HSCs). The LT-HSCs are stem cells that present extensive life long, self-renewing potential and are capable of repopulating the whole hematopoietic system. In contrast, ST-HSCs have a lower self-renewing capacity but a rapid capability of myeloerythroid cell repopulation (Yang et al., 2005). They can be identified according to the expression of cytokine polypeptide deformylase (fms)–like tyrosine kinase receptor 3 (flt3): long term-HSCs are LSK $CD34^-flt3^-$ and short term-HSCs (LSK $CD34^+$) can be further divided into 2 subsets. ST-HSCs $flt3^+$ for cells with great lymphoid reconstitution and ST-HSCs $flt3^-$ for rapid myelopoiesis. Because increased LSK numbers were observed we investigated the differentiation stage of the LSK population. Indeed LSK $flt3^+$ (ST-HSCs) were increased with ZA treatment (Fig.6C) suggesting that cells with great lymphoid reconstitution are augmented by ZA induced changes in the bone microenvironment, as seen in the long term reconstitution assay (Fig. 3 B,C).

To investigate whether ZA influenced bone angiogenesis we also measured endothelial progenitor cells (EPCs) by FACS in both bone and peripheral blood (Fig. 6D, E). ZA treatment significantly decreased $CD11b^-$ $CD34^+$ $ftl1^+$ EPCS at 3 weeks only but at 4 weeks no difference was seen (Fig. 6D) in bone nor in the peripheral blood (Fig. 6E).

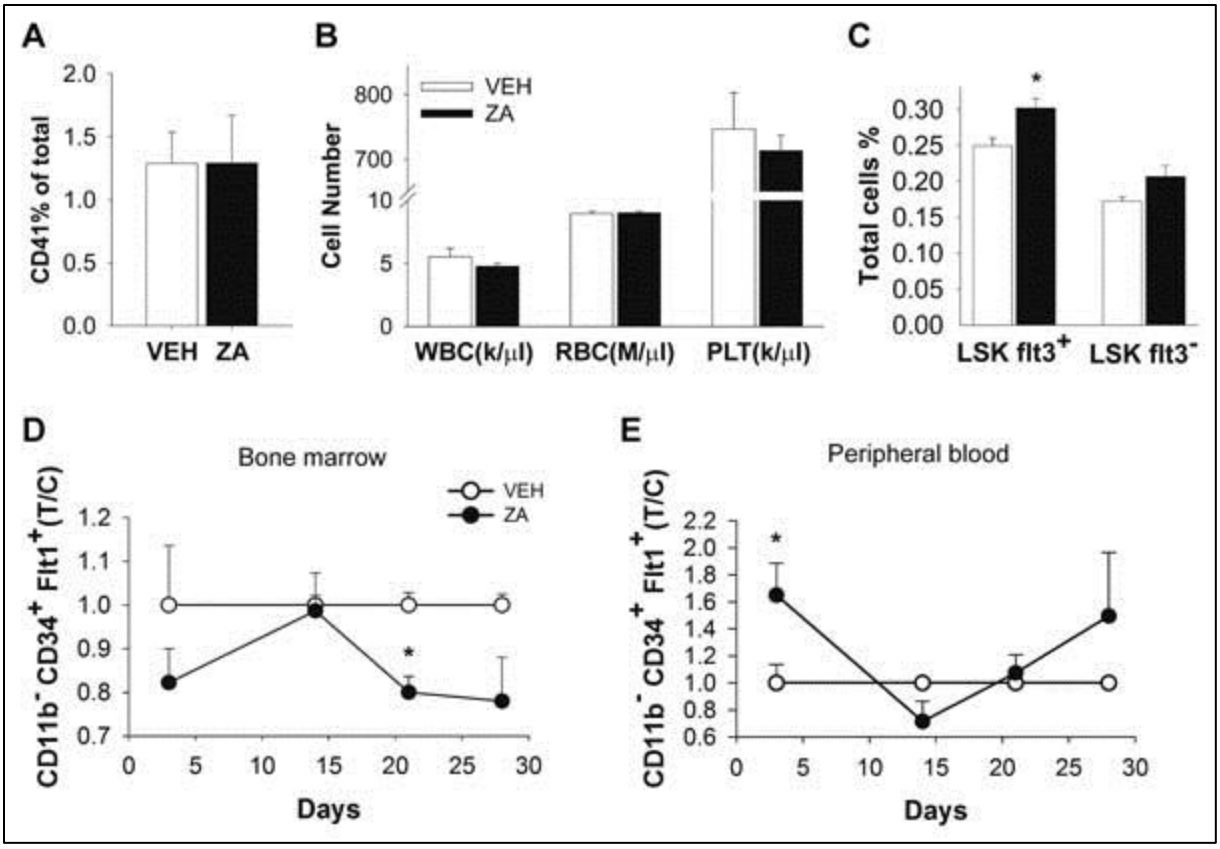

Figure3.6. Effects of ZA on hematopoietic cell composition.

To investigate the effects of ZA on hematopoiesis and progenitor cells, bone marrow and blood cells of 4-week-old mice were analyzed; A: no alteration in the bone marrow $CD41^+$ mature hematopoietic composition with ZA treatment versus vehicle (VEH) control; B: blood cell count of white blood cells (WBC), red blood cells (RBC), and platelets (PLT) were not altered with ZA treatment; C: sixteen-week-old mice were treated with ZA or vehicle (VEH) for 4 weeks and bone marrow cells were collected and LSK $Flt3^+$ and $Flt3^-$ were analyzed by flow cytometry. D–E: Endothelial progenitor cells ($CD11b^-$ $CD34^+$ $Flt3^+$) were analyzed over time to investigate the effects of ZA in bone marrow and mobilization to the peripheral blood; (D) EPCs in the bone marrow were significantly decreased at 3 weeks of ZA treatment but not significantly different at 4 weeks; (E) EPCs in the peripheral blood were not altered by ZA treatment ($*P < 0.05$). Data are presented as mean ± SEM, n = 8/gp (T/C) treatment/control. Permission for figure from J Cell Biochem (Soki et al., 2013)

Gene expression analyses

To further confirm the HSC population commitment, bone marrow cells were sorted into Lin- or Lin^+ cells and gene expression of hematopoietic related genes were analyzed. Lineage negative cells had increased expression of BMP2, BMP6, Bmi1, Tie2, Notch1, but no change in Ink4a, in mice treated with ZA (Fig. 3.7A). BMP2 and BMP6 are proteins known to support endosteal niche maintenance, suggesting an active participation of HSCs in the endosteal niche. Bmi1, Ink4a and Tie2 genes are indicators of self-renewal capacity (Park et al., 2003). Bmi1 is required for HSC self-renewal since $Bmi1^{-/-}$ mice have normal HSCs in fetal liver but decreased HSC numbers in postnatal mice. p16Ink4a is downstream of Bmi1 and participates in the regulation of HSC proliferation (Smith et al., 2003).

Because ZA may affect the calcium resource in bone due to the inhibition of bone resorption we also investigated calcium related genes in linage negative cells and found calmodulin 1(Cam1) and calcium chloride channel (ClCa1) were decreased with ZA treatment (Fig. 3.7B).

Linage positive cells had decreased gene expression for BMP6 but no significant changes were observed in Bmi1, Tie2, Notch1, Ink4a and flt3 (Fig. 3.7C). Total bone marrow cells had decreased gene expression of osteocalcin (OCN) and vascular cell adhesion molecule 1 (VCAM1) genes (Fig. 3.7D). Altogether, the increased LSK population may be in part due to increased self-renewal capacity and suppression of linage commitment towards short term hematopoietic stem cells.

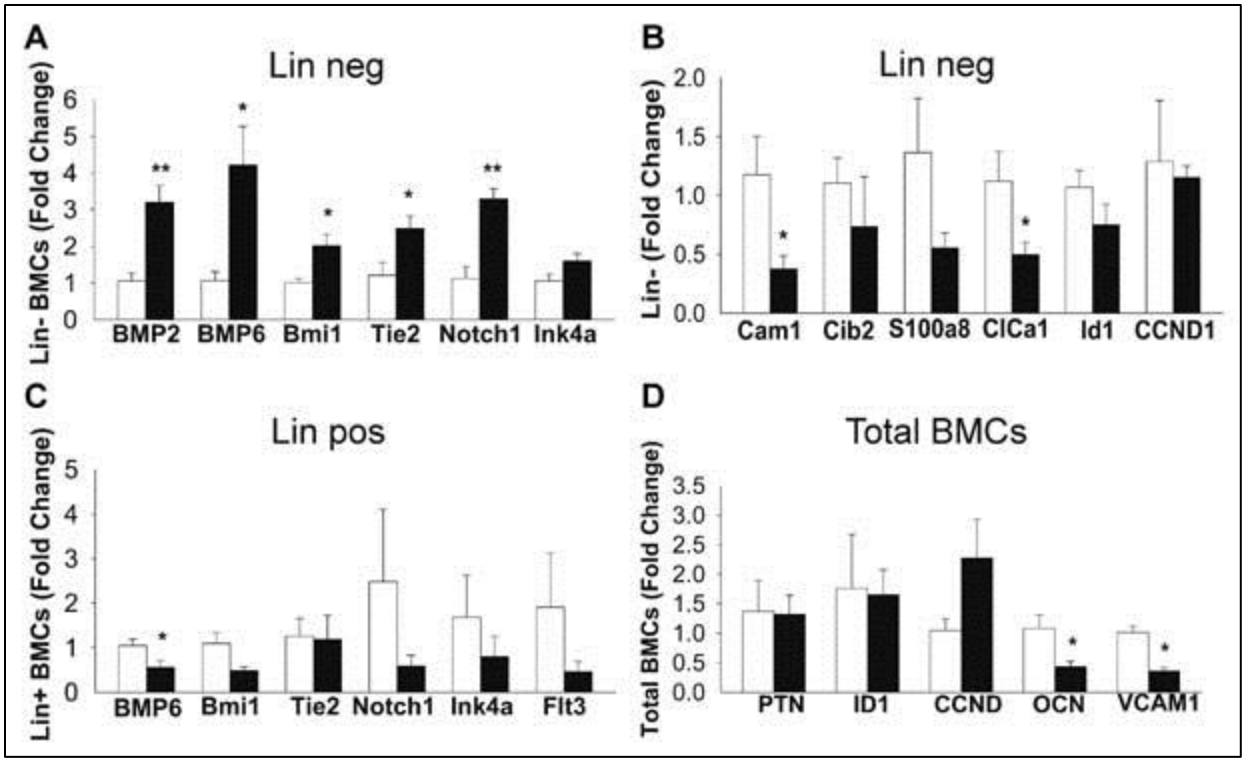

Figure3.7. Gene expression analyses of bone marrow sorted cells:

Lineage negative (A,B), lineage positive (C), and unsorted total bone marrow cells (D). Bone marrow cells of 4-week-old mice treated with ZA for 4 weeks were sorted into Lin^- and Lin^+ cells by magnetic cell sorting beads (MACS) and gene expression analyzed by qPCR. (A) Lineage negative cells had increased expression of BMP2, BMP6, BMi1, Tie2, Notch1, but no alteration of Ink4a, in mice treated with ZA versus vehicle (VEH) control. (B) Lineage negative cells were also analyzed for calcium related genes. Cam1 and ClCa1 were decreased with ZA. (C) Lineage positive cells had decreased gene expression for BMP6 but no significant changes were observed in Bmi1, Tie2, Notch1, Ink4a, and Flt3. (D) Total bone marrow cells had decrease gene expression of OCN and VCAM1 genes (*$P < 0.05$, **p,0.005). Data are presented as mean ± SEM, n = 5/gp. Permission for figure from J Cell Biochem (Soki et al., 2013)

Adult model vasculature and HSCs

Because the experiments were performed in 4 week old mice that are in an active phase of growth and high bone activity, adult mice were also investigated. Four month old mice were treated with ZA or vehicle for 4 weeks and same analyses were performed. LSK numbers were again increased in ZA treated mice (Fig. 3.8A). Micro CT

analyses of Microfil perfused mice presented no changes in vessel volume, thickness or number, suggesting that increased LSK numbers in 4 month old mice were not due to increased vascular niches (Fig. 3.8B-G).

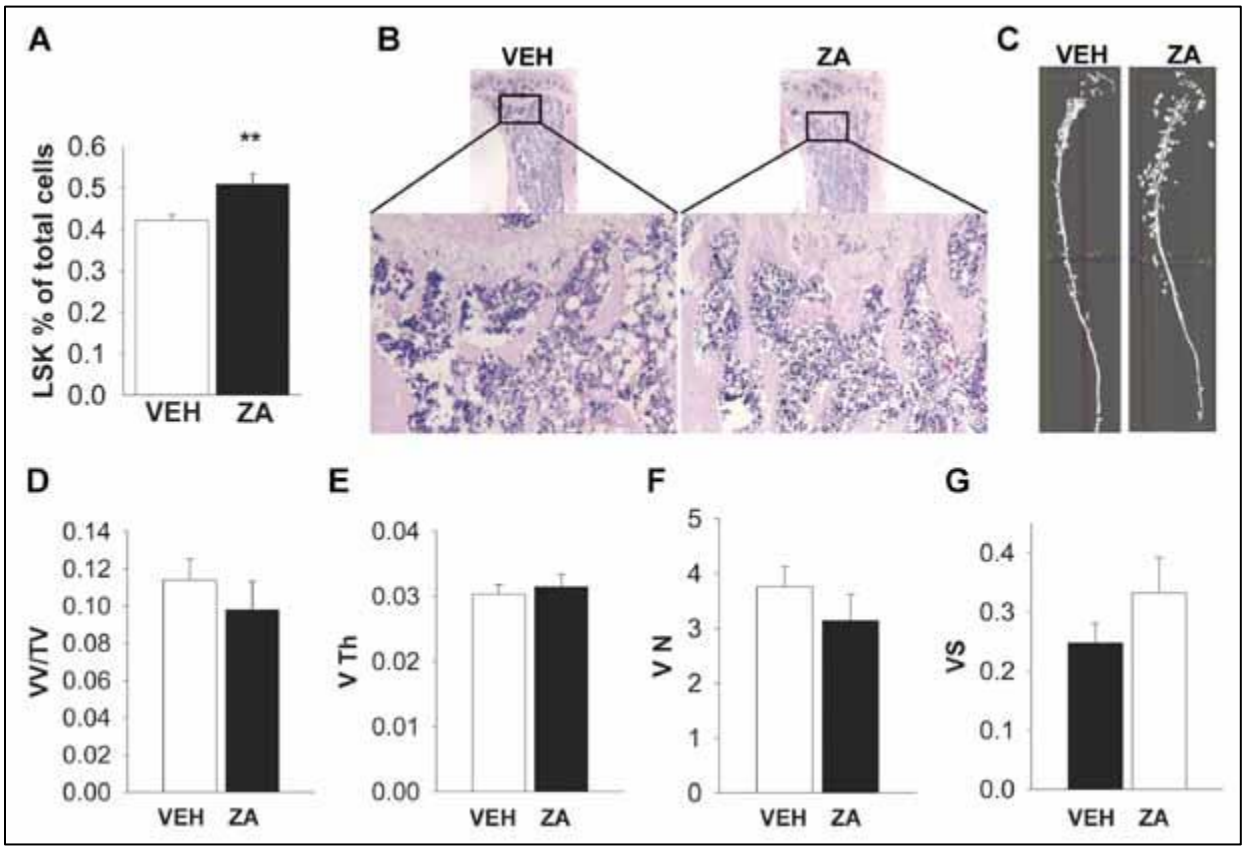

Figure3.8. Effects of ZA on bone and vasculature in 16-week-old mice.

ZA did not alter vessel volume (VV/TV), thickness (V Th), or number(VN) in adult mice, but still increased LSK numbers. Sixteen-week-old mice were treated with ZA or vehicle (VEH) for 4 weeks. A: Bone marrow cells were collected and LSK were analyzed by flow cytometry. B: H&E staining of mice treated with vehicle or ZA shows similar vessels in the trabecular area of tibiae. C: Representative MicroCT images of bones with microfil perfusion. D–G: MicroCT analyses of microfil for vessel volume/tissue volume (VV/TV%), thickness (V Th, mm), number (VN, mm^{-1}), and spacing (VS, mm; $^{**}P < 0.005$). Data are presented as mean ± SEM, n = 8/gp. Permission for figure from J Cell Biochem (Soki et al., 2013)

DISCUSSION

In this study, treatment with ZA in both young and adult mice significantly increased the hematopoietic progenitor population (LSK) in the bone marrow. The augmented LSK population was confirmed functionally by long term reconstitution assays showing increased repopulation of B and T lymphocytes in mice that received bone marrow stromal cells from donor mice treated with ZA. One interesting finding was that reconstitution of Gr-1^{+} population that accounts for myeloid cells was not different. A recent study demonstrated that TGF-β1 can regulate the hematopoietic stem cell long–term differentiation. High levels of TGF-β1 resulted in stimulation of myeloid-biased HSCs and inhibition of lymphoid-biased HSCs (Challen et al., 2010). ZA is known to inhibit bone resorption and consequently reduce the release of TGF-β1 from resorbed bone (Wu et al., 2010). Accordingly, with ZA treatment an increased reconstitution of lymphocyte B and T cells were observed but not the myeloid population suggesting that low TGF-β1 could be modulating the reconstitution of these cells in the bone marrow. Moreover, ZA treatment increased the LSK flt3^{+} population that are known to be short term lymphocytic reconstituting cells, confirming an effect of ZA in hematopoietic reconstitution.

Since LSK cells were increased in ZA treated mice, 3 potential hypotheses were investigated; 1) mobilization of LSK in the bone marrow, 2) increased bone marrow niches supporting increased numbers of LSKs, 3) altered cell composition and stem cell differentiation.

Mobilization depends on detachment of HSCs from their niches concomitantly with cellular migration and egress from bone marrow to the circulation. Mobilization strategies have been used clinically to collect a large number of hematopoietic stem cells from the blood, a less invasive procedure than bone marrow aspiration for transplantation. Regulation of the bone microenvironment and the niches present in the marrow are important to preserve HSCs and therapies that interfere in this balance can affect the stem cell pool. Based on our findings, mobilization of LSKs in ZA treated mice was not altered since the LSK population was unchanged in the peripheral blood as well as the spleens**.** No difference was seen in white and red blood count or in the

platelet numbers in peripheral blood. Studies demonstrated that bone remodeling is altered when HSC mobilization is induced by granulocyte-colony stimulating factor (G-CSF) treatment with decreased bone volume (Lee et al., 1991;Takahashi et al., 1996). Therefore, bone activity is closely associated with HSC commitment for mobilization and differentiation with egress of HSCs from the bone marrow. Interestingly ZA inhibits osteoclast function and subsequently results in increased bone volume, an opposite effect seen in G-CSF therapies.

Since ZA treatment did not stimulate the egress of LSKs from the bone marrow to the circulation two other questions were addressed. First is whether ZA promotion of LSK increase is due to hematopoietic niche alteration and secondly, whether ZA regulates the factors necessary for the retention of HSCs with modulation of cell commitment. ZA is a bisphosphonate clinically utilized for the treatment of bone associated diseases such as osteosporosis and skeletal malignancies due to its inhibitory actions in osteoclast mediated bone resorption. ZA actions in bone were confirmed by histomorphometric analyses of bone sections demonstrating a significant increase bone mass and decrease in osteoclast activity. Hematopoietic niches maintain self-renewing and dormant HSCs in the bone marrow and although still controversial, HSCs are believed to reside in association with bone locals termed endosteal niches and surrounding sinusoids present in the marrow, known as vascular niches. It was also proposed that the distinct niches have different functionalities: the endosteal niche homes and maintains long-term HSCs and the vascular niche is involved in the short term HSC maintenance, promoting the differentiation and circulation of HSCs (Grassinger et al., 2010). However to fully differentiate both niches is difficult due to the close association of the vasculature especially in trabecular bone rich areas, locations where HSCs preferentially lodge (Ellis et al., 2011). An increase in the osteoblastic niche could account for the augmented LSK population, especially for the long-term LSKs that were modestly increased after ZA treatment using the SLAM markers.

Bisphosphonates are being studied for their activities not only in the bone microenvironment but also putative direct effects in tumor cell proliferation, adhesion, invasion, apoptosis and angiogenesis (Rogers and Holen, 2011). The anti-angiogenic effects of ZA have been demonstrated by its actions as a potent inhibitor of endothelial

cell proliferation, adhesion migration and angiogenesis. However, ZA effects in bone vasculature are still poorly defined. Bone vascular biology is an understudied area and the interdependency of vascular physiology and osteogenesis need to be more thoroughly investigated. It is known that angiogenesis and bone formation are intrinsically linked during bone development (Wang et al., 2007) and repair (Wan et al., 2008). Osteoclasts actively participate in this process, excavating the marrow cavity, while sinusoids establish this unique type of bone microcirculation (Bianco, 2011). Although biological evidence is observed in the bone coupling with endothelial cells (Parfitt, 2000), the formation and modulation of the vasculature linked with bone modeling and the effects of stimulators (hormones, drugs and mechanical loading) that affect its microstructure are relatively unexplored. Studies in mechanical loading, demonstrated that bone angiogenesis was accompanied with bone gain in running rats (Yao et al., 2004) and VEGF was the mechanism driving both effects. Recently, an interest study demonstrated that intermittent parathyroid hormone (PTH) induced vascular endothelial growth factor (VEGF A) production that mediated PTH anabolic response (Prisby et al., 2011). PTH treated mice had fewer blood vessel numbers and relocated existing blood vessels near to new bone formation sites. (Wood et al., 2002;Metcalf et al., 2011). Interestingly, ZA treatment decreased the circulating levels of VEGF in cancer patients with bone metastasis (Santini et al., 2003) but their effects on the bone vasculature were not determined.

