OSTEOCLASTS ARE IMPORTANT FOR BONE ANGIOGENESIS

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OSTEOCLASTS ARE IMPORTANT FOR BONE ANGIOGENESIS

Frank Cameron Cackowski, PhD

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Osteoclastogenesis and angiogenesis are correlated in bone during physiological and pathological processes including development, fracture healing, bone metastases and inflammatory bone disease. However, it is unclear if and how these processes are linked. This dissertation investigates a possible causative role for osteoclasts in bone angiogenesis. First, changes in osteoclast formation and activity affected angiogenesis in a parallel fashion. Osteoclast inhibition decreased angiogenesis, while osteoclast stimulation increased angiogenesis in fetal mouse metatarsal explants. Likewise, osteoclast stimulation also increased angiogenesis in mouse calvaria *in vivo*, thus showing that osteoclasts and angiogenesis are linked. Further studies were conducted to determine the mechanism by which osteoclasts may increase angiogenesis. Angiogenic factor expression by osteoclasts was analyzed by reverse-trancriptase PCR and Q-PCR angiogenesis arrays of human bone marrow osteoclasts. MMP-9 was the most highly expressed osteoclast angiogenic factor at the mRNA level. Because MMP-9 is important for osteoclast and blood vessel invasion of the growth plate and fracture calluses, the role of MMP-9 in osteoclast stimulated angiogenesis was studied in depth. Osteoclast stimulation with RANKL or PTHrP failed to stimulate angiogenesis in MMP-9$^{-/-}$ mouse calvaria or metatarsal explants. Surprisingly, osteoclast stimulation was dramatically blunted in MMP-9$^{-/-}$ calvaria or metatarsal explants. However, the number of vessels per osteoclast was not different between WT and MMP-9$^{-/-}$ mice, indicating that osteoclasts lacking MMP-9 do not have an intrinsic angiogenic defect. Further, bone marrow cultures from WT and MMP-9$^{-/-}$ mice formed similar numbers of osteoclasts, demonstrating that osteoclast differentiation or precursor number is not responsible for the inability of PTHrP or RANKL to increase osteoclastogenesis in MMP-9$^{-/-}$ mice. These results suggest that MMP-9 is important for osteoclast-stimulated angiogenesis by affecting the number of osteoclasts at the angiogenic site due to its previously

reported effects on osteoclast migration. These studies greatly increase our understanding of angiogenesis in bone and suggest an important role for osteoclasts in angiogenesis during bone development, fracture healing, bone metastasis, inflammatory bone diseases and the potential effects of osteoclast inhibitory agents on angiogenesis.

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PREFACE

This dissertation would not have been possible without the help and understanding of many people over the past 4 years. My mentor Dr. Roodman was primarily responsible for guiding my progress. He tirelessly supported my scientific progress and intellectual development and was always accessible for advice, professional and otherwise. His ability to manage a successful laboratory and gain a reputation as an expert on the clinical aspects of myeloma bone disease has made him an excellent role model for me.

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1.0 INTRODUCTION

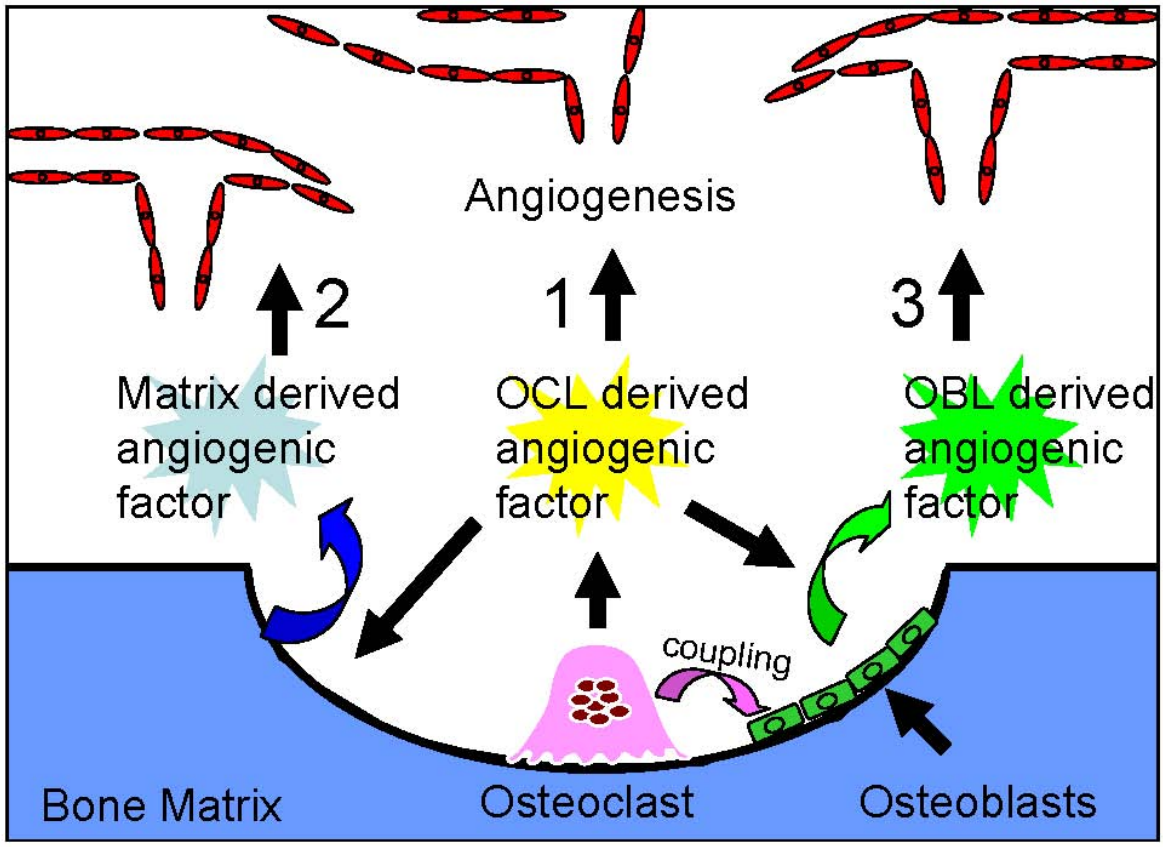
1.1 DISSERTATION GOALS AND OBJECTIVES

Angiogenesis is critical to normal bone development and physiology and disease processes involving bone, such as rheumatoid arthritis and bone metastases. There are many situations where angiogenesis is correlated with osteoclastogenesis including endochondral ossification in development, myeloma and other bone metastases, rheumatoid arthritis and Paget's disease of bone.[1-3] Despite these observations, only two Medline-indexed studies have investigated in depth whether osteoclasts stimulate angiogenesis, and their results were conflicting.[4, 5] Therefore, the role that osteoclasts play in angiogenesis in normal physiology and inflammatory bone disease is unclear. Because of the role that osteoclasts may play in these processes I have undertaken the required studies to test the hypothesis that; **Osteoclasts stimulate angiogenesis in the bone microenvironment by secreting factors that directly or indirectly increase new vessel formation** (Figure 1.1)**.**

A major goal of this dissertation was to select models that would most accurately determine if and how osteoclasts stimulate angiogenesis. As has been suggested by Tanaka et al, osteoclasts may stimulate angiogenesis by secretion of an angiogenic factor such as osteopontin, which acts directly on endothelial cells (mechanism #1). Other reasons suggesting that direct stimulation of angiogenesis by osteoclasts may occur include; their secretion of several

angiogenic factors, and well known direct stimulation of angiogenesis by the closely related cell type, the macrophage.[6-9] To model such a mechanism *in vitro*, I conducted experiments testing the capacity of purified osteoclast culture conditioned media to stimulate tube formation of endothelial cells. Literature supporting this mechanism is discussed in sections 1.2.3.3 and 1.3.2.

Alternatively, osteoclasts may stimulate angiogenesis by more complicated mechanisms, including release of angiogenic factors from bone or marrow extra-cellular matrices (mechanism #2, literature discussed in section 1.2.3.2), or by induction of angiogenic factor secretion by cell types such as osteoblasts (mechanism #3, literature discussed in section 1.3.2). There are several reasons that support such mechanisms. Release of growth factors, especially TGF-β, from bone matrix by osteoclasts is well documented.[10, 11] The matrix metalloproteinase MMP-9 is highly expressed by osteoclasts and is used by macrophages or tumor cells to stimulate angiogenesis by releasing VEGF from matrix.[12, 13] Further, MMP-$9^{-/-}$ mice have delayed blood vessel invasion into the metaphyseal growth plate, which would be expected if osteoclasts stimulate angiogenesis by secreting MMP-9.[14] Alternatively (mechanism #3), osteoblasts are well known to stimulate angiogenesis through secretion of VEGF, and osteoclast formation induces a coupled increase in osteoblast formation and activity.[15, 16] Therefore, osteoclasts may stimulate angiogenesis through interactions with osteoblasts. To model *in vitro* the possible ways that osteoclasts could stimulate angiogenesis, including these complex mechanisms, I used a bone organ culture system, the fetal mouse metatarsal angiogenesis assay, in which endothelial cell tubes grow out from explants of mouse metatarsals, rather than purified populations of various cell types.[17]. I then tested the relevance of the *in vitro* findings *in vivo*.



1. Direct secretion of an angiogenic factor
2. Release of an angiogenic factor from matrix.
3. Induction of an angiogenic factor in osteoblasts or other cells.

Figure 1.1. Hypothesis

Osteoclasts stimulate angiogenesis in the bone microenvironment by secreting factors that directly or indirectly increase vessel formation. Osteoclasts may stimulate angiogenesis by **(1)** direct action of an angiogenic factor on vessels, **(2)** release of an angiogenic factor for calcified or non-calcified matrix and **(3)** induction of an angiogenic factor in second cell type such as an osteoblast or stromal cell.

1.2 BIOLOGY OF OSTEOCLASTS AND BONE ANGIOGENESIS

1.2.1 Osteoclast Biology

1.2.1.1 Osteoclast Differentiation

After almost three decades of intensive study, the cellular origins of the osteoclast (OCL) are clear (Figure 1.2). OCLs are derived from hematopoietic stem cells, which subsequently form common myeloid precursors and granulocyte-macrophage precursors (CFU-GM).[18, 19] CFU-GM can further differentiate into granulocyte precursors or macrophage / osteoclast / dendritic cell common precursors (MODP), which are also referred to as early OCL precursors.[20, 21] These cells are c-Fms^{+} (M-CSF receptor) and RANK^{-}. M-CSF induces the expression of RANK (RANKL receptor) in these cells, thus forming late OCL precursors (Although different authors refer to different cell types as early and late OCL precursors.[20]) Late OCL precursors form committed precursors, which than fuse to form mature OCLs in the presence of RANKL or macrophages in the absence of RANKL.[21] In bone RANKL ligand signaling is regulated by the balance of RANKL and its decoy receptor, osteoprotegerin.[22] The capability of early OCL precursors to form dendritic cells or osteoclasts was first shown by Miyamoto et al.[23] In this study, M-CSF inhibited the formation of dendritic cells and promoted OCL differentiation from early OCL precursors by induction of c-fos, whereas GM-CSF was shown to promote dendritic cell differentiation from early OCL precursors by inhibiting expression of c-fos. There remains some degree of plasticity among the cell types derived from early OCL precursors. Rivollier et al showed that immature dendritic cells can transdifferentiate into functional OCL in the

presence of M-CSF and RANKL.[24] Conversely, several studies have found that differentiated macrophages can also transdifferentiate into OCLs.[25-27]