In this study, we observed the expected increase in bone mass, but we also investigated the changes that occur in the vasculature of mice treated with ZA. To this end, perfusion of a radiopaque microfilm was performed to visualize and quantify changes in the vasculature volume and vessel numbers. Interestingly, young mice (4 week old) treated with ZA had no alteration in overall vessel volume but had increased vessel numbers that were smaller and accumulated between the augmented trabecular areas in the metaphysis. This finding lead us to further investigate if the changes in the bone niches (both endosteal and vascular) microstructures contributed to the increased LSK numbers. Because the experiments were performed in young (4week old) mice where bone is highly active, we also performed experiments in adult (4 month old) mice. Similar to the young mice, LSK cells and bone mass were significantly increased after 4

week treatment with ZA but surprisingly vessel numbers and volumes were not changed in the adult mice. The increase in bone vessel numbers in the young model but not in the adult mice suggests that ZA may act differently depending on the age and bone activity. Moreover, the fact that ZA is a known antiangiogenic factor, but did not reduced vessel number or volume in bone raises interesting questions regarding the mechanisms by which bone angiogenesis occurs and how important their interdependency is with bone modulation and activity. Further studies are necessary to fully elucidate bone angiogenesis and its contribution to the bone microenvironment. Collectively, the findings suggest that ZA indirectly supports the osteoblastic niche and that the vasculature niche may not be a key player in the augmented LSK population in bone.

To account for an increase of LSK in the marrow, the present study sought to determine whether ZA would affect other cell types in the bone marrow. Analysis of hematopoietic $CD41^+$ cells revealed similar levels between ZA and VEH. Moreover, investigation of different LSK populations was performed to determine whether ZA inhibited cell commitment that could account for the increased population in the bone marrow. Indeed short-term lymphocytic reconstituting LSK $flt3^+$ cells were increased with ZA treatment. Lymperi et al. demonstrated that inhibition of osteoclastic resorption with alendronate treatment resulted in expansion of hematopoietic progenitor cells (LSK) but with a decrease in the absolute number of LSK $Flt3^-$ HSCs and long-term culture-initiating cells in the bone marrow (Lymperi et al., 2011). However, alendronate is a second generation bisphosphonate with a lower efficacy of osteoclast inhibition than ZA.

Gene expression of Lin^- sorted cells demonstrated that Bmi1 and Tie2, related with HSC self-renewal (Rizo et al., 2008;Arai et al., 2004), were both increased, suggesting that hematopoietic progenitor cells are acquiring self-renewal capacity. Interestingly, BMP2 and BMP6 were both augmented with ZA treatment in Lin negative cells. Jung et al. demonstrated that hematopoietic stem cells participate in bone formation and niche activity with secretion of BMP2 and BMP6 (Jung et al., 2008). Later, a role of erythropoietin for BMP2 and BMP6 production was reported (Shiozawa et al., 2010). Indeed, the endosteal niche is expanded with ZA treatment and

hematopoietic progenitor cell could be actively participating in the bone formation. It is possible that as osteoclasts are compromised with ZA treatment their coupling factors to stimulate osteoblasts (e.g. ephrins) are compromised and HPCs are serving to compensate. Notch 1, a receptor for ligand Jagged 1 expressed by osteoblasts, was also increased and is involved in the expansion of the HSC pool in vivo (Calvi et al., 2003)

Altogether, accordingly with increased endosteal niches and increased LSKs, our data suggests that ZA indirectly supports the osteoblastic niche LSK expansion that occurs independent of the vascular niche. Moreover, Zoledronic acid has a differential response in the vasculature modulation in young and adult mice, suggesting that age and bone activity may be significant factors defining the vasculature modulation in bone.

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CHAPTER 4

BONE MARROW MACROPHAGES SUPPORT PROSTATE CANCER GROWTH IN BONE

ABSTRACT

Bone is the preferred site of prostate cancer metastasis, and advanced-stage patients commonly develop skeletal metastasis. Still, the mechanisms underlying bone metastasis remain elusive. Resident macrophages in bone, termed “osteomacs”, play important roles in bone remodeling, repair and hematopoietic stem cell niche maintenance, yet their role in skeletal metastasis has been under investigated. The purpose of this study was to determine the role of macrophages in prostate cancer skeletal metastasis. *In vivo* experimental approaches employed a chemically inducible macrophage ablation model (i.e. MAFIA mice) in which AP20187 (AP) injections result in fas-induced apoptosis of c-fms (CSF-1 receptor) positive macrophage lineage cells. A significant reduction in tumor growth was noted in tibiae after intratibial tumor inoculation when macrophages were ablated. Similar results were observed when a different strategy of macrophage ablation was performed with clodronate liposomes. Luciferase tagged PC-3 intratibial tumors were inoculated in athymic mice and BLI imaging showed less growth and smaller tumors overtime in macrophage depleted mice. The presence of tumors was sufficient for the increase in tumor associated macrophages in bone marrow. Macrophage ablation in MAFIA mice also hindered tumor growth in osseous settings when tumors were co-implanted subcutaneously with vertebral bodies (vossicles) as compared to the subcutaneous tumors suggesting that the marrow microenvironment and the macrophages in that environment are particularly

vital for tumor growth. Interestingly, even though tumors were consistently smaller in macrophage depleted mice, paradoxical results of macrophage depletion on the bone were observed. Histomorphometry and μCT analyses demonstrated that clodronate treated mice had increased bone volumes in long bones while MAFIA mice had significant reductions. These results suggest that the effect of macrophage depletion on tumor growth was independent of its effect on bone responses and that macrophages in bone may be more important to tumor growth than the bone itself. In conclusion, resident macrophages play a pivotal role in prostate cancer growth in bone.

INTRODUCTION

The skeleton is a favored organ for metastasis that results in significant morbidity for cancer patients. In skeletal metastasis, the interplay of tumor cells with the bone microenvironment results in bone remodeling and tumor growth (Weilbaecher et al., 2011a;Loberg et al., 2005). Macrophages are myeloid phagocytic cells recruited in response to infection, inflammation and tissue injury, therefore, having a role in the innate and adaptive immune response (Pettit et al., 2008;Pollard, 2009). They are activated differentially according to the stimuli provided: M1 anti-tumorigenic macrophages are classically activated and M2 pro-tumorigenic macrophages (also known as tumor associated macrophages, TAMs) are alternatively activated (Pollard, 2004;Lewis and Pollard, 2006). TAMs are prominently found and involved with cancer initiation, progression and metastasis, facilitating angiogenesis, matrix breakdown and tumor cell-motility (Pollard, 2009;Mantovani and Sica, 2010;Mantovani, 2010;Pollard, 2004). Clinical studies in prostate cancer demonstrated that overexpression of colony-stimulating factor (CSF-1) and its receptor (CSF-1R or c-fms), responsible for monocyte and macrophage expansion, indicated poor prognosis in primary prostate cancers and development of metastasis to the bone (Richardsen et al., 2008). Modification of the myeloid cells in bone has demonstrated influence on tumor metastasis to bone. Prostate cancer cell localization and growth in bone was increased when a single dose of cyclophosphamide, a bone marrow–suppressive chemotherapeutic drug, was administered prior to intracardiac tumor inoculation (Park et al., 2012a). This was

associated with a transient expansion of myeloid cells and increased cytokines with myelogenic potential such as C-C chemokine ligand 2 (CCL2), interleukin-6 (IL-6), and VEGF-A that primed the environment for tumor growth. Moreover, the chemokine CCL2 known to attract and differentiate macrophages towards TAMS and osteoclasts has been shown to increase prostate cancer growth and bone metastasis (Mizutani et al., 2009;Loberg et al., 2007b;Loberg et al., 2007a;Roca et al., 2009;Rozel et al., 2009). TAMs have been implicated in tumor growth, progression and metastasis of different types of cancer, but little is known about their role in skeletal metastasis.

Macrophages residing in bone, termed 'osteomacs' constitute one sixth of the total cells and differ from osteoclasts, by expressing specific surface markers: F4/80 and CD68 (Austyn and Gordon, 1981;Chang et al., 2008). Interestingly, depletion of macrophages compromised osteoblastic bone formation (Pettit et al., 2008;Chang et al., 2008). Bone resident macrophages are found in close association with osteoblasts and are important in bone remodeling, bone healing and hematopoietic niche maintenance (Alexander et al., 2011b;Winkler et al., 2010b;Pettit et al., 2008). Moreover, osteal macrophages have important roles in skeletal homeostasis and PTH anabolic actions on bone remodeling (Cho et al., 2014). Differences in bone remodeling were observed depending on the differentiation stages of macrophages that were targeted. Depletion of early lineage macrophages resulted in osteopenia whereas differentiated macrophage depletion presented the opposite effect of an osteogenic environment and enhanced PTH anabolism. Osteomacs play important roles in the bone niche. However, their role in pathologies such as bone metastasis remains elusive. Surprisingly, the involvement of macrophages and their participation in the modulation of the bone microenvironment during skeletal metastasis have not yet been explored. Given the importance of macrophages in bone homeostasis, we hypothesized that macrophages promote prostate cancer metastatic growth in bone.

MATERIALS AND METHODS

Animal care and cell lines.

All animal experiments were performed with the approval of the University of Michigan Committee for the Use and Care of Animals. C57Bl/6 male wild-type mice and MAFIA mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male athymic mice were purchased from Harlan Laboratories.

Luciferase-labeled PC-3 cells were initially purchased from ATCC and established as previously described (Schneider et al., 2005). RM-1 cells were originally obtained from Dr Timothy C. Thompson (Baylor College of Medicine, Houston, TX).

MAFIA mouse model with intratibial RM-1 tumors.

The macrophage Fas-induced apoptosis (MAFIA) C57BL/6-Tg mouse has a c-fms promoter (CSF-1 receptor) specifically targeting phagocytic cells such as macrophages, and to a lesser extent dendritic cells. Administration of a synthetic dimerizer, AP20187 (Clontech Laboratories, Inc.) activates the suicide gene leading to Fas-mediated apoptosis in resting and cycling macrophage cells. MAFIA mice (12-14 wks) received daily i.p. injections of AP20187 (10 mg/kg) (or vehicle) for 3 days for macrophage depletion as previously described (Cho et al., 2014). Booster injections (1mg/kg) every 3d were administered to sustain macrophage suppression. After initial depletion, RM-1 mouse metastatic prostate cancer cells were inoculated via intratibial injection under anesthesia as previously described (Park et al., 2010). At study end, bones were analyzed histomorphometrically to evaluate tumor, bone area and resorption indices. Serum TRAP5b was measured as an indicator of bone remodeling activity.

RM-1 tumor measurements

X-ray images from intratibial tumor bearing mice were analyzed for radiolucency. Radiolucent areas were delineated utilizing the ImageJ program for total area determination. Blinded measurements were performed by two different people and averaged for each sample.

Clodronate loaded liposomes.

Clodronate (dichloromethylene bisphosphonate)-loaded liposomes (clodronate liposomes) or PBS-loaded liposomes (PBS liposomes; used as control) were purchased from ClodronateLiposomes.com (The Netherlands). Four-week-old male athymic or C57BL/6 mice were treated with clodronate liposomes (10 µl/g) for 3 consecutive injections, then given every third day for 2 weeks (C57BL/6) or every week at a reduced dosage (6 µl/g; athymic mice) for the next 3 weeks. After initial depletion, RM-1 or Luciferase tagged PC-3 cells were injected in the tibia of mice. *In vivo* bioluminescence (BLI) was performed every 3 days for PC-3 luciferase cell evaluation.

Flow cytometric analysis.

Immediately after sacrifice, bone marrow cells were flushed with FACS buffer (2% FBS, 2 mM EDTA in 1X PBS) and 1 x 10^6 cells were incubated with fluorescent-conjugated antibodies (PE 10 µl; FITC 1 µl; APC 5 µl) in 500 µl of FACS buffer for 30 min at 4°C. Antibodies for flow cytometric analyses included: anti-mouse F4/80 FITC (Abcam, CI:A3-1), anti-mouse CD11B APC (eBioscience, M1/70) and CD206 PE (AbD Serotec, MR5D3).

Histology and tartrate resistant acid phosphase (TRAP) staining.

Tibiae were fixed in 4% paraformaldehyde solutions for 24 hr and decalcified in 10% EDTA for 21 days at 4°C. Paraffin-embedded tibial sections were stained with H&E and histomorphometric analyses were performed using Osteomeasure (OsteoMetrics Inc. Atlanta, GA). TRAP staining on bone sections was performed using a TRAP staining kit (Sigma-Aldrich) per manufacturer's instructions.

µCT imaging.

Fixed tibiae and femurs were analyzed by µCT imaging (eXplore Locus, GE Healthcare, London, ON, Canada; Scanco µCT-100, Medical AG, Bruttisellen, Switzerland). Bones were fixed in formalin and embedded in 1% agarose. After placing them in a 19 mm diameter tube, bones were scanned over their entire length using a µCT system (µCT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were: voxel size 12 µm, medium resolution, 70 kVp, 114 µA, 0.5 mm AL filter, and integration

time 500 ms. Trabecular bone parameters were measured over 50 slices using a threshold of 28% at 15 slices distal to the growth plate; cortical bone was measured over 30 slices at 60% of the bone length distal to the tibio-fibular joint (TFJ) using a 28% threshold.

Immunohistochemical F4/80 staining.

Tibiae were fixed in 4% paraformaldehyde/PBS (4°C, 24hr), decalcified in 14% EDTA for 2 weeks, and embedded in paraffin. Immunohistochemical staining was performed, using the Cell & Tissue Staining Kit (HRP-DAB system; R&D systems) and the rat monoclonal anti-mouse F4/80 (1:100, Abcam, Cambridge, UK). Percentages of F4/80+ cells were quantified using NIS Elements software (Nikon). The mean area positive for F4/80+ cells was calculated for each tissue within 4 different areas of the tissue.

Blood and bone marrow serum biochemical assays.

ImmunoAssay kits obtained from IDS were used to measure serum TRAP5b following manufacturer's instructions.

Statistical Methods.

Statistical analysis was performed by two-way ANOVA or unpaired t test using the GraphPad Instat statistical program with significance of $p<0.05$. Data are presented as mean ± SEM. Tumor growth in the mouse models was analyzed using repeated measures mixed models. Growth over time was tested between groups using an interaction of group and time in the model if the growth is linear. Pairwise group differences were explored using post-hoc tests adjusted for multiple comparisons.

RESULTS

Prostate cancer growth in bone of macrophage-ablated MAFIA mice

The macrophage fas-induced apoptosis (MAFIA) mouse provides for conditional macrophage depletion (23). Administration of a synthetic dimerizer, AP20187 activates

the suicide gene leading to Fas-mediated apoptosis in resting and cycling macrophage cells. In addition, bicistronic expression of the suicide gene and EGFP permit tracking of macrophages in all tissues of the mouse. After 3 consecutive daily injections for initial depletion, luciferase-labeled RM-1 mouse metastatic prostate cancer cells (Power et al., 2009) were inoculated via intratibial injection and tumors were monitored for up to 2 weeks (Fig. 4.1A). The efficiency of macrophage ($Gr1^{lo}$, $F4/80^{+}$, c-*fms* intermediate and $CD11b^{hi}$) depletion was validated, before tumor inoculation, and after 3 consecutive daily injections (Fig. 4.1B).

To determine the impact of macrophages on tumor growth in bone, RM-1 cells were injected intratibially in MAFIA mice pre-treated with vehicle or AP. After 14 days, tumor sizes were analyzed by radiolucent area quantification and confirmed by histology (Fig 4.1C). Interestingly, macrophage depleted mice had significantly smaller tumors in bone compared to vehicle treated controls.

As previously reported, macrophage depletion highlights the important roles macrophages play in bone formation, healing, and PTH anabolic actions (Alexander et al., 2011b;Cho et al., 2014) and hence likely in skeletal tumor growth. Since macrophages share precursors with osteoclasts in bone, serum TRAP5b, a bone resorption marker, was analyzed (Fig. 4.1D). Mice had significantly reduced levels of serum TRAP5b after 2 weeks of macrophage depletion compared to vehicle controls. Histomorphometric analyses were performed in tibiae with RM-1 tumors (Fig. 4.1E and F) and in tumor-free femurs. Intratibial tumors presented no significant differences in total bone. In the femurs of macrophage depleted mice, significant reductions in bone volume were observed confirming macrophage depletion effect in bone homeostasis as previously reported (Fig. 4.1F) (Cho et al., 2014). Even though serum TRAP5b levels were significantly reduced with macrophage depletion, osteoclast number per total tissue area (N.Oc/T.ar), osteoclast number per bone perimeter (N.Oc/B.Pm) and osteoclast surface per bone surface (Oc.S/Bs) were not significantly changed in tibiae or femurs with macrophage ablation. These data suggest that macrophage ablation in MAFIA mice hinders tumor growth in bone and significantly decreases total bone volume.

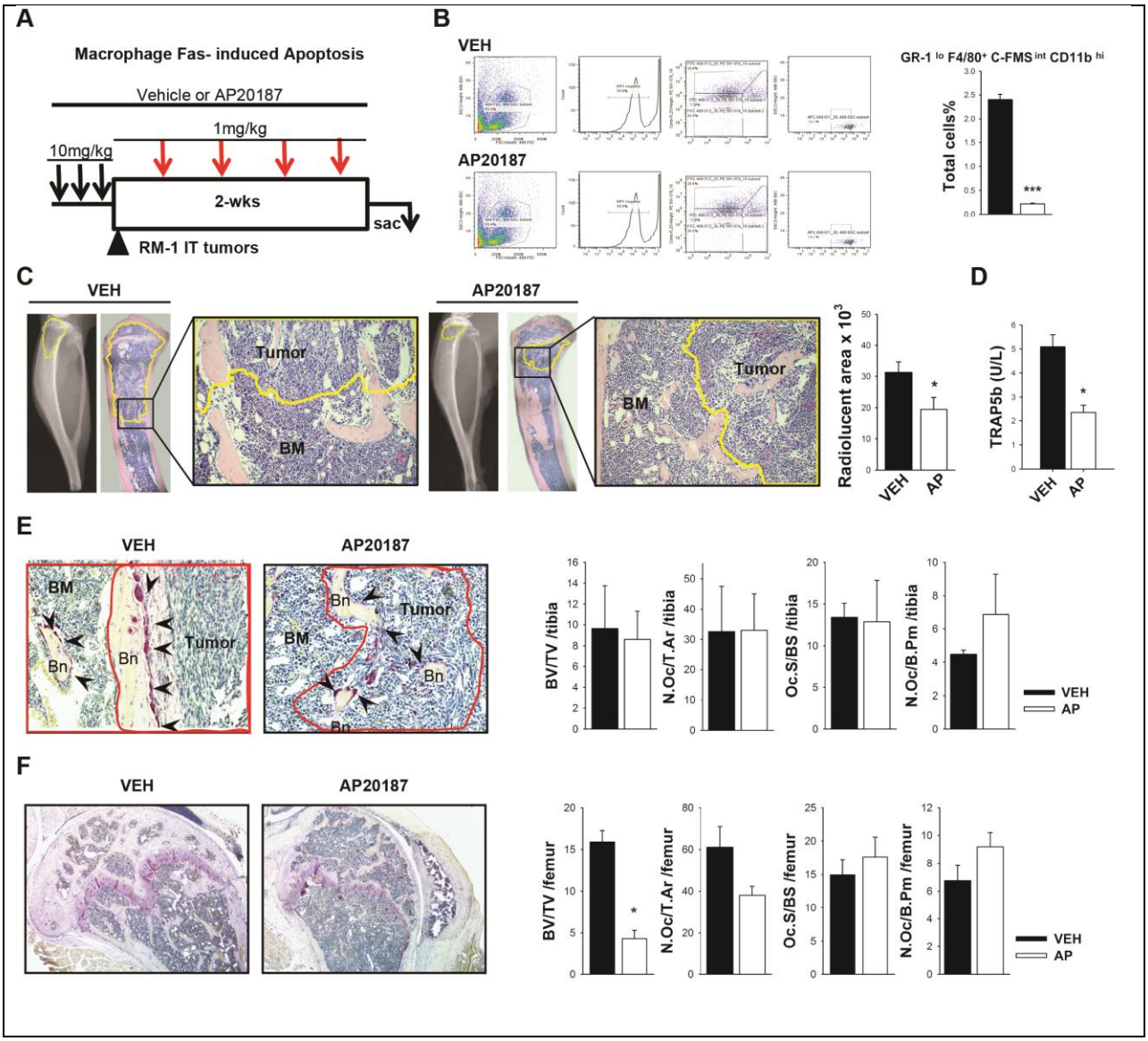

Figure 4.1. Macrophage ablation in MAFIA mice hinders tumor growth in bone and significantly decreases total bone volume

A) Macrophage depletion regimen and intratibial tumor growth. Sixteen week old male MAFIA mice were treated with AP20187 (AP) (10mg/kg) or vehicle control (VEH) for 3 days (black arrows). RM-1 murine prostate cancer cells were injected intratibially (IT) on the fourth day (black triangle) and booster injections were given every third day (1mg/kg) (red arrows) for depletion maintenance. Fourteen days later mice were euthanized and evaluated. B) Representative flow cytometric analysis of the whole bone marrow cells after 3 days initial depletion and quantitative analyses of GR-1^{lo} F4/80^{+} C-FMS^{int} SSC$^{int/lo}$ CD11B^{hi}macrophages. C) Radiographic and histological analyses of intratibial RM-1 tumors in vehicle (VEH) and AP20187 (AP) treated mice. Tumors are highlighted in yellow and 20X images show the edges of tumor and bone marrow cells (BM). Radiolucent areas were quantified in radiographic images of intratibial tumors as shown in graph. D) Serum analyses of TRAP5b (units per liter). E) Histologic images (20X) in the tibia of MAFIA mice in the presence of tumors. Tumors are delineated in red; bone (Bn), bone marrow tumor-free areas and TRAP positive osteoclasts

(black arrows) are indicated. Bone histomorphometric analyses including bone volume (BV/TV), osteoclast numbers per total tissue area (N.Oc/T.Ar), osteoclast surface per bone surface (Oc.S/BS) and osteoclast numbers per bone perimeter (N.Oc/B. Pm) are shown. F) Histomorphometric analyses of tumor-free femurs. Data are mean ±SEM, n=6-8 per group. *P<0.05, *** P<0.001

Macrophage ablation in MAFIA mice hinders prostate cancer subcutaneous growth in vossicle model.