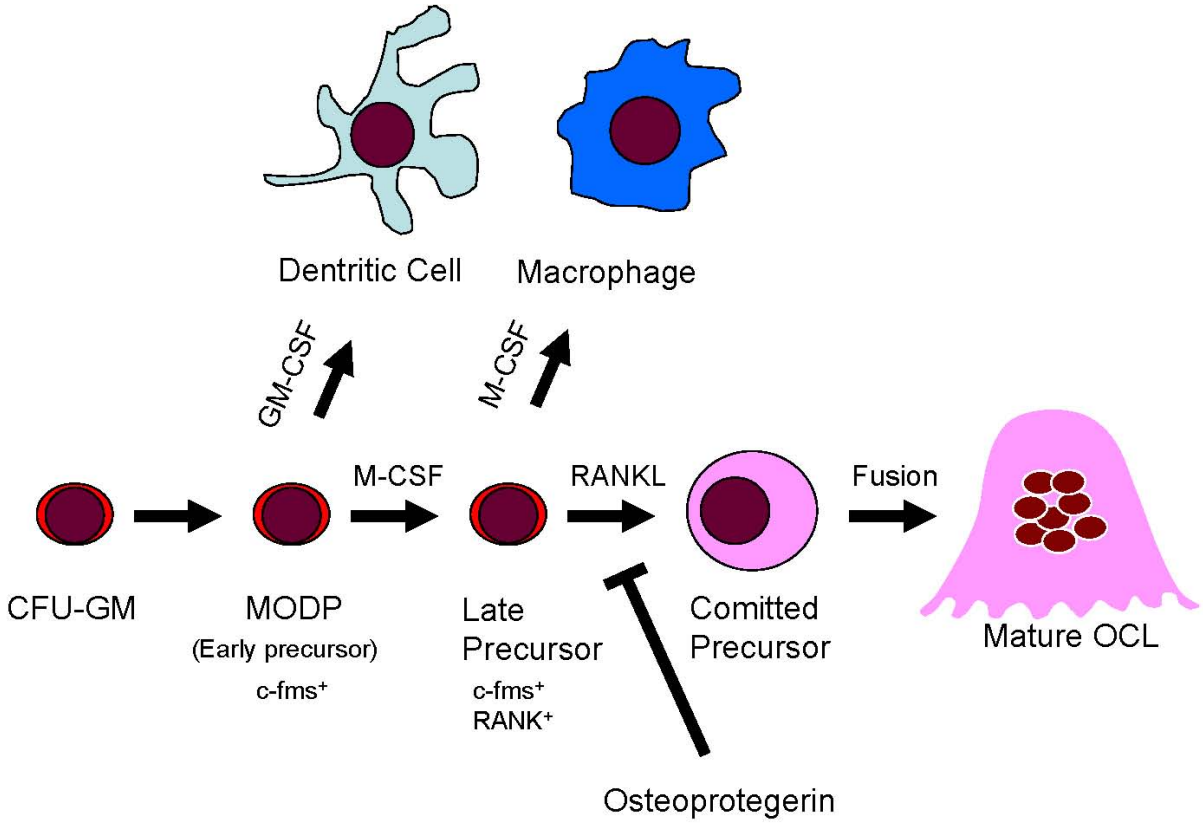


Figure 1.2. Osteoclast differentiation

As described above, M-CSF and RANKL are central regulators of OCL differentiation. However, multiple factors can also regulate OCL differentiation, only some of which will be described here. Many inflammatory cytokines, in particular TNF-α, stimulate OCL formation. TNF-α is a potent stimulator of OCL formation, primarily through induction of M-CSF and RANKL in bone marrow osteoblasts and stromal cells.[28] TNF-α also acts directly on OCL precursors to induce OCL formation. However, it is unclear if TNF-α coupled with M-CSF are sufficient to induce OCL formation, or whether sub-optimal doses of RANKL are also required. Several studies have found that TNF-α and M-CSF are sufficient to induce OCL formation from

precursors.[29-31] However, in a study where the authors used highly purified cell populations to eliminate exposure of OCL precursors to paracrine-produced RANKL, sub-optimal doses of RANKL were required for TNF-α to stimulate osteoclastogenesis[32]. In agreement with this observation that TNF-α requires RANKL to stimulate osteoclastogenesis, transgenically expressed TNF-α was not able to rescue OCL formation in a RANK (RANKL receptor) null mouse.[33] Although TNF-α by itself may or may not be sufficient to induce OCL differentiation in combination with M-CSF, the addition of other cytokines to M-CSF and TNF-α can induce OCL in a RANK independent manner. Kim et al showed that TNF-α and M-CSF can induce OCL formation in cells from a $RANK^{-/-}$ mouse if the precursors are first incubated with M-CSF and TGF-β. Even if TNF-α and M-CSF are sufficient to induce OCL formation, the OCLs that are formed do not resorb bone efficiently. Addition of IL-1α was required for comparable resorption as RANKL derived OCLs.[29-31]

The inflammatory cytokine IL-1 likewise stimulates OCL formation both indirectly and directly, although it was first believed that its effects were all indirect. Induction of IL-1 is required for stimulation of RANKL expression in stromal cells by TNF-α, and is required for maximal OCL formation in whole bone marrow cultures stimulated by M-CSF and RANKL.[34, 35] IL-1 also can stimulate OCL formation by inducing production of PGE2 in osteoblasts.[36] IL-1 induces the fusion of fully differentiated committed precursors (pre-OCLs) and can stimulate OCL formation by acting directly on OCL precursors in the presence of permissive levels of RANKL.[34, 37]

Like the inflammatory cytokines, LPS also stimulates OCL differentiation as well as survival. These effects of LPS on survival are most primarily due to stimulation of osteoblasts through toll-like receptors to increase RANKL production.[38] LPS stimulates OCL survival

through TLR4, but this was not due to production of the inflammatory cytokines TNF-α, IL-1β, and IL-6.[39] This contrasts with the effects of LPS on bone marrow macrophages, where inflammatory cytokines are released in response to LPS. These results suggest that the responses of OCLs and macrophages to LPS are different.

The inflammatory cytokine IFN-γ also stimulates OCL formation, although not directly. The direct effect of IFN-γ upon OCL precursors is to inhibit OCL formation by inducing degradation of the RANK adaptor protein TRAF6.[40] However, the net effect of IFN-γ *in vivo* is to increase OCL formation through activation of T cells which increase secretion of RANKL and TNF-α.[41]

Like IFN-γ, TGF-β also has competing effects on OCL formation. The direct action of TGF-β upon OCL precursors is to stimulate OCL formation. Endogenous TGF-β is required for osteoclastogenesis stimulated by M-CSF and RANKL and addition of TGF-β increases osteoclastogenesis stimulated by TNF-α.[42, 43] Further, TGF-β also blocks the direct inhibitory effect of IFN-γ upon OCL formation.[44] However, TGF-β also acts to decrease OCL formation by increasing the ratio of osteoprotegrin (OPG – a RANKL decoy receptor) to RANKL produced by osteoblasts. Both osteoblast OPG is increased and RANKL is decreased by TGF-β.[45, 46]

Unlike inflammatory cytokines, which in general stimulate OCL differentiation, anti-inflammatory (T_H2) cytokines usually inhibit OCL formation. In contrast to their effects on OCL, anti-inflammatory cytokines induce a pro-angiogenic state in macrophages. IL-10 inhibits OCL formation, possibly by down-regulating NFATc1 in OCL precursors.[47] IL-13 and IL-4 also inhibit OCL formation, both by direct effects on OCL precursors and by increasing OPG

production by osteoblasts. IL-4, like IL-10, likewise down-regulates NFATc1 in OCL precursors.[48]

1.2.1.2 Osteoclast Activation and Resorption

Once formed, OCLs must be exposed to the proper soluble and matrix bound signals to resorb bone. To resorb bone, OCLs must be motile, adhere to bone, polarize their cytoplasm and isolate the section of bone to be resorbed (ruffled border) from the external environment so that their secreted proteases and acid are localized. Thus, "activation" of OCL combines several distinct but related processes.[49] Two organelles are required for a resorbing OCL; the ruffled membrane and the actin ring. The ruffled membrane is the portion of the OCL membrane adjacent to bone. It contains the machinery required for acidification of the resorption space and is formed by fusion of acidified vesicles with the bone-opposed surface. The actin ring, also known as the "sealing zone", is made up of polymerized actin and surrounds the resoption space. It is necessary but not sufficient for OCLs to resorb bone.[50]

Interaction with the extra cellular matrix is central to the formation of a resorbing OCL. $\alpha_v\beta_3$ integrin is required for resorptive OCLs but not for OCL formation as illustrated by the β_3 knockout mouse.[51] $\beta_3^{-/-}$ mice actually have more OCLs *in vivo* but are osteopetrotic (excess bone). $\beta_3^{-/-}$ OCLs fail to undergo the cytoskeletal organization characteristic of resorbing OCLs; they fail to form actin rings when isolated from bone and form abnormal ruffled membranes *in vivo*.

The mechanism by which $\alpha_v\beta_3$ ligation leads to OCL cytoskeletal reorganization and resorption is beginning to be delineated. Src plays a central role in this process as shown by the suprising finding that the predominant phenotype of $src^{-/-}$ mice is osteopetrosis.[52] $Src^{-/-}$ mice

have an almost identical phenotype to $\beta_3^{-/-}$ mice; increased OCL numbers, but lack of resorption pits and ruffled membranes are found *in vivo*.[53] A study of the src family kinase syk, using either global $syk^{-/-}$ or mice lacking syk in hematopoietic cells provided a linear pathway for β_3 stimulation of OCL resorption.[54] $Syk^{-/-}$ mice are osteopetrotic. OCLs in syk hematopoietic chimeras are smaller than wt and not juxtaposed to bone. The authors propose a pathway where $\alpha_v\beta_3$ ligation leads to Src recruitment and phosphorylation. Src then associates with and phosphorylates syk, which requires the ITAM proteins Dap12 and FcRγ. Phosphorylation of the guanine nucleotide exchange factor Vav3 required $\alpha_v\beta_3$ ligation and Syk kinase activity. Vav3, as well as the GTPase Rac1, are required for a normal bone phenotype and OCL resorption and actin ring formation.[55] M-CSF in addition to its role in stimulating OCL formation can cooperate with $\alpha_v\beta_3$ to induce OCL cytoskeletal organization. High doses of M-CSF (100 ng / mL) can compensate for lack of $\alpha_v\beta_3$ in terms of stimulating OCL formation, but cannot restore resorptive ability. [56]

RANKL, in addition to its central role in OCL differentiation, also stimulates OCL activation as shown in cultures of isolated rat OCLs.[57] Likewise, the osteoclastogenic cytokine IL-1 also stimulates OCL cytoskeletal organization and activation.[37] Both of these cytokines stimulate OCL activation through the adaptor molecule TRAF6, although the downstream intracellular signaling pathways are unclear. The TNF receptor associated factor 6 (TRAF6) binding domain of RANK is required for RANKL induced OCL activation. A RANK mutant that could not bind TRAF6 or mediate RANKL stimulated OCL activation was compensated for by addition of IL-1β.[58] IL-1 induced OCL activation occurs through a TRAF6 and c-Src containing complex, suggesting that it cross talks with integrin regulation of OCL activation.[59]

1.2.1.3 PTH and PTHrP in Bone Remodeling

The key paracrine factors described above that regulate OCL formation are in turn primarily regulated at the whole animal level by endocrine factors including 1,25 dihydroxy vitamin D_3 and parathyroid hormone (PTH). PTH acts to increase serum calcium by increasing OCL formation and activity and 1,25 dihydroxy vitamin D_3 synthesis and thus results in increased intestinal calcium absorption, increased calcium retention in the kidney, and release of calcium from the skeleton by way of osteoclastic bone resorption. PTH is a peptide hormone, which is comprised of 84 amino acids. It is homologous to a great extent in the N-terminal 13 amino acids to parathyroid hormone related peptide (PTHrP). Both molecules share a common receptor (PTHR1) that mediates most of their functions.[60]

The physiological effects of PTH and PTHrP are complex, as they can affect both OCLs and osteoblasts. Physiologic concentrations of PTH and PTHrP given continuously act in a catabolic fashion – to activate OCLs and degrade bone. This OCL induction is primarily indirect – through increased RANKL and decreased osteoportegerin expression in osteoblasts.[61] Paradoxically, PTH or PTHrP increase bone mass when given intermittently. This increase in bone mass is primarily due to decreased apoptosis of osteoblasts and reduced adipocytic differentiation of the common mesenchymal precursor.[62]

In contrast to PTH, which functions primarily in adults to maintain calcium homeostasis, PTHrP has important roles in development, as was shown in mice lacking PTHrP or PTHR1.[63] PTHrP is thought to be most important in development due to its effects on chondrocytes. $PTHrP^{-/-}$ mice die post-natally, likely due to asphysxia because of the reduced size of their ribcage.[64] They have shortened epiphyseal cartilages due to reduced chondrocyte proliferation, as was demonstrated by reduced thymidine uptake. In addition, the architecture of

the chondrocyte columns is disrupted.[65] The role of PTHrP in development is likely not completely due to signaling through PTHR1, as PTHR1$^{-/-}$ mice have three additional abnormalities not formed in the PTHrP$^{-/-}$; increased osteoblast number, decreased trabecular bone formation in the primary spongiosa and delayed vessel invasion into the primary ossification center.[66] The delay in vessel invasion was blunted in mice lacking both PTHrP and PTHR1. The authors thus concluded that PTHrP delayed vessel invasion through a different receptor than the classic PTHR1.[66]

In agreement with this observation, PTHrP has been reported to be anti-angiogenic.[67, 68] Full length PTHrP reduced angiogenesis in the chick chrioallantoic membrane and mouse matrigel plug angiogenesis assays. Most of the activity required for PTHrP inhibition of endothelial cell migration or angiogenesis in the matrigel plug assay was located in the first 10 amino acids of the molecule. PTHrP's effects on endothelial cell migration required PKA. This is consistent with PTHR1 being responsible for the anti-angiogenic effects of PTHrP. However, a requirement for PTHR1 in PTHrP inhibition of angiogenesis was not shown. In contrast with these data, an earlier report found that PTHrP was pro-angiogenic.[69] PTHrP knockdown was shown to inhibit angiogenesis induced by tumor cells around a diffusion chamber implant. PTHrP did not affect endothelial cell migration or survival, but increased capillary tube formation on a collagen matrix in a PKA dependent manner. PTHrP also induces vasodilation through a mechanism requiring endothelial cells but not PKA.[70] Therefore, a possible direct role of PTHrP in angiogenesis is unclear.

1.2.2 General Angiogenic Mechanisms

1.2.2.1 Angiogenesis in Biology

Angiogenesis is the development of new blood vessels from a pre-existing vascular network. It occurs in the embryo after a primitive vascular network is laid down through vasculogenesis, is responsible for most post-natal blood vessel formation and is essential for the formation of a functional vascular network in the embryo. Vasculogenesis by contrast is the formation of a vascular bed de novo through differentiation of precursors.[71] The study of angiogenesis is primarily concerned with the growth of capillaries from other capillaries or small venules, which grow either by sprouting or by insertion of tissue pillars into vessels to form branches (intussusception). Larger vessels grow by circumferential growth rather than by sprouting and branching.[72] Angiogenesis occurs in a limited number of circumstances in normal adults including wound healing and the female reproductive organs but is upregulated in disease states such as cancer and rheumatoid arthritis.[73-76] Because of this downregulation of angiogenesis in normal post-natal physiology, its inhibition is an attractive therapeutic approach.[76] Indeed, the number of investigators involved in angiogenesis research has ballooned in recent decades. At this writing, there were over 42,000 PubMed citations on angiogenesis.