To investigate the impact of macrophages in prostate tumor growth in bone, murine RM-1 prostate cancer cells were injected in vertebral bodies (vossicles) (Koh et al., 2005) of MAFIA mice previously treated with vehicle or AP20187 for macrophage depletion and subcutaneously implanted in the back of MAFIA mice hosts with or without initial macrophage depletion. Tumors were measured every 3 days for up to 2 weeks and booster AP or vehicle injections were given every 3 days to maintain macrophage ablation (Fig. 4.2A).

Tumor sizes were significantly smaller in mice with macrophage depletion at 9 and 12 days (Fig. 2B). Vossicles were collected and flow cytometric analysis showed that tumors had decreased c-fms+F4/80+ macrophages but no significant difference in overall tumor weight (Fig. 4.2C and D). Long bones from recipient mice presented significantly fewer macrophages in the bone marrow (c-fms+F4/80+) and decreased levels of both M2 (c-fms+CD206+) and M1 (c-fms+CD86+) macrophages (Fig. 4.2E). This suggests that macrophage ablation in MAFIA mice efficiently decreased macrophage levels in the vossicle model. In the long bone marrow of the recipient mice treated with the AP compound, macrophage ablation was more dramatic with almost 4-fold reduction. Moreover, macrophage ablation decreased both M1 and M2 macrophages in the marrow.

To better understand the macrophage contribution in tumor growth, subcutaneous tumors were injected in the back of the MAFIA mice and after 2 weeks tumors were collected and analyzed (Fig. 4.2F). Interestingly, no difference in size and weight were observed after 14 days (Fig. 4.2G), even though macrophage ablation was efficient in subcutaneous tumors. These data suggest that macrophage ablation in MAFIA mice hinders tumor growth in endogenous osseous settings as compared to the

subcutaneous setting. Collectively, these findings suggest the marrow microenvironment and the macrophages in that environment are particularly vital for tumor growth.

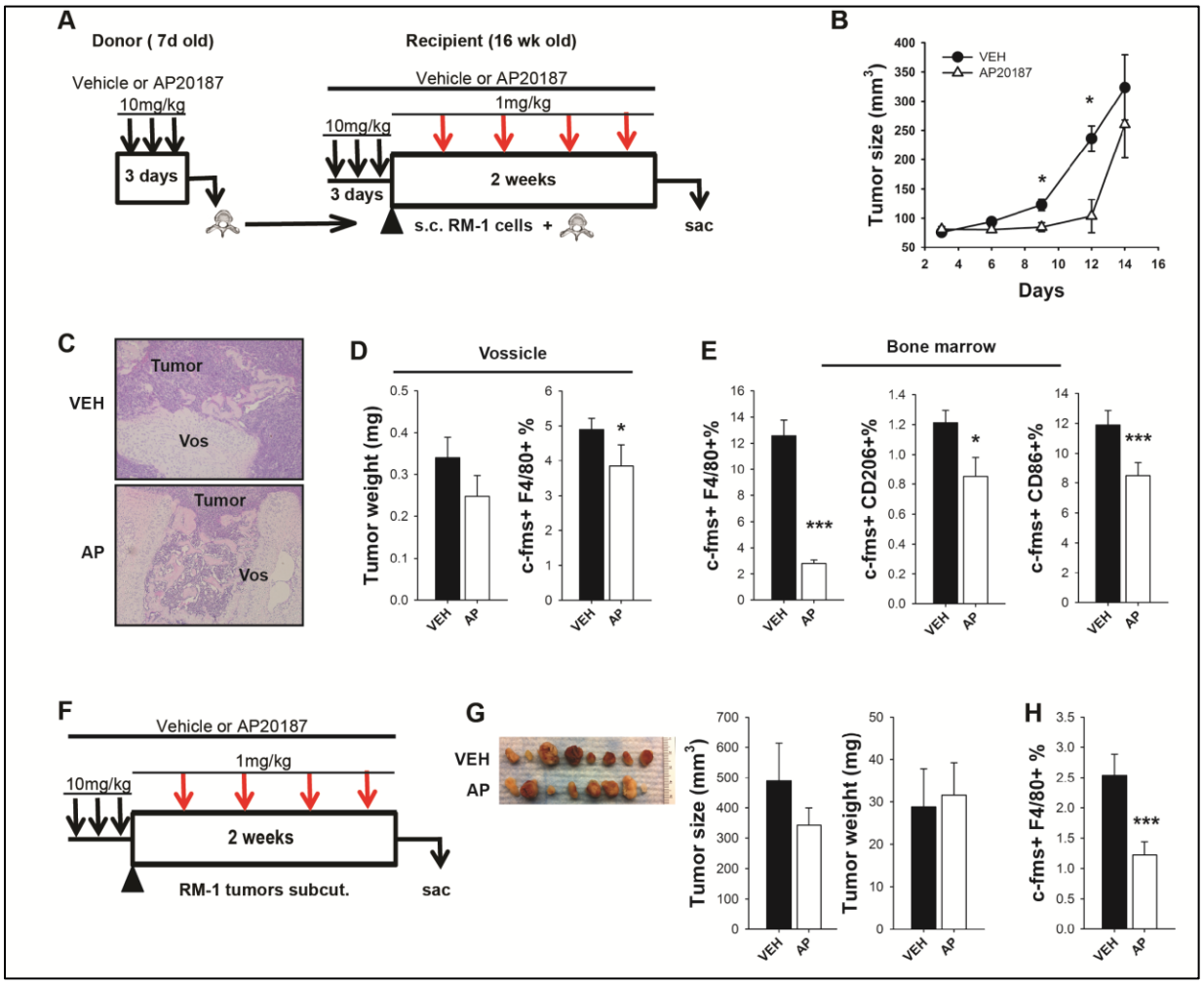

Figure 4.2. Macrophage ablation in MAFIA mice hinders prostate cancer subcutaneous growth in vossicle model.

A) Experimental design for vossicle model. Sixteen week old MAFIA mice (recipients n=6) and 7 day old mice (donors, n=10/group) were treated for 3 days with vehicle (VEH) or AP20187 (AP) (n=6-8). Vertebrae from donor mice were collected and implanted subcutaneously with RM-1 prostate cancer cells (vossicles) in the back of VEH or AP treated recipient mice. Booster injections were given every third day (1mg/kg) for depletion maintenance and tumors were analyzed after 14 days. B) Vossicle tumor growth. Tumor sizes (2 tumors /mouse) were measured by caliper every 3 days for up to 14 days. Tumors in macrophage ablated mice were significantly smaller than controls at day 9 and 12. C) Representative vossicle-tumor H&E sections showing the vossicle (Vos) surrounded by the tumor cells (10X). D) Vossicles were analyzed by FACS for macrophage c-fms+F4/80+% of total cells. Tumor weight (mg) was measured 14 days after implantation. E) FACS analysis of macrophages in the whole bone marrow of tibiae shows macrophage depletion with AP treatment for macrophages c-fms+F4/80+, M2 macrophages F4/80+CD206+ and M1 macrophages F4/80+ CD86+ cells.

Data are mean ± SEM n=6-8 per group. * P<0.05 , *** P<0.001. F) Experimental design for subcutaneous RM-1 tumors in MAFIA mice. Sixteen week old MAFIA mice (n=8) were treated for 3 days with vehicle (VEH) or AP20187 (AP) for initial depletion. In the fourth day RM-1 prostate cancer cells mixed with low growth factor matrigel were injected subcutaneously in the back of vehicle or AP treated mice. Booster injections were given every third day (1mg/kg) for depletion maintenance and tumors were analyzed after 14 days. G) Subcutaneous tumors of VEH or AP treated mice and graphs for tumor size (mm^3) and tumor weight (mg). H) FACS analyses for c-fms+ F4/80+ macrophages in subcutaneous tumors. Data are mean ± SEM, n=8 per group. * P<0.05, *** P<0.001.

Prostate cancer growth in bone of clodronate liposome macrophage-ablated mice

The MAFIA mouse model targets c-fms+ immature macrophages and resulted not only in reduced bone volume but also decreased tumor growth. To investigate whether the presence of tumors in bone would change the bone marrow population, macrophage populations in femurs without tumors were compared to tibiae with tumors. Interestingly, the presence of tumors significantly boosted the macrophage population in the bones when compared to tumor-free femurs (Fig. 4.3A). Nearly a 2-fold increase in M2 macrophages (F4/80+CD206+), monocytes (CD11B+), and CD206+ expression in F4/80+CD11B+ immature macrophages was observed when tumors were present. To better understand the effect of macrophage depletion at different stages of macrophage maturation, an alternate approach was taken utilizing clodronate loaded liposomes to induce apoptosis of mature phagocytic macrophages. Clodronate liposomes were administered to 4 week old male mice for 3 days to initiate depletion. RM-1 prostate cancer cells were injected intratibially on the fourth day and booster injections of clodronate were given every third day to maintain macrophage depletion for 2 weeks (Fig. 4.3B). Consistent with the MAFIA mouse model, clodronate treated mice presented smaller intratibial tumors confirmed by histologic and radiographic analyses of tumor area and radiolucent area quantification (Fig. 4.2C and 4.2D). Efficient macrophage ablation was confirmed by F4/80+ cell staining of tibiae sections (Fig. 4.3E). Figure 4.3F shows that macrophage ablation by clodronate liposome treatment significantly decreased M2 macrophage F4/80+CD11B+CD206+ cells in intratibial tumors. In summary, figure 4.3 suggests that targeting the phagocytic mature M2 macrophages resulted in smaller tumors in bone and confirmed macrophage contribution to tumor growth in bone.

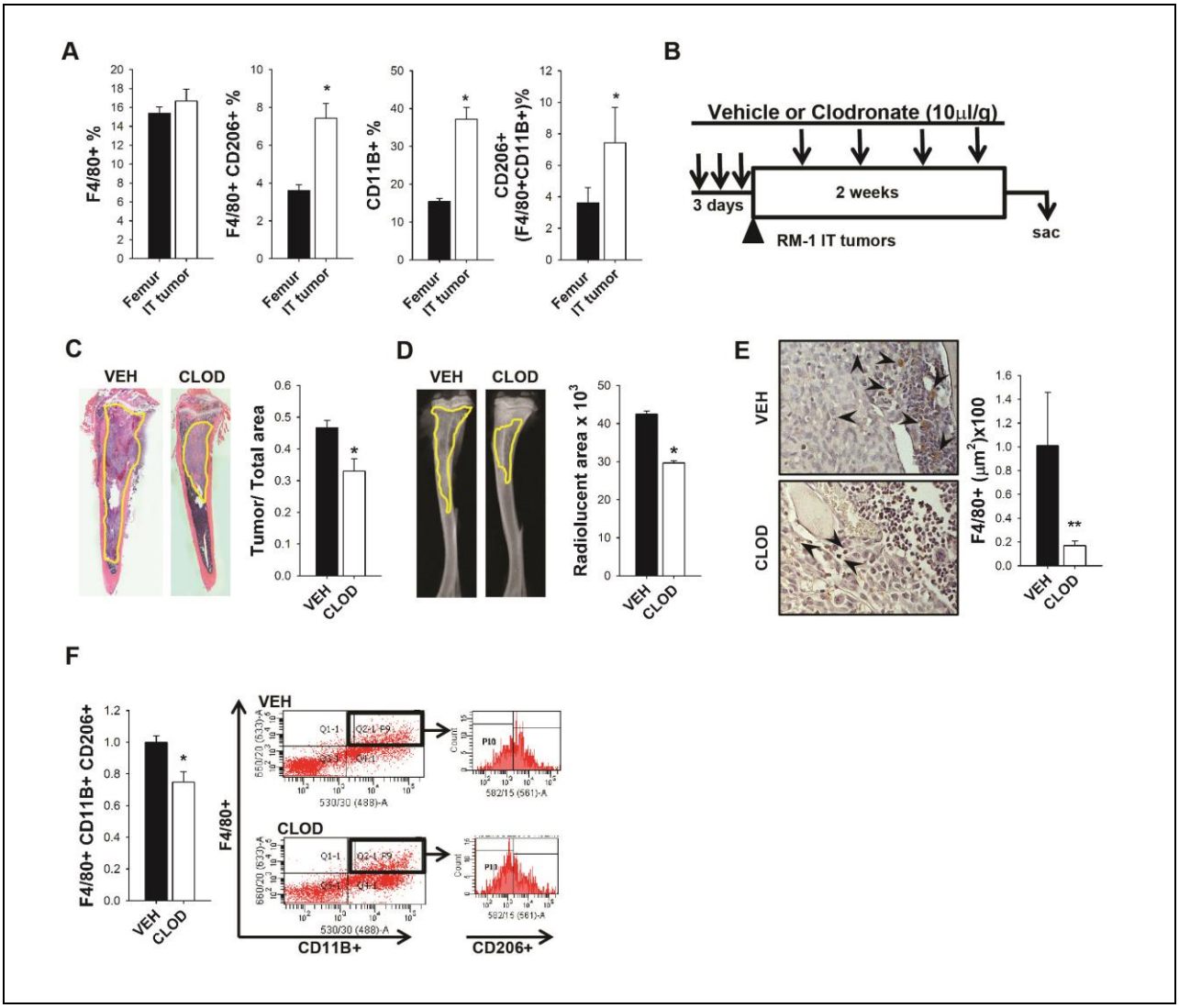

Figure 4.3. Clodronate liposome induced macrophage ablation and hindered RM-1 tumor growth in bone.

A) Representative flow cytometric analysis of the intratibial tumors for M2 macrophage marker CD206 gated of F4/80+/CD11B+ cells over total cells. F) FACS analyses of non-tumor femur versus intratibial tumors for macrophages (F4/80+), M2 macrophages (F4/80+ CD206+), monocytes (CD11B+) and CD206+ cells gated on F4/80+CD11B+ population (n=10). B) Macrophage depletion regimen and intratibial tumor growth for 2 weeks. Four-six week old C57Bl6 male mice were treated with 3 consecutive injections of clodronate loaded liposomes (CLOD) (10μl/g) or vehicle (VEH). RM-1 murine prostate cancer cells were injected intratibially on the fourth day and booster injections were given every third day for depletion maintenance. Fourteen days later mice were euthanized and evaluated. C and D) Histological and radiographic analyses of intratibial RM-1 tumors in vehicle (VEH) and clodronate (CLOD) treated mice. C) Representative image of intratibial tumor (yellow) and tumor area/tibia area quantification. D) Representative X-ray image of tibia and radiolucent area (yellow) quantification. E) F4/80 staining (red-brown) on tibial sections (20X) and quantification. Arrows

show F4/80+ cells. Data are mean ± SEM; n=18 per group, unless otherwise denoted; * P<0.05.

Clodronate liposome macrophage ablation resulted in increased bone volume

Both macrophage ablation strategies, the specific transgenic macrophage MAFIA mice, and the pharmaceutical approach targeting the more mature macrophages with clodronate liposome treatment, resulted in smaller tumors in bone. To better understand the contribution of mature phagocytic macrophages in bone remodeling and how this may influence skeletal tumor growth, bone histomorphometric analyses were performed. In contrast to the MAFIA model, clodronate liposome treatment resulted in increased total bone volume (Fig. 4.4A) even though tumors were significantly smaller (Fig. 4.4B). Short term macrophage ablation for 2 weeks also resulted in decreased serum TRAP5b levels (Fig. 4.4C). Osteoclast TRAP+ staining showed a significant reduction in the osteoclast surface per bone surface (Oc.S/BS) but no changes in osteoclast number per total tissue area (N.Oc/T.Ar) or bone perimeter (N.Oc/B.Pm) (Fig. 4.4D).

To exclude the possibility that differences seen in bone volume could be due to the osteolytic nature of RM-1 murine tumor cells, μCT analysis was performed in tumor-free femurs (Fig. 4.4E). Consistent with previous studies (Cho et al., 2014), clodronate liposome induced-macrophage ablation resulted in increased total bone (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and reduction in trabecular spacing (Tb.Sp). This data shows that targeting the mature macrophage population results in a differential response in bone versus the MAFIA model. Importantly, both macrophage ablation models presented decreased tumor sizes in bone, but had different responses in bone modeling, suggesting that macrophage contribution to tumor growth is independent of its function in bone remodeling.

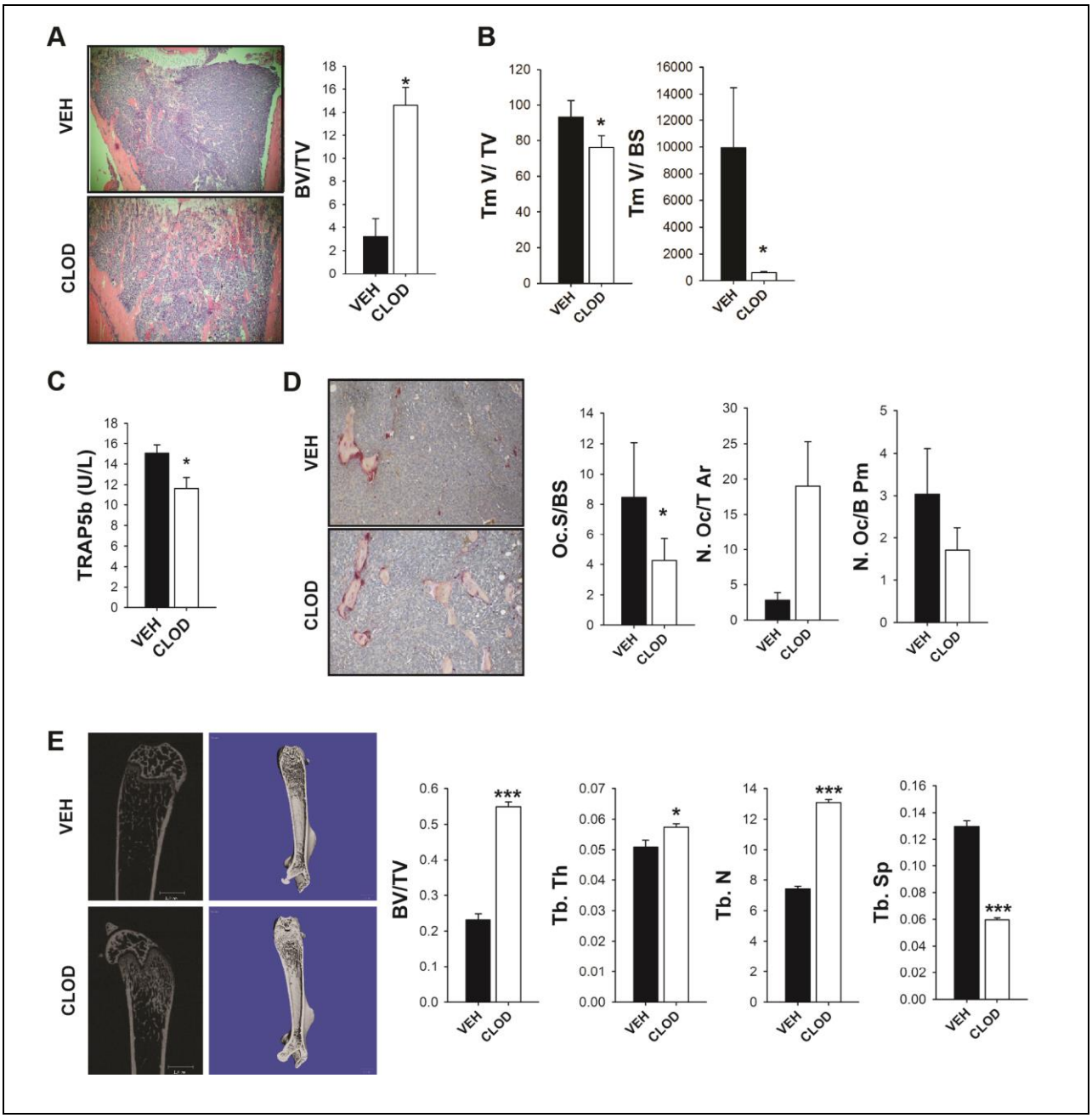

Figure 4.4. Clodronate liposome treated mice had increased bone volume in intratibial tumor sections.