1.2.2.2 Cellular Mechanisms of Angiogenesis

A discussion of bone angiogenic mechanisms first requires an understanding of the cells involved in angiogenesis. Much attention in angiogenesis is focused on the vessel lining cell, the endothelial cell, but other cell types also play important roles. Most vessels are surrounded by cell types generally referred to as mural cells; pericytes on capillaries and vascular smooth muscle cells on larger vessels.[72] Angiogenesis occurs by two principle microanatomic modes,

sprouting and intussusception. For many years sprouting was for the most part synonymous with angiogenesis itself.[77]

In sprouting angiogenesis, an endothelial cell selected to form a new sprout, called a tip cell, is identified. The basement membrane is degraded and the tip cell migrates outward. The tip cell is followed by stalk endothelial cells, which proliferate to lengthen the stalk. The tip cell eventually encounters an appropriate tip cell from another sprout and thus forms a linkage. The endothelial cells reorganize to form a lumen and blood flow begins. Finally the vessel matures, with the stabilization of endothelial cell contacts, recruitment of mural cells and formation of a basement membrane.[72, 78]

The other, less discussed and understood, mode of angiogenesis is intussusception. In intussusception, pillars are inserted into capillaries to form new branches. This allows additional complexity and better perfusion to an existing vascular bed. Unlike sprouting, intussusception does not allow capillaries to extend into previously avascular areas. This reorganization is accomplished in the continuous presence of blood flow, unlike sprouting angiogenesis. Proliferation of endothelial cells, while involved is somewhat less important than in sprouting angiogenesis.[77, 78]

Bone marrow endothelial cell precursors make important contributions to blood vessel growth, though they may not act through truly angiogenic mechanisms. Technically speaking, vascular growth by endothelial precursor cells is vasculogenesis and not angiogenesis because it involves differentiation from precursors rather than sprouting or intussusception from existing capillary beds. In addition, this growth is usually circumferential rather than sprouting or intussusceptive.[72] As an illustration that bone marrow endothelial cell precursors contribute to vasculogenesis rather than angiogenesis, bone marrow endothelial precursor cells were shown to

form blood island structures reminiscent of the blood islands formed during vasculogenesis in the embryo.[79] However, there has been some evidence that endothelial precursor cells can be incorporated into capillaries, which suggests they contribute to sprouting angiogenesis as well.[80, 81]

1.2.2.3 Key Regulators of Angiogenesis

The most well known and perhaps most important family of angiogenic stimulatory molecules are the vascular endothelial growth factors (VEGFs). This large family is the product of five different genes in mammals (*VEGFA (or simply VEGF), VEGFB, VEGFC, FIGF (VEGFD),* and *PGF (PlGF)*), many of which have splice isoforms.[82, 83] Each VEGF form exerts its effects on blood vessel angiogenesis or lymphangiogenesis through binding to one or more of the VEGF receptor tyrosine kinases VEGFR1 (flt-1), R2 (flk-1 / KDR) and R3 (flt-4).[83] VEGFR2 is principally involved in blood vessel angiogenesis, while VEGFR3 is principally involved in lymphangiogenesis. The contribution of VEGFR1 to angiogenesis is more complex. VEGFR1, but not its kinase domain, is required for embryonic angiogenesis. $VEGFR1^{-/-}$ embryos die with disorganized vessels, which suggests that VEGFR1 acts as a decoy for VEGF-A during development. However, the kinase domain of VEGFR1 is required for post-natal angiogenesis.[84, 85]

VEGF-A (or simply VEGF) is alternatively spliced to yield as many as six isoforms, which add another layer of complexity to angiogenic regulation.[82, 86] The longer forms bind heparan sulfate proteoglycans, while the shortest form – $VEGF_{121}$ (or $VEGF_{120}$ in mice) is freely diffusible. The various VEGF isoforms have different biological functions. The matrix bound forms are required for proper vessel structure. In a mouse expressing $VEGF_{120}$ ($VEGF^{120/120}$)but none of the other VEGF isoforms, there are fewer, but more dilated vessels. The defect was due

to branch formation rather than proliferation.[87] A similar phenotype of the $VEGF^{120/120}$ mouse with fewer but larger vessels was observed in bone. Endochondral ossification is delayed in a phenotype resembling that of the $MMP9^{-/-}$ mouse (discussed below) [88]. In addition to molecular genetic regulation, shorter forms of VEGF can be generated by MMPs rather than by alternative splicing, with similar biological effects.[89]

The angiopoietin family makes essential but complex contributions to angiogenesis. The family consists of three ligands; Ang-1 and Ang-2 in most species, as well as Ang-3 in mouse and Ang-4 in human.[90] The angiopoietins exert their biological functions by binding to the Tie2 tyrosine kinase receptor. Ang-1 acts as an agonist, while Ang-2 acts as a receptor antagonist.[91, 92] Although they are orthologs, Ang-3 and Ang-4 have opposite effects on Tie2, with Ang-3 acting as an antagonist and Ang-4 acting as an agonist.[90] Stimulation of Tie2 induces maturation of vessels, including endothelial cell stabilization and mural cell recruitment. This was first illustrated in a knockout of the Ang-1 gene, where knockout vessels had less spread endothelial cells and lacked mural cells.[93] $Ang\text{-}1^{-/-}$ mice are embryonic lethal and die due to cardiovascular abnormalities. Surprisingly, $Ang\text{-}2^{-/-}$ mice are born alive but die by two weeks of age of lymphatic abnormalities. Post-natal blood vessel angiogenesis is also inhibited in $Ang\text{-}2^{-/-}$ mice.[94] These and other observations have led to the following classic model of the role of angiopoietins in post-natal angiogenesis: Ang-2 is required for initiation of angiogenesis by loosening endothelial and mural cell attachements. In the presence of VEGF, Ang-2 leads to angiogenesis, whereas in its absence Ang-2 leads to vessel regression. Ang-1 is required for vessel maturation.[95]

Another family of molecules with affects on both endothelial and mural cells are the TGF-βs. The TGF-β superfamily includes not only the 3 TGF-β forms, but also bone

morphogenetic proteins, activins, and inhibins. Furthermore, the TGF-β family members exert their influences through binding to three different classes of receptors; type I receptors Alk1 and Alk5, the type II receptor TGFβRII, and the type III receptor endoglin, which influences the interaction of type I and type II receptors. Perhaps not-surprisingly given the many molecules involved, the effects of TGF-β on angiogenesis are complex, with many cell and tissue dependent effects.[96]

Because deletion of TGF-β signaling components such as Alk-1 results in lethality from vasculogenic defects at approximately E10.5, TGF-β is obviously important for vessel formation or function.[97] Vessels in TGF-β signaling component knockout mice are hyperdilated and have abnormal mural cell recruitment, suggesting that TGF-β is involved in vessel maturation. Consistent with a role in vessel stabilization, TGF-β increases endothelial cell expression of proteins important for interaction with the basement membrane.[98]

However, the effect of TGF-β signaling on other endothelial cell functions is not as clear. TGF- β signals through heterodimerization of type I and type II receptors. Studies using constitutively active type I receptors in endothelial cells have yielded conflicting results. Studies using different sources of endothelial cells are in agreement that constitutively active Alk-5 inhibits endothelial cell proliferation, sprouting or migration.[99-102] However, while most studies have found that Alk-1 activation also inhibits endothelial cells, one study using mouse embryonic endothelial cells, found that constitutively active Alk-1 stimulated endothelial cell migration.[102] TGF-β's effects on angiogenesis are likely to vary depending on cellular or tissue context. Such inhibitory effects of TGF-β upon endothelial migration are consistent with it playing a role in the maturation phase of angiogenesis, as was first observed in mice lacking TGF- β signaling components.