A) Representative image (4X) of total bone volume (BV/TV) and quantification. B) Total tumor volume (Tm V/TV) and tumor volume per bone surface (Tm V/BS) were analyzed. C) Serum analyses of TRAP5b (units per liter). D) Representative image (20X) of TRAP positive osteoclast surface per bone surface (Oc.S/BS), osteoclast numbers per total tissue area (N.Oc/T.Ar) (p=0.052), and osteoclast numbers per bone perimeter (N.Oc/B. Pm) were quantified. E) Representative 2D and 3D μCT images of tumor free femurs show increased trabecular bone volume in clodronate treated mice. Trabecular total bone volume (BV/TV), trabecular thickness (Tb.Th), number (Tb.N) and spacing (Tb.Sp) were quantified. Data are mean ± SEM; n=10-13 per group; * P<0.05, *** P<0.001.

PC-3 human prostate cancer growth in bone of clodronate liposome macrophage-ablated mice

A human prostate cancer model was employed to confirm the macrophage contribution in skeletal tumor growth using PC-3 cells in the intratibial tumor inoculation experiment, this time using athymic mice. To delineate macrophage involvement in bone tumors, bone marrow flush was compared between the intratibial tumors and tumor-free femurs of mice treated with clodronate or vehicle control, and similar results were observed with significantly increased M2 macrophage (F4/80+ CD206+) cells in the presence of tumors, suggesting the contribution of M2 macrophages in skeletal tumors (Fig. 4.5A)

Three days after initial depletion with vehicle control or clodronate liposome treatment, luciferase tagged PC-3 cells were injected in the tibia and tumors were monitored by bioluminescent imaging every week for 6 weeks. Booster injections were given weekly (5-8 μl/g) for macrophage depletion maintenance during the 6 week period (Fig. 4.5B). After 6 weeks, bioluminescent imaging showed that clodronate liposome treated mice presented significantly smaller tumors in the tibia compared to controls, confirming a role of macrophages in PC-3 tumor growth in bone (Fig. 4.5C). Tibiae with tumors were collected (Fig. 4.5D) and flow cytometry confirmed significant ablation of M2 macrophage (F4/80+CD206+) cells (Fig. 4.5E).

In addition, μCT and histomorphometric analyses of tibiae demonstrated that clodronate liposome treatment increased bone volume and resulted in smaller tumors (Tm/TV) (Fig. 4.5F and G). Serum TRAP5b was not changed after 6 weeks of macrophage ablation (Fig. 4.5H). TRAP staining showed significant reduction in osteoclast surface per bone surface (Oc.S/BS), number of osteoclasts per bone perimeter (N.Oc/B Pm) and increased total number of osteoclasts (N.Oc/T.Ar). Similar to RM-1 prostate cancer cells, these data suggest that macrophages contribute to PC-3 prostate cancer growth in bone independent of the bone response.

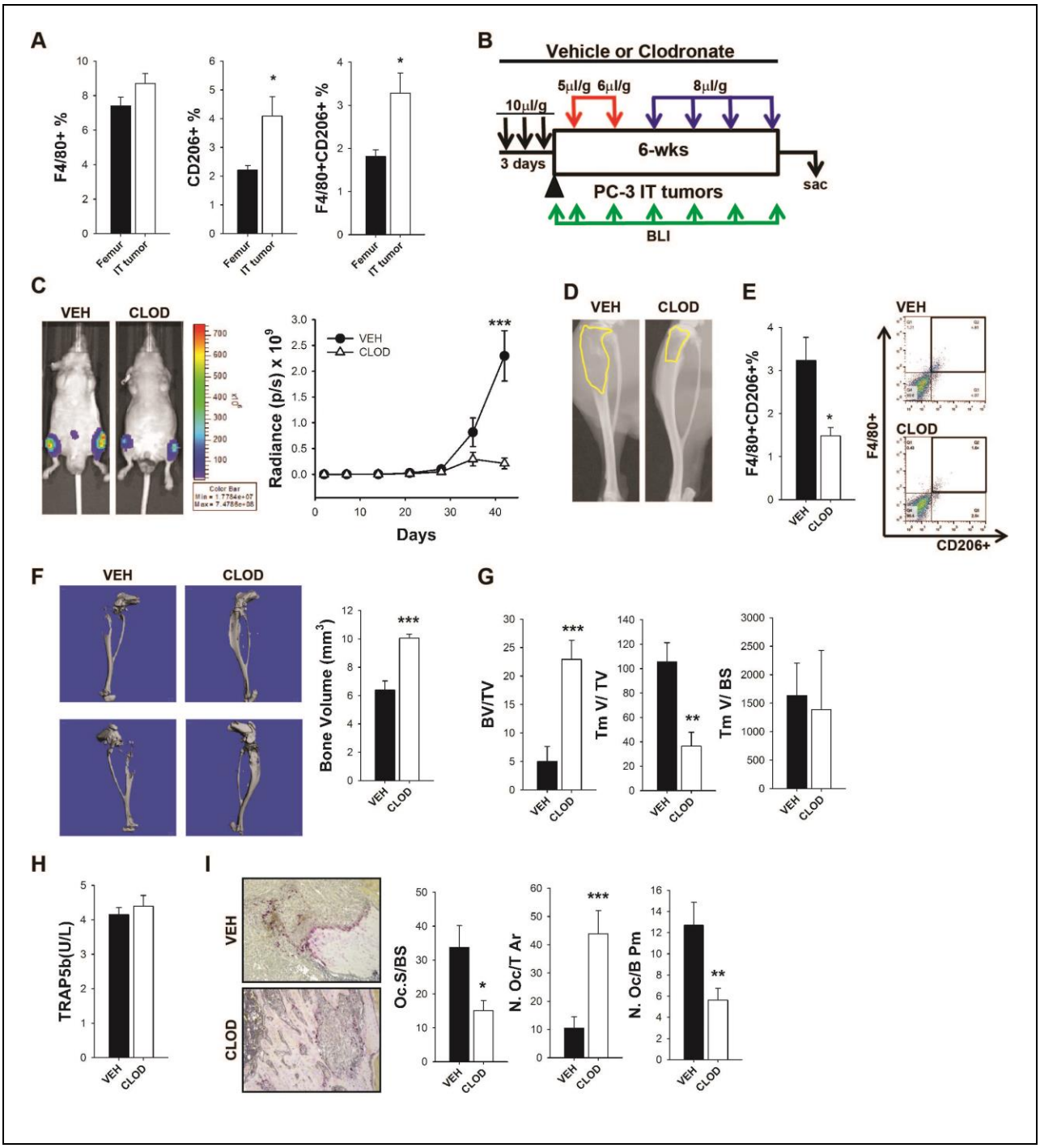

Figure 4.5. PC-3 intratibial tumors in athymic mice increased M2 macrophages that contributes to intraosseous tumor growth.

A) FACS analyses of tumor-free femur whole bone marrow flush and intratibial tumors for macrophages (F4/80+), CD206+ cells and double positive M2 macrophages (F4/80+ CD206+) (n=10/group). B) Macrophage depletion regimen and intratibial PC-3 tumor growth for 6 weeks. Four-six week old athymic male mice were treated with 3 consecutive injections of clodronate loaded liposome (CLOD) (10μl/g) or vehicle (VEH). PC-3 Luciferase labelled human prostate cancer cells were injected intratibially on the fourth day and booster clodronate or vehicle

injections were given every week for depletion maintenance at different doses. BLI images were taken every week for luciferase radiance for the next 6 weeks. C) BLI representative images at 42 days of intratibial PC-3 tumor growth in vehicle (VEH) and clodronate (CLOD) treated mice. Graph represents BLI measurements for 6 weeks. (n=15-17). D) Representative X-ray image of tibia and radiolucent area was delineated in yellow. E) Flow cytometric analysis of intratibial tumors for F4/80+/CD206+ quantification of total cells (%) and representative dot plots. F) μCT analyses of bones in long term depleted mice with intratibial tumors (n=15-17) and representative 3D images shows higher total bone volume (BV/TV) in clodronate treated mice. G) Bone histomorphometric analyses in clodronate or vehicle treated mice in intratibial tumor sections of total bone volume (BV/TV), total tumor volume (TmV/TV) and tumor volume per bone surface (TmV/BS) (n=14-15). H) Serum analyses of TRAP5b (units per liter). I) Representative image (10X) of TRAP positive osteoclasts stained in red. Osteoclast surface per bone surface (Oc.S/BS), osteoclast number per total tissue area (N.Oc/T.Ar), and osteoclast number per bone perimeter (N.Oc/B.Pm) were quantified. Data are mean ±SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

DISCUSSION

Macrophages are heterogeneous myeloid lineage cells that comprise diverse functions towards the immune defenses (Mantovani and Sica, 2010;Mantovani et al., 2004). They can rapidly respond to signs of injury, infection and to changes in the environment, having a diverse differentiation and activation profile according to the stimuli received (Pollard, 2009). Simplistically, macrophages can be described as classically activated (also known as M1) or alternatively activated (known as M2). M1 macrophages respond to infection and generate pro-inflammatory responses to exert their role as cell killers against invaders and pathogens. On the other hand, M2 macrophages are known to participate in wound healing, tissue repair, and as anti-inflammatory cells to moderate inflammatory responses. M2 macrophages are also known as tumor associated macrophages (TAMs) because of the similar immunosuppression roles they have in the context of tumors, contributing to tumor growth, progression and metastasis of different types of cancer (Pollard, 2004;Siveen and Kuttan, 2009). In bone, ‘osteomacs’ are the resident macrophages that line the periosteal and endosteal tissues and have essential roles, supporting osteoblast maintenance and functional activity (Chang et al., 2008). Moreover, these resident macrophages have been shown to impact bone healing, remodeling and hematopoietic stem cell maintenance (Pettit et al., 2008;Alexander et al., 2011b;Winkler et al.,

2010b;Cho et al., 2014). However, how resident macrophages and their impact in the bone microenvironment affect skeletal tumor growth is unclear.

The present study demonstrated that targeting macrophages for depletion hindered tumor growth in bone. Different strategies of macrophage depletion were utilized and similar results were observed. In the transgenic MAFIA mouse, intratibial tumors presented smaller tumors in macrophage depleted mice compared to controls. Depletion of macrophages in the bone marrow was confirmed by flow cytometry looking at very specific macrophage markers ($Gr1^{lo}$, $F4/80^{+}$, c-fms^{int} and $CD11b^{hi}$) prior to tumor inoculation. When subcutaneous tumor growth was analyzed after 2 weeks of inoculation, no difference was observed, even though there was a significant reduction in macrophage c-fms+F4/80+ in both subcutaneous tumors and bone marrow. Interestingly, there was a significant difference in tumor size in the vossicle model at earlier time points in macrophage depleted mice. We hypothesized that when tumors are growing within the vertebrae body, a significant reduction of macrophages have higher impact on the tumor growth, but when tumors outgrow the bone area this difference in growth is no longer the driving factor. This suggests that macrophage depletion has a higher impact in the bone marrow compartment, which is rich in myeloid cells, and which is the case in the intratibial tumor model. To further validate the role of macrophages in skeletal tumor growth, a more narrowly focused macrophage depletion model was introduced with clodronate liposome treatment. Mature macrophage depletion was efficient, and similar to the MAFIA mouse model, there were smaller tumors in macrophage depleted mice. In this model, tibiae with tumors and tumor-free femurs were both analyzed by FACS for macrophage markers. Interestingly, clodronate-induced depletion significantly reduced M2 macrophages (F4/80+ CD206+) in intratibial tumors. Moreover, the presence of tumors in tibiae significantly increased M2 macrophages. Similar results were also confirmed with the human PC-3 prostate cancer cell line inoculated in the tibiae of athymic mice over 6 weeks. These data not only confirmed the macrophage contribution for tumor growth, but also demonstrated that tumor development in bone marrow increased M2-like macrophages, which further suggests the important role of macrophage polarization in tumor progression.

Altering the bone microenvironment, rich in myeloid cells, prior to tumor inoculation affected tumor growth in bone. Park et al demonstrated that a transient expansion of myeloid cells resulted from a single dose of cyclophosphamide prior to intracardiac tumor inoculation of prostate cancer cells increased localization and growth in bone (Park et al., 2012a). Moreover, tumor derived factors such as PTHrP (parathyroid hormone-related protein) and CCL2 may induce direct and indirect changes in bone and tumor microenvironment that contribute for tumor growth (McCauley and Martin, 2012b;Loberg et al., 2007b;Loberg et al., 2007a;Li et al., 2009;Roca et al., 2009;Rozel et al., 2009;Mizutani et al., 2009;Zhang et al., 2010;Fridlender et al., 2010;Park et al., 2012a;Soki et al., 2012). For example, PTHrP coming from solid prostate tumors can prime and recruit the myeloid cells, also known as myeloid derived suppressor cells (MDSCs) that contributed for solid tumor growth. CCL2 is a chemokine also known for its role in both tumor growth and myeloid cell modulation, a contributing factor for M2 macrophage polarization (Loberg et al., 2007b;Loberg et al., 2007a;Li et al., 2009;Roca et al., 2009;Rozel et al., 2009;Mizutani et al., 2009;Zhang et al., 2010;Fridlender et al., 2010;Park et al., 2012a). Specifically, in bone, CCL2 is known to be involved in a destructive cascade, being released after PTHrP stimulation of osteoblasts, which in turn secretes CCL2 in the bone marrow activating osteoclastogenesis and tumor growth (Li et al., 2009).

Osteoclasts are key players in skeletal metastasis, and are specialized cells differentiated from macrophages. Therefore, a special challenge in bone when studying macrophages is the effects that macrophage depletion may have on osteoclast activity. No difference was observed in local osteoclast numbers when tumors were present although systemic osteoclast TRAP5b levels were reduced after 2 weeks of macrophage depletion in MAFIA mice. Clodronate liposome administration for 2 weeks also resulted in reduced TRAP5b levels. A longer macrophage depletion regimen for 6 weeks indeed resulted in decreased osteoclast number per bone perimeter and osteoclast surface per bone surface, but differences were not seen in TRAP5b levels. This may be a reflection of increased bone content while the osteoclast activity is stabilized as well as a differential response in the local environment versus the systemic circulation. Many tumor derived factors are known to promote osteoclastogenesis such

as PTHrP and CCL2 (Soki et al., 2012;Park et al., 2013;Loberg et al., 2007b;Li et al., 2009;Mizutani et al., 2009;Park et al., 2012a). Overall, these data suggest that even though macrophage depletion may suppress in part osteoclast differentiation/activation, tumors may compensate for the suppression, driving the differentiation/activation of the remaining macrophage progenitors towards osteoclasts.

An interesting finding in the present study was the fact that tumors were consistently smaller in macrophage depleted mice, even though paradoxical results of macrophage depletion on the bone were observed. Targeting early progenitor cells under the c-fms promoter in the transgenic MAFIA mouse model resulted not only in reduced tumor growth, but also decreased bone volume in the hind limbs of macrophage depleted mice. In contrast, targeting mature phagocytic macrophages with administration of clodronate liposome resulted in increased bone volume, while tumor growth was also still hindered in macrophage depleted mice in both murine RM-1 and human PC-3 prostate cancer cells. These results suggest that the effect of macrophage depletion in tumor growth was independent of its effect on bone tissue. We have previously demonstrated that osteal macrophages support bone remodeling and PTH anabolic actions in bone of adult murine skeletal system (Cho et al., 2014). Clodronate loaded liposomes triggered an opposite effect resulting in an osteogenic microenvironment and augmented PTH anabolic actions. The main difference observed was that the phagocytic myeloid CD68+ cells that include macrophages, dendritic cells and neutrophils were differential according to the depletion model. In clodronate liposome treated mice there was a significant increase in CD68+ cells which led to the hypothesis that selective depletion of phagocytic macrophages activates a compensatory expansion and activation of the mononuclear phagocytic system for cell clearance of apoptotic cells and debris.

Apoptotic cell clearance through phagocytosis, also known as efferocytosis, has been shown to contribute for tumor growth and modulation of immune responses (Reiter et al., 1999b;Michlewska et al., 2009;Gregory and Pound, 2011a). For example, macrophage efferocytosis of apoptotic tumor cells mediated through milk fat globule EGF-8 (MFG-E8), a protein that facilitates this process, modulates macrophage polarization into M2 tumor associated macrophages (Soki et al., 2014a). This may be a

potential mechanism by which macrophages may play a role not only in tumor growth and polarization, but also in bone responses. How phagocytic macrophages and the mechanisms utilized for cell clearance may influence in tumor growth is an intriguing avenue of future investigation.

CONCLUSIONS

In summary, this study shows strong evidence that resident macrophages contribute to prostate cancer growth in bone. The presence of tumors was sufficient for the increase in tumor associated macrophages in bone marrow. Moreover, targeting earlier progenitor cells or mature phagocytic macrophages hinders tumor growth in bone with a differential effect in bone area. The MAFIA mouse model that targets immature macrophage progenitors displayed reduced bone. On the other hand, clodronate liposome treatment that targets mature phagocytic macrophages resulted in increased bone volume. In conclusion, these data support that macrophages have important roles in tumor growth in bone. Moreover, macrophage depletion hindered tumor growth independently of the paradoxical effect on bone content suggesting macrophages in bone may be more important to tumor growth than the bone itself.

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CHAPTER 5

POLARIZATION OF PROSTATE CANCER ASSOCIATED MACROPHAGES IS INDUCED BY MILK-FAT GLOBULE-EGF FACTOR 8 (MFG-E8) MEDIATED EFFEROCYTOSIS

ABSTRACT

Tumor cells secrete factors that modulate macrophage activation and polarization into M2 type tumor associated macrophages (TAMs), which promote tumor growth, progression and metastasis. The mechanisms that mediate this polarization are not clear. Macrophages are phagocytic cells that participate in the clearance of apoptotic cells, a process known as efferocytosis. Milk fat globule - EGF factor 8 (MFG-E8) is a bridge protein that facilitates efferocytosis and is associated with suppression of pro-inflammatory responses. The current study investigated the hypothesis that MFG-E8 mediated efferocytosis promotes M2 polarization. Tissue and serum exosomes from prostate cancer patients presented higher levels of MFG-E8 compared to controls, a novel finding in human prostate cancer. Co-culture of macrophages with apoptotic cancer cells increased efferocytosis, elevated MFG-E8 protein expression levels, and induced macrophage polarization into an alternatively activated M2 phenotype. Administration of antibody against MFG-E8 significantly attenuated the increase in M2 polarization. Inhibition of STAT3 phosphorylation using the inhibitor Stattic, decreased efferocytosis and M2 macrophage polarization *in vitro,* with a correlating increase in SOCS3 protein expression. Moreover, MFG-E8 knockdown tumor cells cultured with wild type or MFG-E8 deficient macrophages resulted in increased SOCS3 expression with decreased STAT3 activation. This suggests that SOCS3 and phospho-STAT3 act

in an inversely dependent manner when stimulated by MFG-E8 and efferocytosis. These results uncover a unique role of efferocytosis via MFG-E8, as a mechanism for macrophage polarization into tumor promoting M2 cells.

INTRODUCTION

The skeleton is a favored organ for metastasis that results in significant morbidity for cancer patients (Bubendorf et al., 2000;Weilbaecher et al., 2011a). Tumor associated macrophages (TAMs) have been investigated in the context of cancer (Li et al., 2009;Loberg et al., 2007b) yet their role in bone metastasis remains elusive (Loberg et al., 2005;Park et al., 2011b;Li et al., 2009). Macrophages are activated differentially according to the stimuli provided: M1 anti-tumorigenic macrophages are defined as classically activated, and M2 pro-tumorigenic macrophages (also known as TAMs) are defined as alternatively activated (Pollard, 2009;Siveen and Kuttan, 2009). TAMs are prominently involved with cancer initiation, progression and metastasis, facilitating angiogenesis, matrix breakdown, and tumor cell-motility (Pollard, 2004;Colotta et al., 2009;Murdoch et al., 2008). While the role of TAMs in skeletal metastasis is emerging, the mechanisms remain to be defined.

In addition to their high rate of cell proliferation, tumors have high apoptosis rates, an often forgotten but important process in tumor dynamics (Gregory and Pound, 2011b). The large number of apoptotic cells is under appreciated due to rapid cell clearance performed by macrophages and other phagocytic cells, a process known as efferocytosis. Indeed, not much is known regarding the interactions of apoptotic tumor cells with macrophages, and subsequently, how this affects the remaining viable tumor cells in skeletal metastasis. Efferocytosis elicits changes in immune responses and can result in immunosuppression of anti-tumor defenses (Reiter et al., 1999a;Savill et al., 2002a), production of anti-inflammatory mediators including transforming growth factor–β (TGF-β) and interleukin-10 (IL-10), and inhibition of pro-inflammatory tumor necrosis factor (TNF) (Savill et al., 2002a;Michlewska et al., 2009).