1.2.3 Normal Bone Angiogenesis

1.2.3.1 Vascular Structure of Adult Bone

OCLs are in close proximity to capillaries and are thus in a good position to signal to blood vessels. The vasculature of adult long bones has a complex structure with extensive anastamoses. Arterial blood supply is derived from six sources; proximal and distal epiphyseal arteries, proximal and distal metaphyseal arteries, diaphyseal nutrient arteries and periosteal arteries. Venous drainage is paired with the corresponding arteries including the central venous sinus which is drained by the nutrient vein[103]. Bone marrow sinusoidal vessels are part of the reticuloendothelial system and have structures very different than bone marrow arterial or capillary vessels, or most vessels in the body. The surface phenotype of bone marrow sinusoidal endothelial endothelial cells is also different than most endothelial cells, with reduced or absent CD31 expression.[104]

The microanatomy of capillaries is characterized by their close association with bone cells. Cortical bone is organized into Haversian systems where bone is concentrically organized around a neurovascular bundle which is lined by osteoblasts and osteoprogenitors. Blood vessels are closely associated with resorption of cortical bone. OCLs form a resorption cavity or cutting cone, into which a capillary then invades.[105] Likewise, capillaries are also closely associated with bone cells in trabecular bone. Long ago, Burkhardt et al showed that capillaries are closely associated with the cells lining trabeculae and observed that microvessel density in bone is associated with increased remodeling.[106] More recently the existence of a microstructure in trabecular bone termed a bone remodeling compartment (BRC), has been proposed. (Figure 1.3)[107] In this model, the OCLs and osteoblasts involved in bone remodeling are enclosed in a dome of osteoblast-like cells that are in close association with a capillary. The interior of the

BRC may or may not be in continuity with the circulation. Another model proposed for the relationship of osteoblasts, OCLs and capillaries is the "basic multicellular unit" (BMU) (Figure 1.4). In the BMU model, in both trabecular and cortical bone, osteoblasts and OCLs are located in close proximity to a blood vessel, except that the lining layer of osteoblast-like cells is not present.[16] Therefore, in both cortical and trabecular bone, osteoclasts are closely opposed to vessels and have the potential to regulate blood vessel formation by paracrine mechanisms.

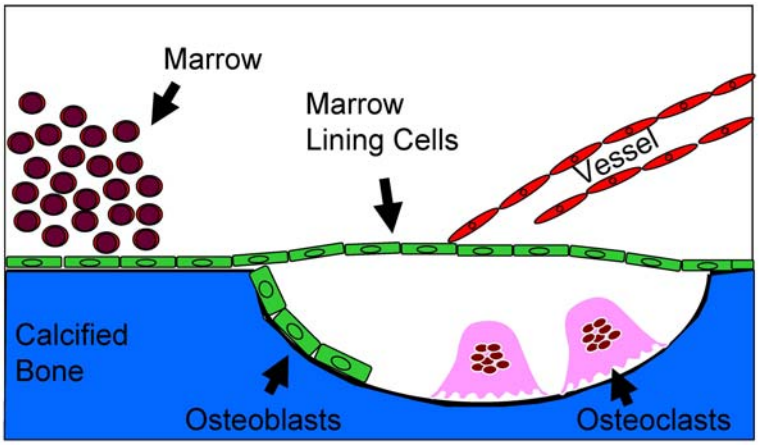


Figure 1.3. Bone remodeling compartment model

Illustration of the bone remodeling compartment model of bone resorption. Bone at bottom, marrow space at top of image. OBL; osteoblasts. Adapted from Hauge et al.[107]

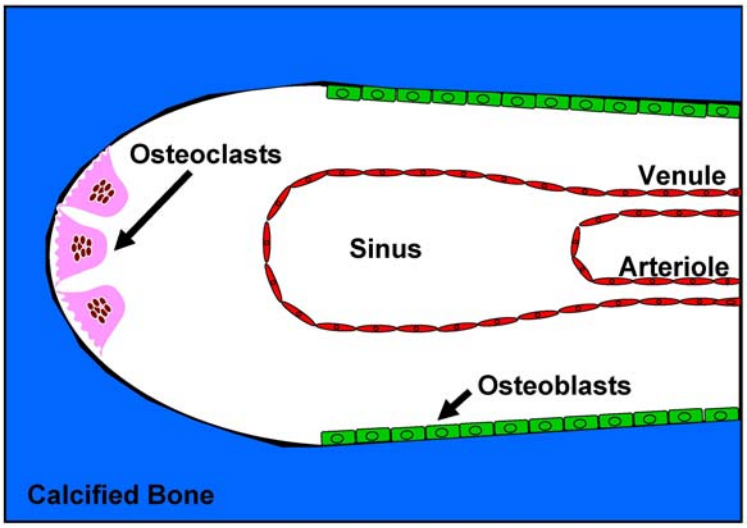


Figure 1.4. Basic multicellular unit model

Illustration of the basic multicellular unit (BMU) model of bone resorption in cortical bone.[16] Note that the endothelial cell lined vascular sinus is closely opposed to both osteoblasts and osteoclasts.

1.2.3.2 Regulation of Bone Angiogenesis by Osteoblasts and Stroma

Most cells in bone have a regulatory role in angiogenesis, many through production of angiogenic factors. I discuss here the mechanisms used by osteoblasts to stimulate angiogenesis, because of the possibility that OCLs stimulate angiogenesis through interaction with osteoblasts or stroma, and that the angiogenic factors acting directly on endothelial cells may be non OCL-derived. OCLs may stimulate angiogenesis through induction of angiogenic factor secretion in osteoblasts, such as the ability of TGF-β, which can be released from matrix by OCLs, to stimulate osteoblast VEGF expression.[108, 109] Alternatively, OCLs may stimulate angiogenesis by way of osteoblasts by increasing osteoblast formation. Except in a small number of pathologies, OCL and osteoblast formation are coupled. Increased OCL formation or

activity leads to increased osteoblastic formation, and possible effects on angiogenesis.[16] To provide a summary of angiogenesis regulation in bone, several of the angiogenic factors expressed by bone cells are listed in Table 1.1.

Most osteoinductive factors, many of which are derived from endothelial cells, are capable of inducing the production of VEGF by osteoblasts.[110] For example, BMP-2 stimulates angiogenesis by inducing osteoblast VEGF production.[111] BMP-2 also stimulates production of the VEGF family member PlGF in mesenchymal stem cells.[112] Likewise, IGF-1 and prostaglandins also upregulate osteoblast VEGF expression.[113, 114] The endothelial derived osteogenic molecule angiotensin-II has also been shown to up-regulate osteoblast VEGF.[115] Endothelin-1, although it is an endothelial cell derived osteogenic factor, has been reported to down-regulate osteoprogenitor VEGF expression *in vitro* in a fetal rat calvarial culture model.[116] However, this discrepancy may have occurred because the cells were not mature osteoblasts. Consistent with this hypothesis, the inhibitory effect of endothelin-1 was less pronounced after longer culture periods.