Milk fat globule-EGF factor 8 (MFG-E8) is a protein secreted by numerous cells including macrophages and is known as a phagocytosis “eat me” signal (Hanayama et al., 2002;Hanayama et al., 2004). MFG-E8 functions as a tether between macrophage and apoptotic cells via a bi-motif function, binding to both phosphatidylserine (PS) externalized on apoptotic cells and the $\alpha v\beta 3$/ $\alpha v\beta 5$ integrin expressed on macrophages (Hanayama et al., 2002). MFG-E8 mutant mice are deficient in efferocytosis, resulting in increased apoptotic cell accumulation, which was implicated in autoimmunity and inflammatory related diseases (Miksa et al., 2008;Asano et al., 2004;Thorp and Tabas, 2009).

Interestingly, phagocytes activated by their interaction with apoptotic cells via MFG-E8 share similar anti-inflammatory and tumor promoting properties as seen in M2 TAMs (Lewis and Pollard, 2006;Gregory and Pound, 2011b;Pollard, 2009). Pre-treatment of LPS stimulated macrophages with rMFG-E8 results in activation of suppressor of cytokine signaling 3 (SOCS3), via the STAT3 pathway (Aziz et al., 2011b). SOCS3 proteins are known to inhibit the JAK/STAT signaling pathway, creating a negative feedback loop to prevent excessive activation of the pathway (Yoshimura et al., 2007). This modulatory role has also been suggested as a mechanism for macrophage polarization.

Despite these suggestive links, efferocytosis via MFG-E8 and its role in tumor efferocytosis and M2 polarization has not been investigated in the context of prostate cancer or skeletal metastasis.

Given that prostate cancer has a high propensity to metastasize to the bone, which is rich in macrophages and anti-inflammatory factors that contribute to tumor growth, we hypothesized that MFG-E8-mediated efferocytosis modulates the bone marrow derived macrophage SOCS3/STAT3 pathway, inducing an M2 switch, and promoting tumor growth. In the current study, the impact of efferocytosis mediated by MFG-E8 on macrophage polarization into M2 TAMs was investigated, and the underlying mechanisms that could be developed as potential therapeutic targets were delineated.

MATERIAL AND METHODS

Primary cells and cell lines

All animals were maintained in accordance with institutional animal care and use guidelines and experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan. MFG-E8 mutant mice were provided by Kamran Atabai from the University of California, San Francisco. Briefly, MFG-E8 mutant mice have an insertion of the pGT1-pfs gene trap-vector in intron 7 of Mfge8, trapping the secretory protein, resulting in fusion protein degradation in the ER (Atabai et al., 2005).

Primary bone marrow cells were collected from male C57BL/6J (Jackson Laboratory, Bar Harbor, ME) and MFG-E8 mutant mice at 4-6wks of age for *in vitro* experiments. Mouse bone marrow macrophages were differentiated *in vitro* from bone marrow cells cultured in α-MEM media with 30 ng/ml murine M-CSF (eBioscience) for 6 days. At day 7, macrophages or RAW 264.7 cells were plated into 12 well plates. Cells were treated with recombinant murine MFG-E8 (R&D Systems) at indicated concentrations. Anti-MFG-E8 (D161-3, MBL International) and IgG control (DAKO) were used at 20μg/ml to treat macrophages *in vitro* as described (Jinushi et al., 2009).

Three prostate cancer cell lines were used in this study. Two human bone metastatic prostate cancer cell lines: C42B and PC-3, were obtained from the American Type Culture Collection (Rockville, MD). RM-1, a murine cell line was originally obtained from Dr.Timothy C.Thompson (Baylor College of Medicine, Houston, TX). C42B cells, originally derived from LNCaP tumors maintained in castrated and intact athymic male mice, are androgen-independent, metastasize to the bone, and form mixed osteoblastic/osteolytic lesions (Lin et al., 2001). PC-3 cells, derived from human vertebral prostate cancer metastasis, are androgen-independent, highly metastatic, and produce osteolytic lesions. Human PC-3 cells were stably transfected to express dsGREEN immunofluorescence.

Apoptosis of prostate cancer cells (RM-1, PC-3 and C42B) was induced by overnight serum starvation followed by 24 hour treatment with cobalt chloride (RM-1 150μM; PC-3 and C42b 200μM) (Sigma-Aldrich). Cells were washed at least 3 times

with PBS after apoptosis induction and enumerated. A 60-70% range of trypan blue positive cells was designated high apoptotic (HAP). UV exposure of tumor cells for 20 minutes in PBS was also utilized to induce apoptosis avoiding chemical-derived effects. Untreated tumor cells contained 5-10% trypan blue positive cells and were designated as basal apoptotic cells (BAP).

Stable-shRNA constructs

Mouse GIPZ lentiviral shRNAmir were transfected into RM-1 prostate cancer cells to produce MFG-E8- shRNA stable contructs. Cells were designated as RM-1 m_08 (clone ID: V2LMM_277508), RM-1 m_30 (clone ID: V2LMM_39830), RM-1 m_41 (clone ID: V3LMM_432041), RM-1 m_43 (clone ID: V3LMM_432043) RM-1 m_45 (clone ID: V3LMM_432045) and RM-1 GIPZ scramble control (Lenti-pGipZ-scramble-VSVG; University of Michigan Vector Core).

Efferocytosis assay

RAW 264.7 cells or primary bone marrow macrophages were plated at 50,000 cells/cm^2 and incubated at different time points with low or high apoptotic prostate cancer cells (RM-1, PC-3 and C42b) at a 1:3 ratio of macrophage to tumor cells, or fluorescent carboxyl beads (Bangs Laboratories, INC) at a 1:2 ratio. Cells were washed with PBS and attached cells were collected for further analyses.

Confocal microscopy

Bone marrow macrophages, RAW 264.7 cells and prostate cancer cells were stained with 0.2μl/ml orange or green CellTracker™ or CellTrace™ CFSE (Invitrogen) for 20 minutes in serum free medium followed by incubation in complete medium for 60 minutes. Macrophages were plated in 1.5mm cover glass chambers for confocal microscopy. High apoptotic cancer cells or carboxylated fluorescent beads (Bang's Labs) were added at a 1:1 or 1:2 ratio to the attached macrophages for up to 24 hours and fixed with ice cold methanol for 20 minutes. Cells were then washed with PBS and covered with Vectashield® mounting medium containing DAPI (Vector Laboratories, Inc). Confocal microscopy images were analyzed using the Leica Inverted SP5X

Confocal Microscope System with 2-Photon FILM and Leica Software (Leica Microsystems Germany).

Flow cytometry

Flow cytometry was performed as described (Cho et al., 2013). Briefly, cells were collected in FACS buffer (PBS, 2% FBS, 2mM EDTA) and 1 million cells were stained for 30 minutes with the following macrophage specific antibodies: F4/80 Alexa Fluor 647 (Abcam, CI:A3-1) and CD206 PE (AbD Serotec, MR5D3). Cells were fixed and permeabilized with Leucoperm™ (AbD Serotec) and incubated with anti-Ym1 rabbit antibody (StemCell Technologies, #01404). Cells were then incubated with secondary antibody, Alexa Fluor® 700 Goat Anti-Rabbit IgG (Molecular Probes®) 700. For confirmation of apoptosis, prostate cancer cells were stained with the FITC Annexin V Apoptosis Detection kit (BD Pharmigen™). After antibody incubation, cells were washed twice with FACS buffer, fixed with 1% formalin and evaluated for FACS analyses (BD FACSAria™ III).

Western blot analyses and quantification

For protein collection, cells were washed twice with cold PBS and lysed with CelLytic™ M (Sigma). SDS-PAGE was performed in 4-20% gradient Novex Tris-Glycine gels (Invitrogen) loaded with 50μg of protein per well. After electrophoresis, proteins were transferred to membranes (Duralon UV membrane; Stratagene) and blocked in 5% milk for 20 minutes. The membranes were then incubated with the primary antibody in 5% milk overnight on a shaker at 4°C**.** Antibodies against β–actin (C4) 1:50,000 (Santa Cruz Biotechnology, Inc), SOCS3 1:500 dilution (Cell Signaling Technology), Phospho-STAT3 (3E2) and STAT3 (124H6) 1:1000 dilution (Cell Signaling Technology), and MFG-E8 1:500 (R&D Systems) were used. After washing, membranes were incubated with secondary antibodies, washed and chemiluminescence (via SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) imaged on X-ray film . Protein quantification was performed using the Scion Image software and calculated relative to control protein expression (β–actin).

RNA extraction and quantitative RT-PCR

RNA isolation was performed as described (Koh et al., 2011) using an RNeasy Mini Kit (QIAGEN, Valencia, CA). cDNA was synthesized using 0.5□g of total RNA in 50□l of reaction volume using the TaqMan Reverse Transcription Kit (Applied Biosystems). Quantitative real time-PCR (qPCR) was performed using the ABI PRISM 7700 using a ready-to-use mix of primers and FAM-labeled probe assay systems (Applied Biosystems) for *Il10* (Mm00439614_m1), transforming growth factor beta1 (*Tgf-β1*) (Mm03024053_m1) and *Ym1* (*Chi3l3)* (Mm00657889_mH), arginase 1 (*Arg1*) (Mm00475988_m1). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Mm99999915_g1) was used as an internal reference and the delta delta CT method was used to calculate the data as described (Novince et al., 2012).

Immunohistochemistry

A prostate cancer tissue microarray (TMA) was obtained through the rapid autopsy program at the University of Michigan Prostate Cancer Specialized Program of Research Excellence Tissue Core. The Institutional Review Board at the University of Michigan approved the use of all tissues. A total of 30 benign (n=12) and malignant (n=18) paraffin embedded tissue specimens were analyzed with a Gleason score of 7-8. Areas of malignant and benign prostatic hyperplasia (BPH) were taken from the same patients (matched paired tissues). Immunohistochemical detection of human MFG-E8 and CD68 (KP1) was carried out in de-paraffinized and rehydrated sections using the HRP-AEC Cell & Tissue Staining Kit (R&D Systems) according to the manufacturer's protocol. Tissue was permeabilized with 0.2% Triton-X 100 and incubated overnight at 4°C with anti-mouse CD68 (1:200) and anti-human MFG-E8 (1:50) (Abcam). Samples were counterstained with hematoxylin and mounted with paramount aqueous mounting medium (Dako). Negative controls were used to detect non-specific staining. MFG-E8 staining was quantified using NIS Elements Software (Nikon). Briefly, the region of interest (ROI) tool was used to set the target area that was positively stained for MFG-E8 over the total area (%) of the tissue. The mean area positive for MFG-E8 was then calculated for each tissue within three different areas of tissue. The proximity of CD68+ cells to MFG-E8 expression was determined by analyzing three tissue sections at 100x

magnification per specimen per TMA. Cells overlapping or in close proximity that were positive for both CD68 (macrophage) and MFG-E8 expression were quantified using Image J software.

Serum exosomes

Serum samples were obtained from subjects through a University of Michigan Institutional Review Board-approved protocol. Serum exosome proteins were isolated from patients with primary (n=6), metastatic prostate cancer (n=7), or control tumor-free (n=3). Human serum was centrifuged at 500*g* for 10 min, after which the resultant supernatant was centrifuged at 20,000g for 20 min. Exosomes were then harvested by centrifugation at 100,000g for 70 min (Sorvall S100-AT5 rotor). The exosome pellet was resuspended in PBS, filtered through 0.2-μm nylon filters (GE) and collected by ultracentrifugation at 100,000*g* for 70 minutes. Exosomes were lysed with RIPA lysis buffer (Millipore, Billerica, MA) containing a complete protease inhibitor tablet (Sigma). Lysates were cleared by centrifugation at 14,000*g* for 20 min. 50μg of protein were loaded in 4-20% gradient Novex Tris-Glycine gels and western blot analysis was performed as described above. MFG-E8 protein expression was quantified using the Scion Image software and calculated relative to control GAPDH.

Mouse inflammation antibody array

Bone marrow macrophages were plated into 6 well dishes ($3x10^6$ cells/well). After 24 hours cells were washed with PBS and 1% FBS α-MEM media was added to the wells with or without high apoptotic RM-1 cells at a 1:1 ratio. The supernatant was collected after 24 hours and proteins were analyzed using the Mouse Inflammation Antibody Array C1 (Cat#AAM-INF-1-8, RayBiotech, Inc) following the manufacturer's instructions.

Statistical Analyses

Statistical analysis was performed by unpaired student's t-test to compare two groups with significance of $p<0.05$. Data are presented as mean ± SEM.

RESULTS

MFG-E8 is highly expressed in prostate cancer

MFG-E8 has been correlated with tumor growth and progression of different types of cancers such as melanoma and breast, but its expression in prostate cancer is still unclear. To elucidate the pattern of MFG-E8 in prostate cancer, immunohistochemistry was performed on serial sections of prostate cancer patient derived tissue microarrays (TMAs), containing a total of 30 benign (n=12) and malignant (n=18) tissue specimens combined (Fig. 5.1*A* and *B*). MFG-E8 expression was significantly higher in malignant (31.93% ± 1.27) compared to benign (4.89% ± 0.68) tissues (Fig. 5.1*A*)**.** MFG-E8 appears to be localized on the plasma membrane and the cytoplasm; however, invasive specimens revealed intense MFG-E8 at the leading edge of tumors and in the apical and basal areas of luminal epithelial cells. The proximity of CD68+ cells to MFG-E8 expression was determined to be two times higher in malignant specimens (Fig. 5.1*B*)**.** The increased proximity of CD68+ cells in areas of high MFG-E8 expression indicates a potential interaction between MFG-E8 and macrophages in prostate cancer.

Protein expression levels were investigated in blood exosomes isolated from patients with prostate cancer or non-cancer controls (Fig. 5.1*C*). Blood was collected from patients with localized primary prostate cancer or castrate resistant patients with metastases (Vlassov et al., 2012). Western blot analyses revealed that MFG-E8 levels were significantly increased in patients that presented with primary and metastatic prostate cancer.

Taken together, these data suggest MFG-E8 is a potential marker for prostate cancer progression.

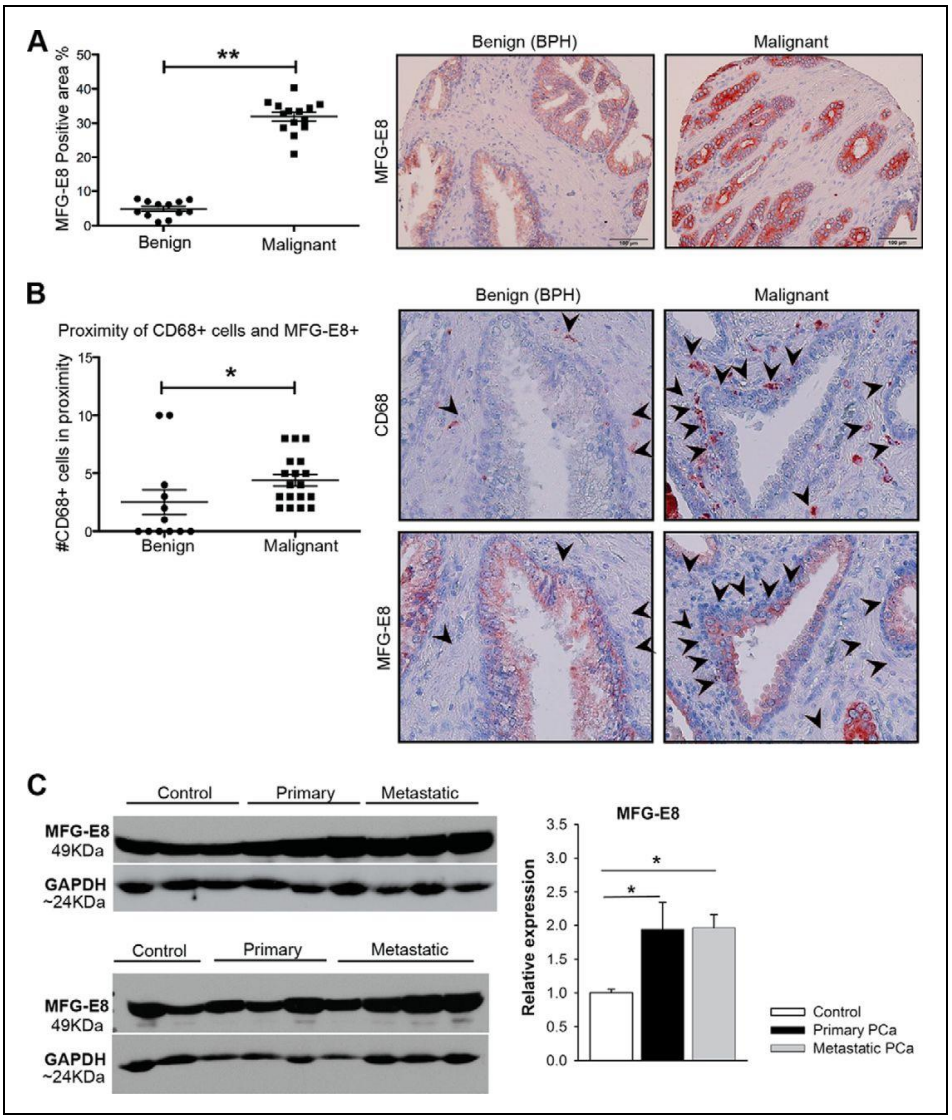

Figure 5.1. MFG-E8 expression is increased in prostate cancer patients.
A and *B*, immunohistochemistry was performed on serial sections of prostate cancer TMAs containing 30 benign (*n* = 12) and malignant (*n* = 18) matched tissue specimens combined. *A*, MFG-E8 expression in benign and malignant prostate cancer TMAs was analyzed by calculation of percent positive MFG-E8 expression in the region of interest. Data are mean ± S.E. **, $p < 0.0001$. *B*, proximity of CD68-positive macrophages and MFG-E8 was determined by analyzing three tissue sections at ×200 magnification per specimen per TMA. Overlapping or cells in close proximity positive for CD68 and MFG-E8 were counted, as indicated by *arrowheads*. The number (#) of CD68+ cells in proximity to MFG-E8 was counted as indicated in the graph. The mean of all three images was calculated, and the overall mean was obtained for each tissue type. Data are mean ± S.E. *, $p < 0.05$. *C*, serum exosome proteins were isolated from patients with primary (*n* = 6) and metastatic (*n* = 7) prostate cancer (*PCa*) or control tumor-free individuals (*n* = 3). The first two controls from the *top blot* were also included in the *bottom blot* for standardization and relative calculation. MFG-E8 protein expression was quantified using Scion Image software and calculated relative to control GAPDH. Data are mean ± S.E. *, $p < 0.05$. Permission for figure from J Biol Chem (Soki et al., 2014b)

MFG-E8 is increased in macrophages during efferocytosis of apoptotic cells

MFG-E8 is a protein that mediates the interaction of macrophages and apoptotic cells, thereby facilitating efferocytosis. To evaluate efferocytosis of apoptotic tumor cells by macrophages, bone marrow macrophages were cultured with tumor cells and efferocytosis was investigated. RM-1 cells stained with Cell Tracker dye (CFSE+ green) and treated with $CoCl_2$ to induce apoptosis, were designated as high apoptotic cells (HAP) (>60% apoptosis). Untreated cells (<10% apoptosis) were designated as basal apoptotic cells (BAP) and used as controls. Figure 5.2*A* shows representative confocal microscopic images of macrophages (red) co-incubated with RM-1 cells (CFSE+ green). Efferocytosis was quantified relative to the number of nuclei (DAPI) and was significantly increased when macrophages were cultured with HAP cells versus control BAP tumor cells (Fig. 5.2*B*). Confocal microscopic images of bone marrow macrophages confirmed efferocytosis as shown in supplemental videos 1 and 2 (Z stack videos)**.** Increased efferocytosis of high apoptotic cells (HAP) compared to control (BAP) was also observed by flow cytometry quantification. F4/80+ bone marrow macrophages that engulfed CFSE+ tumor cells were analyzed as shown in Figure 5.2*C***.**

Efferocytosis was also analyzed in unstained RAW 264.7 (RAW) macrophages that were co-cultured with RM-1 HAP or BAP for 5 hours. Efferocytosis of RM-1 cells by macrophages was measured by flow cytometric analysis based on RAW cell engulfment of Cell Tracker stained RM-1 cells (Fig. 5.2*D*). Efferocytosis was increased when macrophages were cultured with RM-1 HAP compared to control BAP tumor cells.

MFG-E8 functions as a facilitator of efferocytosis, binding to phosphatidyl serine (PS) expressed on apoptotic cells and to the $\alpha v\beta 3$/ $\alpha v\beta 5$ integrin expressed on macrophages (Hanayama et al., 2002). To determine whether increased efferocytosis would affect MFG-E8 expression in macrophages, RAW cells were cultured with low or high apoptotic tumor cells at different time points and MFG-E8 protein expression was determined **(**Fig. 5.2*E***)**. Western Blot analyses showed that MFG-E8 protein expression increased over time and was augmented when cells were cultured with HAP tumor cells. Taken together, these data suggest that co-incubation of macrophages with apoptotic tumor cells elicits efferocytosis and increased MFG-E8 levels.

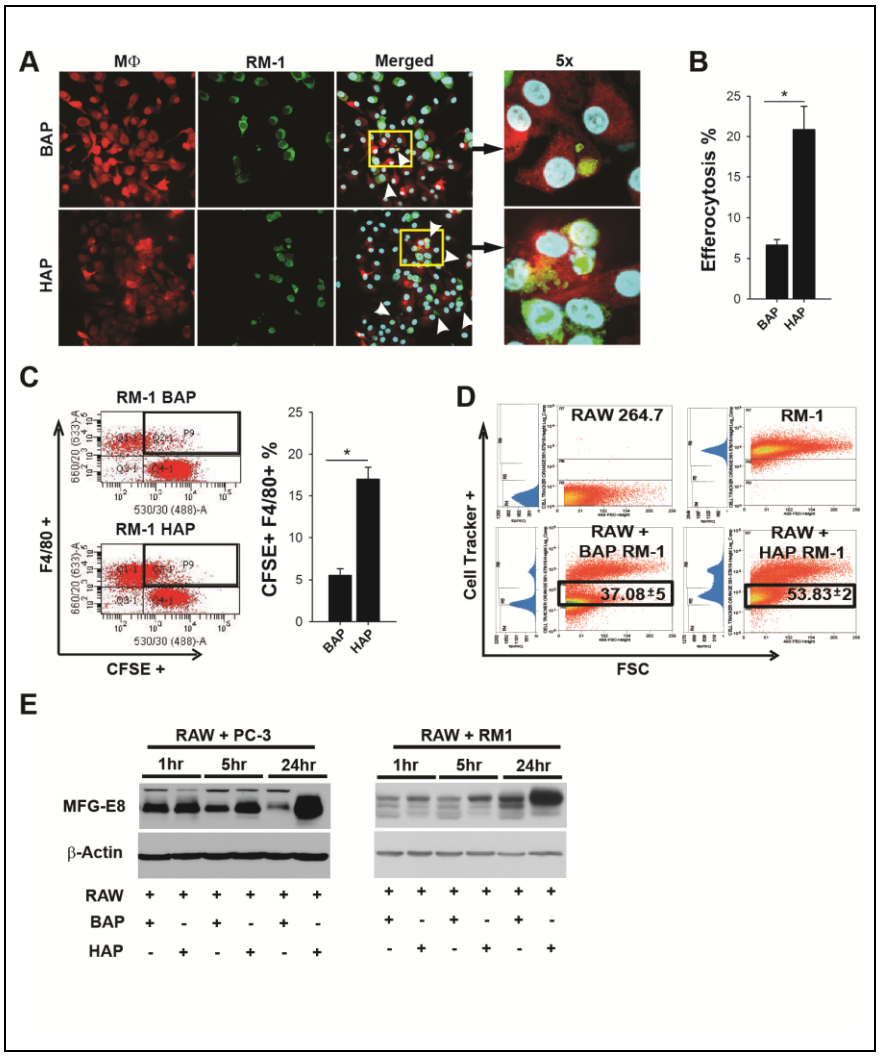

Figure 5.2. Efferocytosis of apoptotic tumor cells and MFG-E8 expression.
A, representative confocal images (×63, 1. 4 oil objective) of stained macrophages (Mφ, red) cocultured for 5 h with CFSE-stained HAP (>60% apoptosis) and BAP (<10% apoptosis) RM-1 prostate cancer cells (green) at a 1:1 ratio. DAPI nuclear staining is shown in blue. White arrowheads show colocalization suggesting efferocytosis. B, quantification of efferocytosis in four fields of confocal microscopic images. Macrophages were cocultured with HAP or BAP RM-1 cells. Data are mean ± S.E. (n = 4/group). *, $p < 0.05$. C, flow cytometric analyses of bone marrow macrophages cultured with CFSE-stained RM-1 cells with basal or high apoptosis for 5 h. A representative plot shows cells double-positive for CFSE and the macrophage marker F4/80 indicating efferocytosis. The graph shows collective data (mean ± S.E., n = 5/group). *, $p < 0.05$. D, flow cytometric analyses of efferocytosis. Unstained RAW 264.7 cells cocultured for 5 h with Cell Tracker-stained HAP (>60% apoptosis) and BAP (<10% apoptosis) RM-1 cells. Efferocytosis was measured on the basis of unstained macrophages engulfing the RM1 Cell Tracker+ cells described as percent total cells ± S.E. (n = 5/group, $p < 0.05$). FSC, forward scatter. E, MFG-E8 is increased in macrophages cultured with high apoptotic RM-1 and PC-3 cells. Shown is a Western blot analysis for MFG-E8 protein expression when RAW 264.7 cells were cultured with BAP or HAP for 1, 5, and 24 h of incubation. Permission for figure from J Biol Chem (Soki et al., 2014b)

MFG-E8 expression in macrophages and tumor-derived MFG-E8

Although MFG-E8 is expressed in many tissues and cells, its expression in prostate cancer cell lines was unknown (Aziz et al., 2011a). To determine whether MFG-E8 is expressed in prostate cancer cells, protein was collected and analyzed from RAW 264.7 (RAW) macrophages and three different prostate cancer cell lines: a prostate cancer murine-derived cell line, RM-1, and two bone metastatic human-derived cell lines, PC-3 and C42B (Fig. 5.3*A*). MFG-E8 expression was increased in HAP versus BAP RM-1 cells but did not significantly change for HAP PC-3 and C42b cells versus BAP cells. Overall, all cell lines tested highly expressed MFG-E8.

Since high apoptotic RM-1 cells express high levels of MFG-E8, the contribution of macrophage-derived MFG-E8 during efferocytosis was further investigated. Bone marrow macrophages from C57BL/6J wild type (WT) or MFG-E8 mutant mice lacking functional secreted MFG-E8 (KO) were expanded and MFG-E8 protein levels were confirmed (Fig. 5.3*B*). To determine whether efferocytosis increased MFG-E8 expression in macrophages without the interference of tumor derived MFG-E8, stable-shRNA RM-1 constructs were generated using five different clones and a pGIPZ scramble clone as control (RM-1 GIPZ) (Fig.5.3*C*). The clones with higher efficiency of MFG-E8 knock down, RM-1 m_08 and RM-1 m_30, were selected for further analyses. Bone marrow macrophages from wild type (WT) and mutant (KO) mice were co-cultured with basal (BAP) or high (HAP) apoptotic cells from shRNA or scramble controls for 5 hours (Fig. 5.3*C*). Interestingly, MFG-E8 protein levels were increased when wild type (WT) macrophages were cultured with high apoptotic cells (HAP) from control (GIPZ) and shRNA clones m_08 and m_30, but not detected when the KO were cultured with high apoptotic cells (Fig. 5.3*D* and *E*). This suggests that even though RM-1 cancer cells express MFG-E8, efferocytosis elicits the production of MFG-E8 in macrophages.

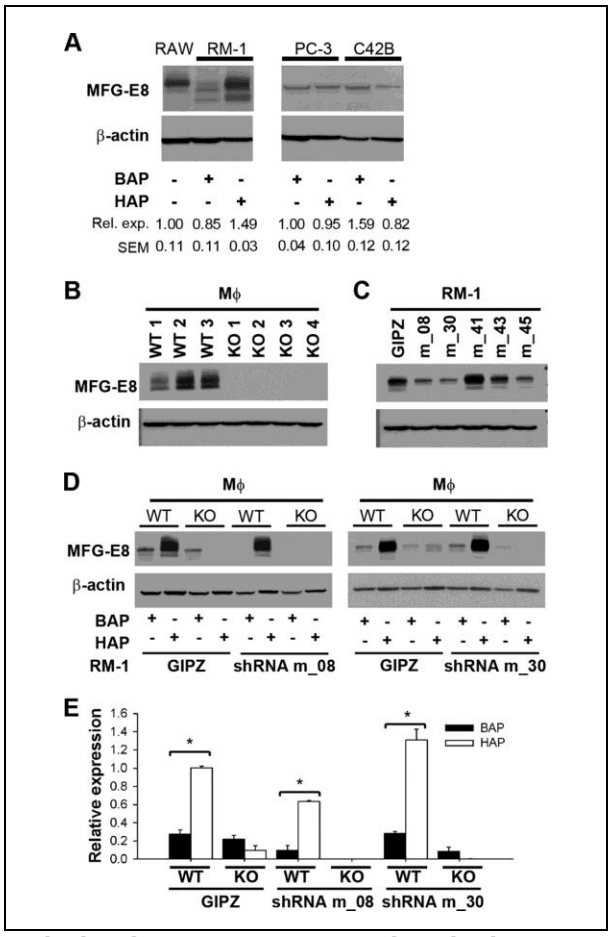

Figure 5.3. Macrophage-derived MFG-E8 expression is increased during efferocytosis regardless of tumor-derived MFG-E8 expression.

A, MFG-E8 expression in prostate cancer and macrophage cell cultures. Shown is expression of MFG-E8 in cells alone. RAW 264.7 macrophages (*RAW*), RM-1, PC-3, and C42B prostate cancer cells express MFG-E8. Prostate cancer cells were treated with $CoCl_2$ for 24 h (>60% apoptosis) and designated as HAP. BAP cells were left untreated (<10% apoptosis). Proteins were analyzed by Western blotting. Expression is reported relative to corresponding β-actin levels. Shown is a representative blot of two independent experiments where values correspond to fold change relative to RAW control (for RM1) or to PC-3 (for PC-3 and C42B). Data are mean ± S.E. (*SEM*) (*n* = 2/group). *Rel. exp.*, relative expression. *B*, MFG-E8 protein expression from bone marrow macrophages collected from C57Bl/6 WT or MFG-E8 KO. *C*, MFG-E8 stable knockdown in cells was generated using five different clones as described under "Experimental Procedures." Protein expression indicated the greatest knockdown for MFG-E8 in clones m_08 and m_30. *D*, WT or KO macrophages were cultured with BAP or HAP RM-1 cells containing GIPZ (negative control) or clone shRNA (*m_08* and *m_30*) for 5 h at a 1:1 ratio. A representative image for Western blot analyses is shown, and MFG-E8 expression was determined. Experiments were repeated twice with similar results. *E*, quantification for MFG-E8 expression was determined by relative expression of MFG-E8 to the control β-actin. Data are mean ± S.E. of two independent experiments and normalized to HAP WT GIPZ control (*n* = 2/group). *, $p < 0.05$. Permission for figure from J Biol Chem (Soki et al., 2014b)

Efferocytosis of apoptotic prostate cancer cells induces M2 polarization in macrophages

Since efferocytosis stimulates MFG-E8 in macrophages, and given the role of MFG-E8 as a potential immune modulator in macrophages, it was hypothesized that MFG-E8 mediated efferocytosis could be a possible mechanism for macrophage polarization into M2 type. To better elucidate whether efferocytosis of tumor cells plays a role in M2 polarization, bone marrow macrophages were co-cultured with CFSE stained BAP or HAP RM-1 cells. After 5 hours, flow cytometric analyses were performed to determine total efferocytosis, the population of F4/80+ macrophages that engulfed CFSE stained RM-1 cells (CFSE+F4/80+), and M2 polarization (F4/80+CD206+) (Fig. 5.4*A*). Efferocytosis was significantly greater for HAP cells and occurred in conjunction with an increase in M2 macrophages. Similar results were also observed when bone marrow macrophages were co-cultured with PC-3 cells (Fig. 5.4*B*). Although addition of rmMFG-E8 did not affect efferocytosis of tumor cells (data not shown), neutralizing antibody against MFG-E8 significantly inhibited efferocytosis of HAP cells and decreased M2 polarization of macrophages when compared to IgG controls (Fig. 5.4*C*). In summary, flow cytometric analyses suggested that increased efferocytosis contributes to M2 polarization via MFG-E8. Moreover, neutralizing MFG-E8 antibody treatment significantly inhibited efferocytosis of HAP cells and reduced the HAP induced M2 polarization, suggesting a unique role for MFG-E8.

To better determine the effect of cancer-efferocytosis in macrophage activation and its participation in cancer inflammation, inflammatory protein array analyses were performed. Bone marrow macrophages were cultured in the presence or absence of HAP RM-1 for 24 hours and the supernatant proteins were analyzed (Fig. 5.4*D*). Interestingly, M2 related proteins (Mantovani et al., 2004;Locati et al., 2013) such as interleukin-6 (IL-6), chemokine (C-C motif) ligand 2 (CCL2, also known as MCP-1), and chemokine (C-C motif) ligand 1 (CCL1, also known as TCA-3) were significantly augmented with efferocytosis. The production of CXC-chemokine family members such as KC, Lix and XCL-1, as well as interleukin-13 (IL-13) and interleukin-17 (IL-17) were also significantly higher when macrophages were cultured with HAP RM-1 cells (data

not shown). However, M1 related proteins such as GM-CSF, INF-γ, IL-1α and β, TNF-α, and IL-12 p40/70 and IL-12P70 were not significantly changed (data not shown).

To further validate the M2 polarization, M2 macrophage associated genes were investigated when macrophages were cultured with HAP RM-1 tumor cells, and at 4°C to block efferocytosis (Fig. 5.4*E*). Expression of M2 macrophage associated genes such as interleukin 10 (*Il10*), transforming growth factor-beta 1 (*Tgfb1*), Ym-1 (*Chi3l3*) and arginase 1 (*Arg1*) were significantly decreased when efferocytosis was blocked at 4°C. Incubation at 4°C did not alter gene expression, except arginase which was significantly increased (Fig. 5.4*F*). Collectively, these data suggest that macrophage alternative activation into an M2 phenotype is facilitated by efferocytosis

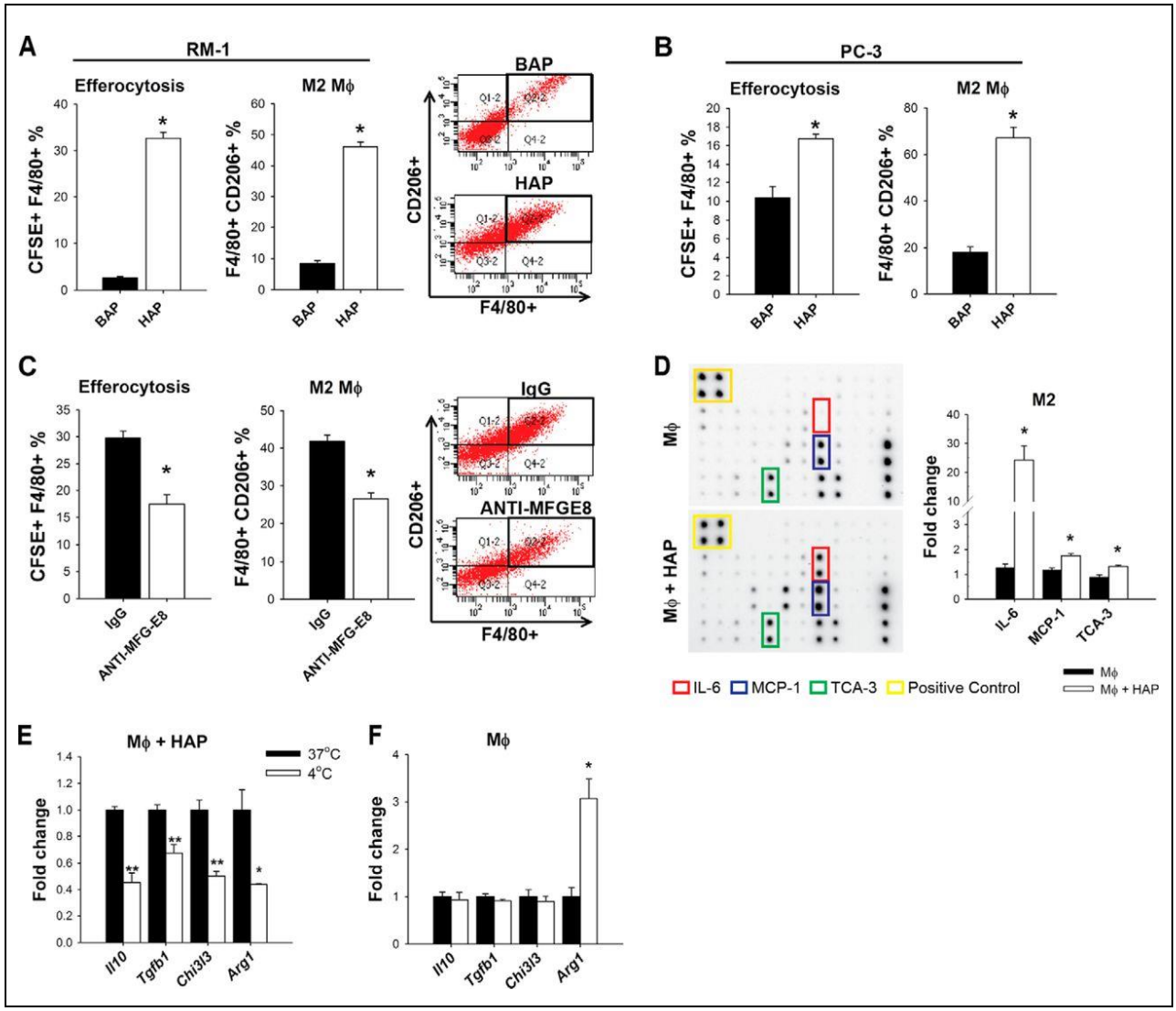

Figure 5.4. Efferocytosis via MFG-E8 induces M2 polarization.

A and *B*, FACS analyses of efferocytosis in bone marrow macrophages (*M*φ) cultured with BAP or HAP RM-1 (*A*) or PC-3 (*B*) cells. Efferocytosis was demonstrated as double-positive cells (*CFSE+F4/80+*) indicating macrophages (*F4/80+*) that engulfed tumor cells (*CFSE+*). Representative FACS and M2 polarization is shown as F4/80+CD206+ cells. Data are mean ± S.E. (*n* = 4/group from three independent experiments). *, $p < 0.05$. *C*, macrophages were treated with IgG or anti-MFG-E8 (20 μg/ml) and then cocultured with HAP RM-1 cells. FACS analyses of efferocytosis, reported as double-positive cells (*CFSE+F4/80+*) and M2 polarization (*F4/80+CD206+*) and representative FACS are shown. Data are mean ± S.E. (*n* = 4/group). *, $p < 0.05$ from three independent experiments. *D*, bone marrow macrophages were cultured for 24 h with HAP RM-1 cells or not cultured. The supernatant was collected, and proteins were analyzed using a mouse inflammation antibody array. The fold increase in densitometry was calculated relative to the positive controls (*yellow*) and according to the protocol of the manufacturer. Shown are IL-6 (*red*), chemokine (C-C motif) ligand 2 (CCL2 or MCP-1, *blue*), and chemokine (C-C motif) ligand 1 (CCL1 or TCA-3, *green*). Data are mean ± S.E. (*n* = 3/group). *, $p < 0.05$ from three independent experiments. *E*, bone marrow macrophages were cultured for 5 h with HAP RM-1 cells at 37 °C or at 4 °C to block efferocytosis. Gene expression levels were analyzed by quantitative PCR relative to *Gapdh*, and fold change was calculated. Shown are interleukin 10 (*Il10*), transforming growth factor β 1 (*Tgfb1*), Ym-1 (also known as chitinase 3-like protein 3, *Chi3l3*), and arginase1 (*Arg1*). Data are mean ± S.E. (*n* = 6/group from two independent experiments). *, $p < 0.05$; **, $p < 0.001$. *F*, bone marrow macrophages incubated at 4 °C did not alter gene expression. Gene expression relative to *Gapdh* and fold change was calculated for interleukin 10 (*Il10*), transforming growth factor β 1 (*Tgfb1*), Ym-1(*Chi3l3*), and arginase1 (*Arg1*). Data are mean ± S.E. (*n* = 3/group). *, $p < 0.05$. Permission for figure from J Biol Chem (Soki et al., 2014b)

MFG-E8 direct role in M2 macrophage polarization

Since rmMFG-E8 is also known to modulate macrophage responses (Aziz et al., 2011b), its direct role in macrophage polarization was investigated (Fig. 5.5). Bone marrow macrophages expanded for 3 days with M-CSF were treated with different concentrations of rmMFG-E8 protein for 50 hours and analyzed by flow cytometry for the M2 markers, mannose receptor CD206 and intracellular Ym-1 (Fig. 5.5*A*). Interestingly, bone marrow macrophages showed a dose dependent increase in M2 polarization, reaching the peak at 0.5μg/ml and decreasing at 2μg/ml, an MFG-E8 concentration which has been reported to inhibit efferocytosis (Hanayama et al., 2002). To better clarify the direct role of MFG-E8, macrophages were treated with interleukin 4 (IL-4) to induce M2 polarization, in the presence of anti-MFG-E8 antibody or IgG control (Fig. 5.5*B*). M2 polarization was significantly reduced with MFG-E8 neutralization. These data suggest that MFG-E8 may directly activate and modulate macrophage polarization into an M2 type.