Like many other cell types, osteoblasts increase production of angiogenic factors in response to hypoxia. Hypoxia upregulates production of VEGF and causes nuclear accumulation of HIF-1α in an osteoblast derived cell line. *In vitro* angiogenesis stimulated by these cells was reduced by antibodies to bFGF and VEGF.[117] Wang et al showed that osteoblast derived VEGF induced by HIF-1α and HIF-2α contributed to developmental angiogenesis and bone formation in mice.[15] HIF isoforms may also play a role in osteoblasts' contribution to angiogenesis stimulated by other processes. For example, HIF-1α was reported to be important in shock wave stimulated osteoblast VEGF expression.[118]

Table 1.1. Expression of pro- angiogenic factors by bone cells

Cell	Molecule	Reference
Osteoclast	VEGF (mouse)	[119]
	VEGF (neonatal human)	[120]
	IL-8	[121]
	Osteopontin	[122]
	bFGF	[6]
	PDGF	[6]
	HGF	[123]
	Angiopoietin-1	[120]
	Angiopoietin-2	[120]
Bone marrow stromal cells	VEGF	[124]
	b-FGF	[125]
Osteoblasts	OPN	[122]
	VEGF	[119]
	Angiopoietin-1	[120]
	Angiopoietin-2	[120]
	IL-8	[126]
	PDGF	[127]
	HGF	[128]
	bFGF	[129]

Table 1.2. Regulation of osteoblast angiogenic factor production

Regulator	Direction of regulation	Angiogenic factor induced	Reference
BMP-2	↑	VEGF	[111]
IGF-1	↑		[113]
Prostaglandins	↑		[114]
HIF-1α, HIF-2α	↑		[15]
	↑		[118]
Shock waves	↑		[130]
PDGF-BB	↑		[108]
TGF-β			
bFGF	↑	HGF	[128]
TNF-α and IFN-γ	↓	Angiopoietin-1	[131]

Osteoblasts may also stimulate angiogenesis by various other mechanisms. Following are examples from the literature. RANKL, which is primarily derived from osteoblasts, can increase angiogenesis as well as vascular permeability *in vitro* and *in vivo* in matrigel plug and chick chrioallantoic membrane *in vivo* models.[132, 133] VEGF was reported to increase the expression of RANK by endothelial cells and their angiogenic response to RANKL.[134] However, this series of papers used high (μg/mL) RANKL concentrations, thereby calling into question the physiological relevance of RANKL stimulated angiogenesis. In contrast, other investigators reported that RANKL inhibits angiogenesis *in vitro*.[135] In addition, studies reported that the osteoblast derived RANKL decoy receptor osteoprotegerin stimulated angiogenesis through endothelial cell apoptosis induced by TRAIL.[135, 136] Therefore, a possiblility of direct effects for the osteoblast derived regulatory molecules RANKL and osteoprotegerin remains unresolved.

Additional possible mechanisms used by osteoblasts to stimulate angiogenesis include the following. PDGF-BB was shown to induce VEGF production in neonatal rat calvarial osteoblasts.[130] bFGF induced HGF expression in fetal rat calvariae derived osteoblasts, which was lessened by cortisol.[128] TGF-β upregulated VEGF expression in an osteoblastic cell line involving signaling through p38 and Erk 1/2 MAP kinases.[108] Co-stimulation of human osteoblasts with TNF-α and IFN-γ resulted in downregulation of angiopoietin-1 expression, mediated in part through nitric oxide signaling.[131]

1.2.3.3 Evidence For and Against Osteoclast-Stimulated Angiogenesis

There are several papers reporting that angiogenesis is closely associated with OCL formation and activity, and that endothelial cells stimulate osteoclastogenesis. Angiogenesis is closely

associated with increased OCL formation in multiple myeloma and other cancers, as well as inflammatory conditions such as rheumatoid arthritis.[2, 137] Addition of bFGF or inhibition of NO production increases angiogenesis, OCL number and resorption on a bone chip implanted in the chick chorioallantoic membrane model.[138, 139] Endothelial cells can stimulate OCL formation by several mechanisms including increased RANKL expression, and stimulate OCL formation in co-cultures by a RANKL dependent mechanism.[140] Endothelial cells may also regulate the recruitment of OCL precursors to remodeling sites from the vascular compartment. Kindle et al showed that $CD14^+$ cell adherence to and migration through endothelial cells, as well as subsequent OCL formation was increased by IL-1 and TNF-α.[141]

Observations from patients with Gorham-Stout syndrome also support an association between osteoclastogenesis and angiogenesis. Gorham-Stout syndrome is characterized by massive osteolysis and blood and lymphatic vessel infiltration. Its pathogenesis is very poorly understood, but osteoclasts are believed to play a role in the resorption based on reports of successes using bisphosphonates for treatment and the ability of serum from a Gorham-Stout patient to induce osteoclastogenesis.[142, 143] Cells isolated from Gorham –Stout patients, that showed some characteristics of OCL precursors, stimulated angiogenesis in the mouse matrigel plug assay.[144] These cells expressed TRAP and $\alpha_v\beta_3$ integrin and adhered to osteopontin. Their production of VEGF and IL-8 was upregulated by TNF-α. However, they were incapable of forming mature OCLs when stimulated with M-CSF, RANKL, or TNF-α.

One study of Paget's disease of bone suggested that OCLs stimulate angiogenesis. Paget's disease is characterized by large, hyper-multinucleated and highly activated OCLs and elevated but disorganized bone remodeling. Vascularity of Paget's disease lesions was reduced after clodronate treatment, which inhibited OCL formation and activity.[3] Furthermore, Paget's

disease is also associated with high output congestive heart failure, which is consistent with tremendously increased bone vascularity.[145]

However, there are few papers directly studying whether OCLs can stimulate angiogenesis. Most prominent of these is Tanaka et al.[5] The authors reported that conditioned media from human OCL cultures stimulated angiogenesis *in vitro* and that co-culture of OCLs with myeloma cells produces more *in vitro* angiogenesis than either OCLs or myeloma cells alone. Further, they reported that neutralizing antibodies to osteopontin abrogated the OCL derived angiogenic activity and neutralizing antibodies to VEGF abrogated the myeloma cell derived angiogenic activity. In addition, co-cultures of OCL and myeloma cells stimulated HUVEC migration and survival, and that osteopontin and VEGF caused release of a soluble osteoclastogenic factor by HUVECs. However, since the authors did not purify the OCLs in their cultures, a significant contribution from macrophages or T-cells cannot be ruled out. Secondly, the neutralizing antibody approach may over emphasize the importance of any one factor and identify required factors in the mix of pro and anti-angiogenic factors, rather than identify the one most important factor. For example using the same angiogenesis assay and a neutralizing antibody approach, other authors have identified either angiopoietin-1 or osteopontin as the most important angiogenic factor secreted by myeloma cells.[146, 147] In addition, endothelial cells express osteopontin and therefore neutralizing antibodies to osteopontin could act on endothelial cells directly.[122] In parallel, with these studies, VEGF-C expressed by osteoclast precursors stimulated lymphangiogenesis in an mouse arthritis model.[148]

In contrast to the report of Tanaka et al, there are two published papers that asked whether OCLs were required for angiogenesis and found that they were not.[4, 149] Deckers et

al studied blood vessel invasion into caudal vertebrae of neonatal mice in which OCL formation or activity was abolished by treatment with the bisphosphonate clodronate or using osteopetrotic mice (op/op or c-fos$^{-/-}$) that lacked OCLs. Blood vessels were able to invade the vertebrae under all three osteoclast-free conditions. Similarly, in another study in osteopetrotic mice, vessels were able to invade the (non-mineralized) epiphyses of mouse tibiae.[149] The results of these two papers are not definitive because the data were not quantitative, so small differences may have been missed. In addition, different bone anatomic locations may respond differently to OCL pro-angiogenic effects. As discussed in more depth below, in the paper characterizing the knockout mouse for the highly expressed OCL proteinase MMP-9, ossification and angiogenesis were delayed in growth plates of long bones. Importantly, there was no defect in epiphyses or bones formed by intramembraneous ossification.[14] These authors also reported (data not shown) that there was delayed vessel invasion into the long bones from op/op and c-fos$^{-/-}$ osteopetrotic mice. The results of 3 key papers addressing whether OCLs play a role in angiogenesis are summarized in Table 1.3.