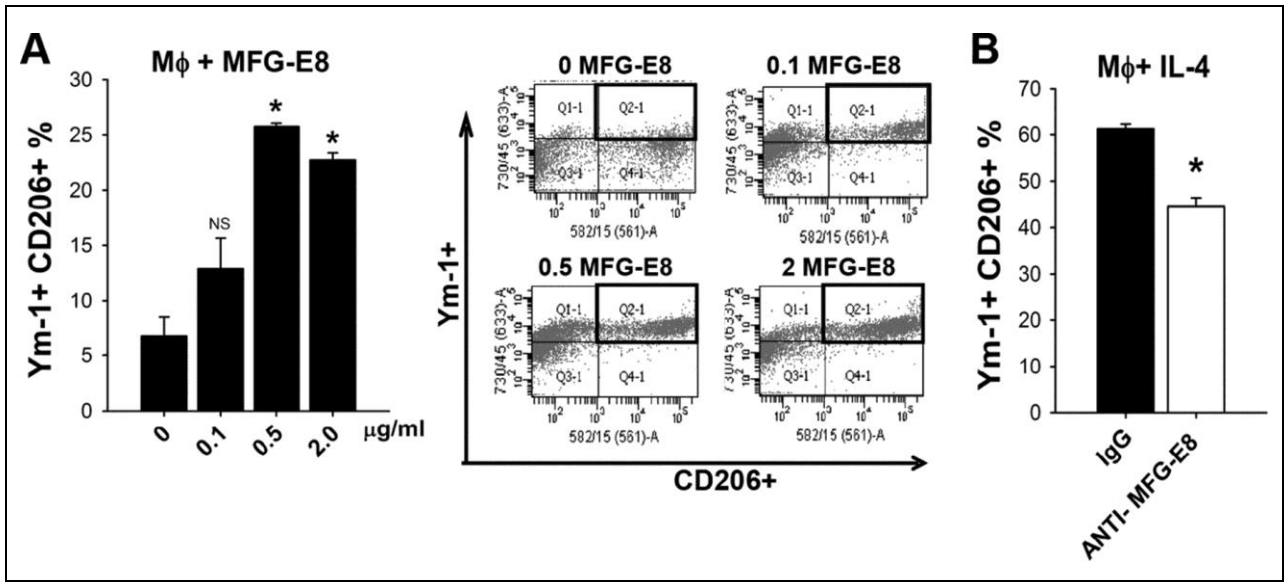

Figure 5.5. MFG-E8 has a direct role in M2 macrophage polarization.
A, rmMFG-E8 treatment increased the M2 population in bone marrow macrophages. Bone marrow cells were expanded for three days with M-CSF and treated with rmMFG-E8 for 50 h at different concentrations (0–2 µg/ml). Shown are FACS analyses of M2 cells double-positive for CD206 and intracellular Ym-1. A representative FACS analysis is shown. Data are mean ± S.E. (n = 4/group). Two independent experiments were performed with similar results. *, $p < 0.05$. *NS*, not significant; *M*φ, bone marrow macrophages. *B*, IL-4-induced bone marrow macrophage M2 polarization (*Ym-1+ CD206+*) was reduced when cells were treated with anti-MFG-E8 antibody. Bone marrow macrophages were treated with 10 units/ml of IL-4 for 24 h in the presence of anti-MFG-E8 antibody (20 µg/ml) or IgG control. Data are mean ± S.E. (n = 4/group). *, $p < 0.05$. Permission for figure from J Biol Chem (Soki et al., 2014b)

STAT3 pathway in MFG-E8 mediated efferocytosis and M2 polarization

MFG-E8 activates the JAK/STAT3 pathway in macrophages (Aziz et al., 2011b). Therefore, to determine whether the STAT3 pathway is important for MFG-E8 mediated efferocytosis, macrophages were treated two hours with the STAT3 inhibitor Stattic prior to co-culture with tumor cells (Fig. 5.6*A* and *B*). Stattic targets the STAT3-SH2 domain and prevents its association with upstream kinases, inhibiting cellular phosphorylation of STAT3 at Tyr705 (Schust et al., 2006). Flow cytometric analyses revealed that phospho-STAT3 (P-STAT3) inhibition with Stattic pre-treatment, not only inhibited efferocytosis of RM-1 (Fig. 5.6*A*) and PC-3 (Fig. 5.6*B*), but also decreased M2 F4/80+CD206+ cells. To better delineate the role of MFG-E8 in efferocytosis and STAT3, macrophages from mutant MFG-E8 (KO) and control (WT) were given apoptotic

mimicking PS-coated carboxylated beads and the STAT3 pathway was investigated (Fig. 5.6*C-E*)**.** Phagocytosis of PS-coated beads was significantly reduced in MFG-E8 mutant (KO) mice as expected **(**Fig. 5.6*C* and *D*). The STAT3 pathway was further investigated in macrophages challenged with STAT3 inhibition or vehicle, prior to phagocytosis with PS-coated carboxylated beads (Fig. 5.6*E*). Interestingly, macrophage phagocytosis stimulated phosphorylation of STAT3 and suppressor of cytokine signaling-3 (SOCS3) activation in WT compared to control non-stimulated macrophages. Inhibition of STAT3 phosphorylation by pre-treatment with Stattic resulted in decreased STAT3 phosphorylation and further increase in SOCS3 expression. MFG-E8 KO macrophage P-STAT3 levels were not changed after phagocytosis of PS-coated beads, and even though inhibition of STAT3 was significant, no effect was observed in SOCS3 activation, suggesting that decreased phagocytosis in MFG-E8 KO blunted P-STAT3 activation and the SOCS3 response.

Collectively, Figure 5.6 suggests that the STAT3 pathway is important for macrophage efferocytosis and M2 polarization. Furthermore, efferocytosis elicits phosphorylation of STAT3.

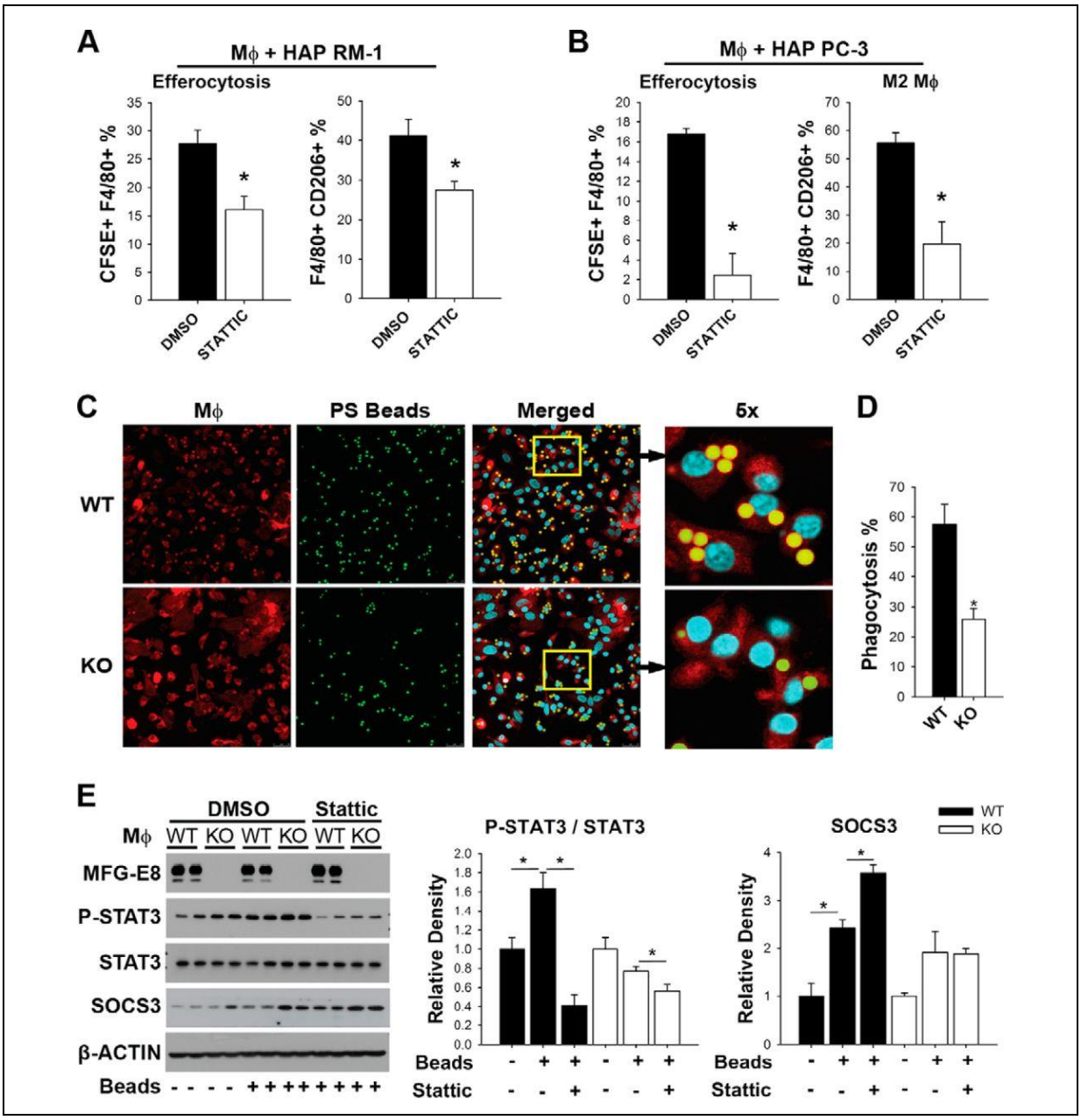

Figure 5.6. Macrophage efferocytosis and activation of phospho-STAT3 signaling.
A and B, flow cytometric analyses for efferocytosis (dual CFSE+F4/80+) and M2 polarization (F4/80+CD206+). Macrophages (Mφ) were pretreated with the phospho-STAT3 inhibitor Stattic 2 h prior to culture with RM-1 (A) and PC-3 (B) HAP tumor cells at a 1:3 ratio (Mφ:HAP). Data are mean ± S.E. (n = 4/group). *, $p < 0.05$. Experiments were performed three times with similar results. DMSO, dimethyl sulfoxide. C, macrophages were cocultured with PS-coated carboxylated beads (1:2 ratio) for 1 h. Shown are representative confocal images of bone marrow macrophages (red) from WT or MFG-E8 mutant mice (KO) engulfing fluorescent carboxylated beads. DAPI (blue) was the nuclear stain. D, quantification of phagocytic index was calculated using the following formula: (number of engulfed beads / number of total Mφ) ×

(Mφ that engulfed beads/number of total Mφ) × 100. Data are mean ± S.E. from two independent experiments, a total of n = 7/group. *, $p < 0.05$. E, representative Western blot showing two independent samples per group and the density quantification. Phospho-STAT3 expression levels were normalized to total STAT3 (p-STAT3/STAT3, n = 4/group), and SOCS3 (n = 2) expression levels were normalized relative to β-actin levels. Fold change was calculated for WT or MFG-E8 mutant mice (KO) dimethyl sulfoxide controls. Experiments were repeated twice, and data are mean ± S.E. *, $p < 0.05$. Permission for figure from J Biol Chem (Soki et al., 2014b)

MFG-E8 and efferocytosis in STAT3/SOCS3 pathway

The STAT3/SOCS3 pathway is important for cytokine expression in macrophages, playing essential roles in tumorigenesis and inflammation (Hiwatashi et al., 2011). SOCS3 is a negative regulator of STAT3 (Dimitriou et al., 2008). Interestingly, Figure 5.6*E* shows that STAT3 inhibition resulted in increased SOCS3 expression. Therefore, the relationship between P-STAT3 and SOCS3 was further investigated in the context of efferoctyosis of tumor cells (Fig. 5.7). Macrophages were pre-treated with Cytochalasin D, a potent inhibitor of actin polymerization and phagocytosis, or with DMSO as a control for 2 hours prior to incubation with high apoptotic (HAP) PC-3 cells. Efferocytosis inhibition with Cytochalasin D, significantly suppressed P-STAT3 activation in macrophages stimulated with PC-3 HAP cells **(**Fig. 5.7*A***)**. SOCS3 demonstrated an opposite response to Cytochalasin D inhibition, with significantly increased SOCS3 expression in both WT and KO macrophages.

To further clarify the STAT3/SOCS3 pathway and the role of MFG-E8 in this process, WT and KO bone marrow macrophages were cultured with HAP RM-1 tumor cells expressing reduced MFG-E8 levels (GIPZ-control, versus knockdown shRNA m_08 or m_30 clones) (Fig. 5.7*B*). The purpose of modulating tumor-derived MFG-E8 was to determine whether paracrine MFG-E8 also played a role in the activation of macrophages and in the STAT3/SOCS3 pathway. Strikingly, macrophages cultured with shRNA knockdown clones presented significantly reduced P-STAT3 activation and exacerbated SOCS3 activation, compared to cells cultured with control GIPZ RM-1 cells, which had an opposite response. These data suggest that tumor derived MFG-E8 is important for macrophage activation of the STAT3 pathway and that the SOCS3 pathway negatively regulates STAT3 activation indicating that tumor cell efferocytosis impacts the STAT3/SOCS3 pathway in macrophages.

Collectively, the important roles of tumor cell efferocytosis in STAT3 activation suggest SOCS3 as a negative regulator of this pathway. Moreover, tumor derived MFG-E8 may play important roles not only in efferocytosis, but may also have a significant paracrine effect in the regulation of the STAT3/SOCS3 pathway.

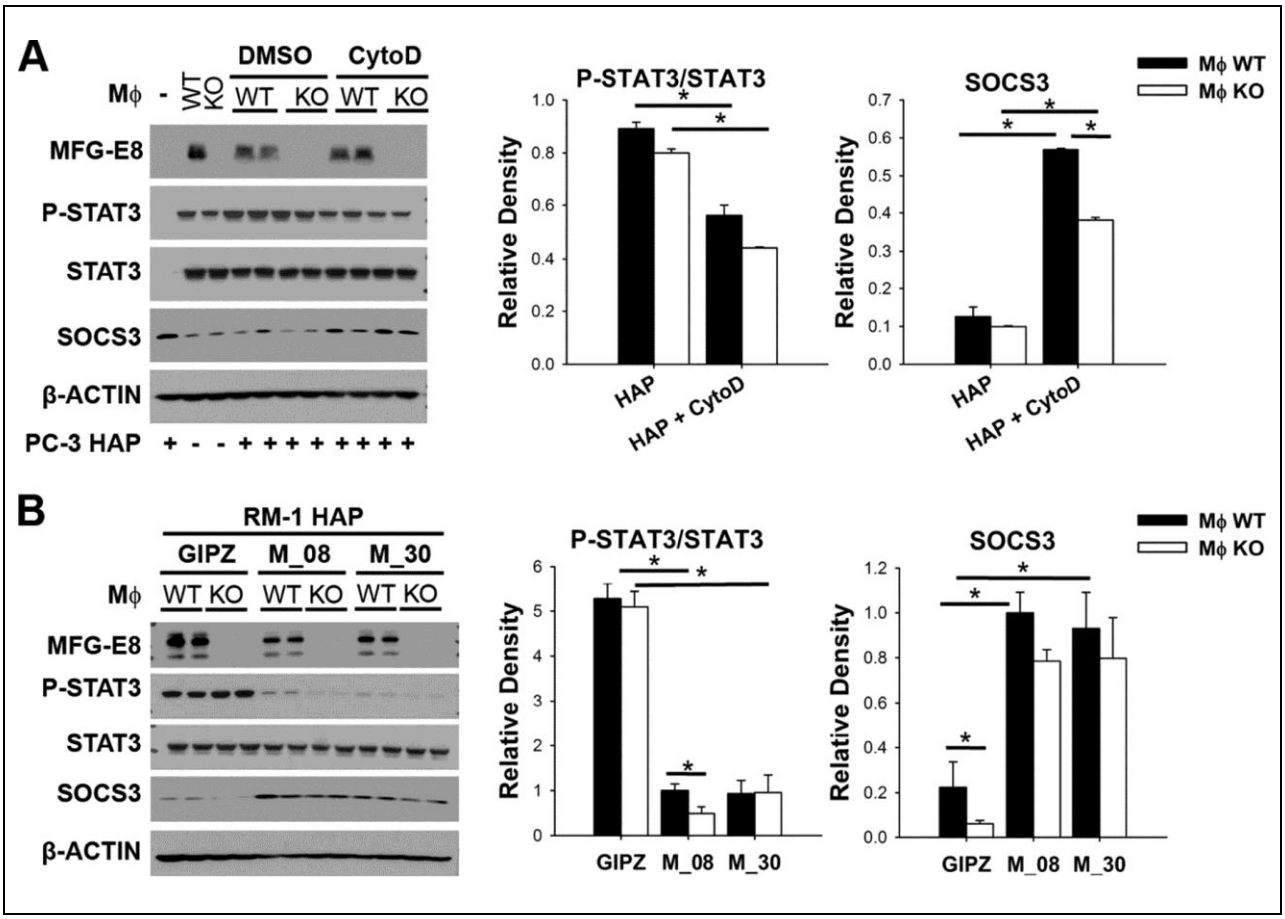

Figure 5.7. MFG-E8 and efferocytosis in the STAT3/SOCS3 signaling pathway.
A, WT or MFG-E8 mutant (KO) macrophages were pretreated 2 h with the efferocytosis inhibitor Cytochalasin D (2 μm) or control dimethyl sulfoxide. Cells were then incubated with HAP PC-3 cells at a 1:3 ratio for 6 h. Western blot analysis was performed in two independent samples per group, and the relative expression to β-actin control was quantified. Phospho-STAT3 expression levels were normalized to total STAT3 (p-STAT3/STAT3). Data are mean ± S.E. (n = 2/group). *, $p < 0.05$. B, high apoptotic GIPZ (negative control) or MFG-E8 knockdown shRNA (m_08 and m_30) RM-1 cells were incubated for 5 h at a 1:1 ratio. A representative image for Western blot analyses is shown, and p-STAT3 and SOCS3 expression was determined. Data are mean ± S.E. of two independent samples per group repeated twice (total n = 4/group). *, $p < 0.05$. Permission for figure from J Biol Chem (Soki et al., 2014b)

DISCUSSION

Efferocytosis is an important process for maintaining homeostasis, but may also have deleterious effects. Its role in the context of cancer and specifically skeletal metastases is intriguing yet unexplored. In normal bone, macrophages, known as 'osteomacs', constitute one sixth of the total cells and express specific markers: F4/80 and CD68 (Austyn and Gordon, 1981;Pettit et al., 2008). Interestingly, depletion of macrophages influences osteoblastic bone formation, bone healing, and hematopoietic niche maintenance (Pettit et al., 2008;Chang et al., 2008;Winkler et al., 2010a;Alexander et al., 2011a;Cho et al., 2014). While there is a growing body of literature on tumor associated macrophages (TAMs), little attention has been given to the phagocytic function of TAMs in the tumor environment and the mechanisms that control this process. In this study, the interactions of macrophages and tumor cells were investigated, with focus on efferocytosis and its effects on macrophage polarization via MFG-E8.

Clearance of apoptotic cells is essential to preserve tissue integrity and prevent the accumulation of harmful products released from dying cells. Deficient efferocytosis is associated with autoimmune diseases due to exacerbated and abnormal inflammatory responses (Hanayama et al., 2004;Hanayama et al., 2002). Efferocytosis via MFG-E8 influences phagocytic cells such as macrophages and dendritic cells to down regulate pro-inflammatory responses (Aziz et al., 2011b;Aziz et al., 2009). Furthermore, MFG-E8 has anti-inflammatory roles in homeostasis, evidenced by its modulation of pro-inflammatory signaling during efferocytosis, through activation of the $\alpha v\beta 3$/ $\alpha v\beta 5$ integrin receptors expressed by macrophages (Brissette et al., 2012;Miksa et al., 2008;Aziz et al., 2009). Pre-treatment of macrophages with the MFG-E8 recombinant protein resulted in reduced pro-inflammatory responses induced by LPS stimulation (Aziz et al., 2011b). Interestingly, in cancer, the pro-tumorigenic macrophage also known as M2 alternatively activated (or TAMs), share many similarities with the phagocytic macrophage. TAMs have an anti-inflammatory role in the tumor microenvironment, expressing phagocytic markers such as mannose

receptors (CD206), and anti-inflammatory cytokines and factors, which not only participate in immunosuppression but also promote tumor growth (Park et al., 2011b) .

In the present study, MFG-E8 was expressed in three different prostate cancer cell lines and co-culture of macrophages with cells undergoing apoptosis increased efferocytosis as well as MFG-E8 production.

MFG-E8 levels were found to have a direct effect on macrophage polarization. A key question in the activation of M2 TAMs is: Which signals drive pro-tumorigenic polarization? MFG-E8 also has anti-inflammatory roles, capable of modulating inflammatory signaling during efferocytosis (Miksa et al., 2008). Interestingly, phagocytes activated by the interaction with apoptotic cells via MFG-E8 share similar anti-inflammatory and tumor promoting properties as seen in M2 TAMs (Gregory and Pound, 2011b). Co-culture of macrophages with apoptotic cells induced macrophage polarization as demonstrated by both flow cytometric analyses and qPCR quantification of M2 related genes. Interestingly, when efferocytosis was decreased via incubation at $4^{o}C$, or neutralizing MFG-E8 antibody treatment, M2 polarization was also reduced, suggesting that efferocytosis is important for macrophage polarization into tumor promoting M2 cells. When macrophages were challenged with efferocytosis, increased secretion of M2 related cytokines such as IL-6 and CCL-2 (MCP-1) was observed (Roca et al., 2009;Fernando et al., 2014;Li et al., 2009;Roca et al., 2009;Zhang et al., 2010). Indeed, the observation that IL-6 is dramatically increased (> 20 fold) with efferocytosis further supports and explains our findings of STAT3 activation in this pathway. M1 related proteins were not significantly changed with efferocytosis. Furthermore, MFG-E8 may also directly mediate macrophage responses. Addition of rmMFG-E8 protein augmented macrophage polarization into an M2 macrophage and this polarization was significantly decreased in the presence of the neutralizing antibody against MFG-E8.

The involvement of the STAT3/SOCS3 activation pathway in macrophage polarization was also investigated. The increase in MFG-E8 levels when bone marrow macrophages were co-cultured with apoptotic cells was accompanied by SOCS3 downregulation. SOCS3 deficiency in myeloid lineage cells has been shown to prolong activation of the JAK/STAT pathway and M1 polarization, suggesting that SOCS3 is required for M1 activation (Aziz et al., 2011b;Dimitriou et al., 2008;Qin et al., 2012).

SOCS3 downregulation in macrophages by siRNA induced M2 polarization and activation of STAT3 (Liu et al., 2008). In addition, myeloid cell-specific SOCS3 conditional knockout mice (cKO) had fewer liver and lung metastatic nodules than wild type in a mouse melanoma metastatic model (Hiwatashi et al., 2011). SOCS3 cKO myeloid cells stimulated with tumor lysates exhibited prolonged STAT3 phosphorylation with increased CCL8 (MCP2) production associated with the anti-tumor metastatic effect.

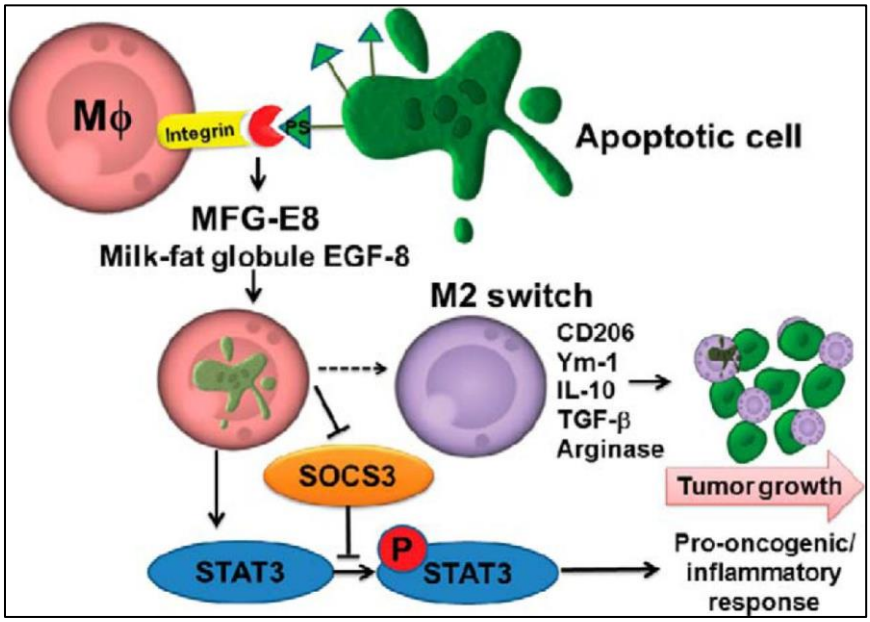

Figure 5.8. Proposed model of MFG-E8-mediated efferocytosis of tumor cells and macrophage polarization.
MFG-E8 is a protein that functions as a bridge binding to the $\alpha_v\beta_3/\alpha_v\beta_5$ integrin expressed by macrophages and to PS externalized on apoptotic cells. Macrophages (*M*φ) interact with apoptotic cells, resulting in increased MFG-E8 expression, therefore mediating the efferocytosis of apoptotic tumor cells. This interaction activates the phosphorylation of STAT3, leading to a polarization of macrophages into M2 tumor-promoting macrophages with increased expression of M2 markers such as CD206 and Ym-1 as well as M2-related genes of cytokines and growth factors known to contribute to tumor promotion. Moreover, MFG-E8 and efferocytosis may inhibit SOCS3, a negative regulator of STAT3, therefore keeping STAT3 signaling activated and promoting M2 polarization. Permission for figure from J Biol Chem (Soki et al., 2014b)

Here, we demonstrated that inhibition of STAT3 signaling resulted in decreased efferocytosis and M2 polarization. In contrast, SOCS3 presented an opposite effect, with reduced expression when STAT3 was activated by efferocytosis and increased expression with efferocytosis and STAT3 inhibition. Taken together, we hypothesize

that STAT3 participates in both efferocytosis and M2 polarization and SOCS3 functions as a negative regulator of this pathway (Figure 5.8).

A notable finding in this study was the fact that tumor-derived MFG-E8 may also play an important role in macrophages. The striking result of STAT3 reduction and SOCS3 activation in macrophages cultured with MFG-E8 knockdown cells indicates the importance of a paracrine effect that MFG-E8 may exert in macrophage activation and signaling. Still, the data suggest that MFG-E8 could be mediating not only efferocytosis and cell-cell interactions, but could also be eliciting macrophage responses in an autocrine and paracrine manner.

Finally, MFG-E8 is not only expressed in multiple tissues, but is also highly expressed in cancers such as malignant melanoma and has been associated with tumor progression and growth (Jinushi et al., 2008;Jinushi et al., 2009). Jinushi*, et. al*., reported that MFG-E8 signals through Akt and Twist-dependent pathways to promote tumor growth and metastasis in malignant melanoma (Jinushi et al., 2008). They proposed MFG-E8 blockage as a potential therapeutic strategy. MFG-E8 antibody administration resulted in improved anti-tumor activities, such as radiation and chemotherapies, and elicited immune activation against tumor cells (Jinushi et al., 2009). Until now, MFG-E8 expression and involvement in prostate cancer had not been explored. In the present study, it was demonstrated that patients with primary and metastatic prostate cancer had higher MFG-E8 expression in prostate tissue and blood exosomes compared to control patients. CD68+ macrophages in close proximity to MFG-E8 enriched areas of tumors were also augmented in malignant prostate cancer tissue. BPH tissues, which present a high turnover rate of prostate epithelia, are associated with increased macrophages and other inflammatory cells (Wang et al., 2012;Fujii et al., 2013). Macrophages contribute to the high proliferation rates and aggravate the chronic inflammatory state (Josson et al., 2010;De et al., 2011). While little is known about the efferocytic macrophages present in the BPH tissue regarding cell clearance, it is possible that they could be active players in this process. Interestingly, in the present study, MFG-E8 expression was dramatically increased in the malignant tissues compared to BPH. When macrophages were quantified, it was clear that malignant tissue presented an increased number of these cells in close

proximity to the MFG-E8+ epithelial cells, which suggests potential efferocytic interactions compared to what is observed in the BPH. Therefore, a role for MFG-E8 in prostate cancer progression and growth is likely and findings from the present study suggest it is a potential candidate for therapeutic targeting.

In conclusion, we report a novel mechanism by which MFG-E8, by mediating efferocytosis of prostate cancer cells, can support tumor growth through facilitation of M2 macrophage polarization and regulation of SOCS3/STAT3 activation.

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CHAPTER 6

CONCLUSIONS

The skeleton is a favored organ for prostate cancer metastasis and one that results in significant morbidity for cancer patients. The bone microenvironment provides a rich soil comprised of bone cells and a diverse population of cells in the bone marrow that interact with metastatic tumor cells. This interaction results in the disruption of a tightly regulated environment, generating a conducive environment for tumors to thrive. Many tumor derived factors influence these events. For example, tumors secrete PTHrP that activates osteoblasts to secrete osteoclastogenic factors, such as RANKL, driving osteoclast bone resorption. As a result, many growth factors and cytokines are then released to the environment, feeding the tumor cells and completing the “vicious cycle” present in skeletal metastasis.

The role of PTHrP in skeletal metastasis was reviewed and extensively discussed in Chapter 2. Briefly, PTHrP was first identified as an etiological factor of hypercalcemia, but plays important roles in normal physiology, bone development and tumor growth, progression and metastasis. PTHrP can function in different manners: with endocrine, paracrine, autocrine and intracrine actions. Therefore, it plays different roles in various cellular processes. PTHrP is highly expressed by cancers with high bone tropism and associated with skeletal metastasis development and progression. The dual function of PTHrP applied in the “seed and soil” hypothesis is demonstrated by its actions in the tumor cell autonomous processes (seed) and the modulation of the bone microenvironment (soil), contributing for tumor growth, progression and bone metastasis. Its actions in tumor cell autonomous processes include promotion of tumor cell proliferation, evasion of apoptosis and anoikis, survival, invasion and migration. Furthermore, PTHrP also plays important roles in the metastatic soil microenvironment.

Evolving PTHrP participation in the modulation of the bone metastatic environment includes regulation of the cellular content and promotion of angiogenesis. In summary, PTHrP has multiple functions that contribute to tumor growth, progression and metastases. Further investigation of the role of PTHrP in the modulation of the bone microenvironment is necessary to elucidate the earlier steps in the mechanism and progression of skeletal metastasis.

The high morbidity associated with skeletal metastasis reflects the lack of effective anti-metastatic therapies (Rogers and Holen, 2011;Gartrell and Saad, 2014). Available therapies to decrease the morbidity associated with the disease are palliative and include bisphosphonates, with zoledronic acid being the most potent, and other osteoclast-targeting agents (Gartrell and Saad, 2014). Hematopoietic stem cell niches such as the endosteal and vascular sites home and regulate the fate of hematopoietic cells for self-renewal. Interestingly, tumor cells can mimic hematopoietic stem cell homing to endosteal niches through chemokine receptors (CXCR4 and CCR7,) and their cognate ligands (expressed in metastatic recipient tissues), thus colonizing the bone (Muller et al., 2001). Other hematopoietic homing mechanisms such as CXCL12/SDF1 and its receptor CXCR4 and annexin II are the axis that regulates the prostate cancer bone tropism and homing to the endosteal niches (Taichman et al., 2002;Jung et al., 2007;Shiozawa et al., 2008;Sun et al., 2010;Shiozawa et al., 2011). In this way, hematopoietic stem cells compete with the metastatic tumor cells. Therefore, therapies that modulate the bone microenvironment such as zoledronic acid, may affect the hematopoietic niches. The effects of osteoclast inhibition in HSC niche and progenitor cell expansion are yet unclear. Chapter 3 investigated the effects of zoledronic acid (ZA), a potent bisphosphonate that inhibits bone resorption, on bone marrow cell populations focusing on HSCs, and the endosteal and vascular niches in bone. In this study, mice were treated with ZA, and HSCs in bone were analyzed. Increased bone volume and HSCs were both observed with ZA treatment. Increased vessel numbers were observed in 4 week young mice but not in 4 month old adult mice suggesting that the ZA induced HSCs expansion was mediated via the osteoblastic niche and not the vascular niche. Moreover, long term self-renewal and osteoblastic related gene expression were increased with ZA treatment suggesting a role in the

modulation of cell commitment and differentiation. In summary, chapter 4 shows that HSCs expansion mediated by osteoclast inhibition with ZA treatment is independent of the vascular niche and its effect is by indirect modulation of the osteoblastic niche. Altogether, osteoclast inhibition and changes in the bone microstructure may play a role in the increased hematopoietic stem cells in the bone marrow. Understanding the effects of cancer therapies in bone is of extreme relevance for tumor metastasis, since it may affect tumor colonization and interaction with the modified microenvironment. Studies demonstrated that increased bone remodeling with PTH treatment resulted in increased prostate cancer localization in the skeleton (Schneider et al., 2005). A significant reduction in prostate cancer skeletal metastasis was observed when concurrent administration of ZA and PTH (parathyroid hormone) were administered suggesting that active bone turnover offers a congenial microenvironment that facilitates metastatic cell colonization in bone. However, a recent study investigated the effects of ZA pre-treatment *in vivo* in the modification of the bone microenvironment and initiation of tumor metastasis of breast cancer (Haider et al., 2014). They showed that pre-treatment did not affect overall number of tumor cells homing to bone, even though it appeared to induce changes in their preferential localization to osteoblasts rich areas. Randomized clinical trials showed that adjuvant ZA did not reduce the incidence of skeletal metastasis (Wirth et al., 2014) even though it proved to be very effective in the reduction of skeletal related events such as pain and bone resorption (Gartrell and Saad, 2014).

Macrophages actively participate in wound healing and tissue homeostasis. Although their protective functions in immune responses are well explored, only recently studies have demonstrated their participation in bone formation and wound healing. Moreover, since cancer has been likened to a “wound that never heals” the mechanisms by which these “wounds” modulate the immune responses are intriguing and as of yet unclear (Dvorak, 1986). Macrophages are modulated according to the different stimuli they receive. Exposure to tumor-derived factors polarizes macrophages into M2-type cells that promote tumor growth, progression and metastasis. Yet, little is known about their role in skeletal metastasis. In chapter 4 we investigated the role of macrophages in prostate cancer tumor growth in bone. We demonstrated utilizing

different strategies of macrophage depletion that macrophages play important roles in tumor growth in bone. Both strategies of macrophage depletion in the transgenic MAFIA mouse model and clodronate loaded liposome model resulted in smaller intratibial tumors compared to vehicle controls. When subcutaneous tumor growth was analyzed in the vossicle model after 2 weeks of inoculation a significant difference in tumor size at earlier time points was observed in macrophage depleted mice. We hypothesized that when tumors are growing within the vertebrae body, a significant reduction of macrophages have higher impact on the tumor growth, but when tumors outgrow the bone area this difference in growth is no longer the driving factor. This suggests that macrophage depletion has a higher impact when the bone marrow compartment, rich in myeloid cells, is present, and which is the case in the intratibial tumor model.

The involvement of tumor associated macrophages was also further investigated. Macrophage depletion significantly reduced M2 macrophages (F4/80+ CD206+) in intratibial tumors. Moreover, macrophage analyzes comparing tibiae with tumors and tumor-free femurs showed that the presence of tumors in tibia significantly increased M2 (F4/80+ CD206+) macrophages in bone. These data not only confirmed the macrophage contribution to tumor growth, but also demonstrated that tumor development in bone marrow increased M2-like macrophages, which further suggests the important role of macrophage polarization in tumor progression. Because macrophages are progenitors for osteoclasts and given the important roles osteoclasts play in skeletal metastasis, osteoclast activity and numbers were analyzed. Even though macrophage depletion resulted in decreased TRAP5b levels, osteoclasts were still observed in the histological sections, especially in areas where bones were in close proximity with tumors as seen in histological sections. Many tumor derived factors that are known to promote osteoclastogenesis such as PTHrP and CCL2 (as discussed in chapter 2) could act in the environment. Therefore it is thought that even though macrophage depletion may suppress in part osteoclast differentiation/activation, tumors may compensate for the suppression, driving the differentiation/activation of the remaining macrophage progenitors towards the osteoclast lineage. Even though tumors were consistently smaller in macrophage depleted mice paradoxical results of

macrophage depletion on the bone were observed. The MAFIA mouse model had decreased bone volume in the hind limbs of macrophage depleted mice. In contrast, clodronate liposome that targets mature phagocytic macrophages presented increased bone volume. These results suggest that the effect of macrophage depletion in tumor growth was independent of its effect in bone responses. Differences in the stages of macrophage depletion may account for the differential response in bone (Cho et al., 2014). It is hypothesized that clodronate liposomes selectively deplete mature phagocytic macrophages, resulting in a compensatory expansion of CD68+ myeloid cells that includes macrophages, dendritic cells and neutrophils. The compensatory expansion and activation of the mononuclear phagocytic system may be a response for cell clearance of apoptotic cells and debris. Apoptotic cell clearance through phagocytosis, could be a possible mechanism for the osteogenic response observed in clodronate liposome induced macrophage depletion. In conclusion, Chapter 4 shows that macrophages play important roles in prostate cancer tumor growth in bone. Macrophage depletion hindered tumor growth independently of the paradoxical effect in bone content suggesting macrophages in bone may be more important to tumor growth than the bone itself.

Tumors have a high rate of cells undergoing apoptosis producing a number of different chemokines and factors that mediate the recruitment of phagocytic cells to their site. In this context, macrophages actively participate as scavengers cells phagocytosing the apoptotic cells, an underexplored mechanism also known as efferocytosis. Proteins such as milk fat globule-EGF factor 8 (MFG-E8) that recognize and bind to both apoptotic and phagocytic cells, are released by macrophages and function as a bridge protein that facilitates efferocytosis (Hanayama et al., 2004;Hanayama et al., 2002). In addition to its role in apoptotic cell clearance, MFG-E8 is also known for anti-inflammatory modulation in macrophages, a potential mechanism for cell polarization into an M2 tumor promoting macrophages (Aziz et al., 2011b;Aziz et al., 2009). Efferocytosis of tumor apoptotic cells and their effect in macrophages have not been investigated in the context of prostate cancer skeletal metastasis. In this manner, the tumor is co-opting the host's immunomodulatory mechanisms to support its growth. Although this concept of co-opting the host operating system has been

considered, it has not been considered in the context of the bone marrow microenvironment. The role of macrophages in skeletal metastasis and the mechanisms involved focusing on the phagocytic function in efferocytosis mediated by MFG-E8, and its effects on immune modulation were investigated and discussed in Chapter 5.

The involvement of MFG-E8 in prostate cancer was demonstrated in tissue and blood exosomes of patients with primary, metastatic and BPH controls. Patients with primary and metastatic prostate cancer had higher MFG-E8 expression in compared to control patients in blood serum samples. Moreover, MFG-E8 expression was dramatically increased in malignant tissues compared to BPH controls. When CD68+ macrophages were quantified, it was clear that malignant tissue presented an increased number of these cells in close proximity to the MFG-E8+ epithelial cells, which suggests potential efferocytic interactions compared to what is observed in the BPH. In vitro and in vivo studies also demonstrated that MFGE8 had a direct effect on macrophage polarization. Efferocytosis of apoptotic tumor cells via MFG-E8 increased expression promoted macrophage polarization into M2 phenotype. Co-culture of macrophages with apoptotic cells induced macrophage polarization as demonstrated by both flow cytometric analyses and qPCR quantification of M2 related genes. Efferocytosis inhibition with neutralizing MFG-E8 antibody resulted in reduced M2 polarization, suggesting that efferocytosis is important for macrophage polarization into tumor promoting M2 cells. Macrophages challenged with efferocytosis had increased secretion of M2 related cytokines such as IL-6 and CCL-2 (MCP-1) (Roca et al., 2009;Fernando et al., 2014;Li et al., 2009;Roca et al., 2009;Zhang et al., 2010). MFG-E8 can also directly induce macrophage polarization. Addition of rmMFG-E8 protein augmented macrophage polarization into an M2 macrophage and this polarization was significantly decreased in the presence of the neutralizing antibody against MFG-E8.

The mechanisms for macrophage activation were further delineated and the involvement of the STAT3/SOCS3 activation pathway in macrophage polarization was observed. Increased MFG-E8 levels when bone marrow macrophages were co-cultured with apoptotic cells was accompanied by SOCS3 downregulation. Inhibition of STAT3 phosphorylation, using the inhibitor Stattic, resulted in decreased efferocytosis

and M2 macrophage polarization with a correlating increase in SOCS3 protein expression. Moreover, MFG-E8 knockdown tumor cells cultured with wild type or MFG-E8 deficient macrophages resulted in increased SOCS3 expression with decreased STAT3 activation. This suggests that SOCS3 and phospho-STAT3 act in an inversely dependent manner when stimulated by MFG-E8 and efferocytosis. In conclusion, chapter 5 reports a novel mechanism by which MFG-E8, by mediating efferocytosis of prostate cancer cells, can support tumor growth through facilitation of M2 macrophage polarization and regulation of SOCS3/STAT3 activation

FUTURE DIRECTIONS

Skeletal metastasis is associated with the high morbidity and mortality of different types of cancer and the lack of effective therapies reflects our incomplete understanding about the mechanisms tumors utilize to colonize and thrive in the bone microenvironment. The role of bone cells such as osteoblasts and osteoclasts play in skeletal metastasis has been extensively explored. However, when tumors metastasize to bones, they encounter a diverse milieu comprised of different cell populations that also interacts with tumor cells. Emerging data support the involvement of macrophages in tumor growth, progression and metastasis. Interestingly most of the studies focus on the indirect role macrophages plays, mediated by cytokines and growth factors secretion that support macrophage's contributions in tumorigenesis. Macrophages are important phagocytic cells, an often underappreciated function, especially when studying their role in the context of tumors. Here we demonstrated that macrophages play important roles in prostate cancer skeletal growth, and that the interaction of macrophages with tumor cells during efferocytosis mediates macrophage polarization into tumor promoting cells. Identifying the mechanisms that tumors use to evade the immune responses are critical and relevant to the advancement of understanding immune cell interactions with metastatic cells and hence development of improved therapeutic strategies.

Many important questions still remain in cancer metastasis studies. To better understand the earlier steps of metastasis and how the bone microenvironment contributes in this regard is a relevant question to be addressed. For example, if a pre-metastatic niche is formed before the arrival of the disseminated tumor cell to the bone. Which cells could be providing this conducive microenvironment for tumors to evade the immune surveillance and thrive? Improved animal models and specific molecular markers are needed to investigate these novel theories and concepts. Understanding the earlier steps of tumor progression and metastasis is necessary for the development of new therapeutic targets to prevent and overcome cancer.

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