Table 1.3. Key osteoclast and angiogenesis literature

First Author	Are OCLs angiogenic ???	Key Findings
Tanaka[5]	Yes	OCL conditioned media stimulated angiogenesis *in vitro* due to osteopontin. Synergistic with myeloma cell-derived VEGF
Deckers[4]	No	OCLs not required for vessel invasion of mouse tail vertebrae *in vivo*.
Sugiura[149]	No	OCLs not required for vessel invasion of epiphyses *in vivo*.

Several papers have analyzed OCL gene expression profiles using genomics and proteomics approaches and provided data supporting a role for OCLs in stimulating

angiogenesis. Kiesel *et al* used microarrays to analyze gene expression of mouse bone marrow cultures at various timepoints of OCL differentiation induced with RANKL and M-CSF.[150] They made theoretical predictions as well as verified two processes experimentally. They observed increased expression of angiogenic factors at 24 and 48 hours after induction of OCL differentiation, including members of the semaphorin family, VEGF-A and VEGF-C. Like Tanaka et al, they showed that conditioned media from bone marrow cultures containing OCLs stimulated HUVEC tube formation on matrigel more than control media or conditioned media from cultures treated with M-CSF alone.

However, other investigators using different cellular systems and genomic or proteomic techniques have not seen the same profile of angiogenic factors expressed by OCLs. RANTES was increased in human peripheral blood derived OCLs at the mRNA level, and mouse marrow derived OCLs at the protein level.[151, 152] TNF-α was likewise upregulated in human peripheral blood derived OCL and RAW264.7 (mouse cell line) derived OCLs both at the message level.[151, 153] Other possible angiogenic factors were also upregulated during OCL differentiation including; urokinase plasminogen activator receptor [153], IGF-1 and PDGF-A [152], and osteopontin and MIP-1α..[154] However, three other genomic or proteomic studies did not report upregulation of any angiogenic factors in OCLs.[155-157]. Therefore in several studies examining OCL gene expression with genomic and proteomic approaches, increased angiogenic factor gene expression during OCL differentiation has not been consistently detected.

1.2.3.4 Bone Angiogenesis During Development

The close spatial and temporal association between OCLs and capillaries in bone development and fracture healing suggests communication between OCLs and vessels, and OCL regulation of angiogenesis. Physiologic bone angiogenesis occurs primarily during development and fracture

healing. In endochondral ossification, cartilage is converted into bone and angiogenesis is required for the ossification process.[158] In endochondral ossification, an avascular cartilage model (anlagen) of the final bone is first formed. The chondrocytes then begin to hypertrophy and ossify in the two eventual centers of ossification; midshaft (primary) and epiphysis (secondary). The marrow cavity is then formed by the concurrent invasion by osteoclasts (or chondroclasts) and blood vessels.[159] This blood vessel invasion is essential for bone development and has been proposed to be the source of osteoblasts.[160] Angiogenic factors are required for OCL entry into the anlagen. **A key finding for the mechanistic studies of this dissertation is; MMP-9 was shown to be required for both blood vessel and OCL entry into the primary ossification center.[161]** Furthermore, the study reporting the phenotype of the MMP-9$^{-/-}$ mouse reported in their discussion section that osteopetrotic (op/op and c-fos$^{-/-}$) mice had delayed developmental vessel invasion into the primary ossification center.[14]

In agreement with the MMP-9 data, other investigators directly implicated VEGF or hypoxia pathways in bone development. VEGF has been proposed to be the primary factor regulating this vessel ingrowth because systemic neutralization of VEGF inhibited vessel ingrowth into epiphyses in a mouse model.[162] Hypoxia upregulates VEGF production by chondrocytes and requires HIF-1α for its effect.[163] Osteoblasts have also been shown to be important in stimulating developmental bone angiogenesis. The HIF pathway is important with contributions from both HIF-1α as well as other HIF-α forms.[15] Angiogenic factors are also required for OCL entry into the anlagen. Thus, bone development, angiogenesis and OCL invasion are closely linked.

1.2.3.5 Angiogenesis During Fracture Healing

Fracture healing shares many features with developmental angiogenesis, so not surprisingly angiogenesis is also important to its progression. A soft cartilaginous callus is first formed from a hematoma, a process analogous to the cartilage model of bone formed during development. The cartilage of the soft callus is then converted to woven bone primarily by way of endochondral ossification, where the cartilage hypertrophies and then is resorbed by OCLs. Finally, as in development, the woven bone is remodeled to its final form.[110]

Because long bone fracture healing is analogous to development, it should not be surprising that the molecular regulation of the two processes is similar. Notably, MMP-9 is also important for healing of long bone non-stabilized fractures, and this defect can be rescued by recombinant VEGF.[164] As in the studies of MMP-9 in development, vessels and OCLs failed to or had delayed invasion of the hypertrophic cartiliage callus. This led to enlarged calluses and non-unions. However, it is unclear if the MMP-9 important for these observations was OCL derived because MMP-9 is also expressed by many macrophages and granulocytes in fractures. In agreement with the studies of MMP-9 in fracture healing, neutralization of VEGF inhibits fracture healing in mouse models.[165]

Likewise, others have suggested that OCLs may stimulate angiogenesis in fracture healing due to heparanase mediated release of VEGF. However, this study is purely descriptive.[1] VEGF was previously known to bind heparan and be released by heparanase. These authors observed OCL expression of heparanase both during endochondral ossification and in formation of woven bone. They also observed that OCLs were located between vessels and hypertrophic chondrocytes. Thus, OCLs are in the correct anatomical position to stimulate angiogenesis. However, no experiments were performed that showed OCL release of matrix

VEGF was important for angiogenesis, and no additional studies have been reported that support this hypothesis.

In support of the hypothesis of this dissertation, lack of OCL or OCL inhibition with bisphosphonates impairs fracture healing. $RANK^{-/-}$ mice have increased non-unions of non-stabilized long bone fractures. Even more importantly, they have reduced numbers of vessels in the fracture calluses.[166] Likewise, OCL inhibition with bisphosphonates delays fracture healing. Bisphosphonates were studied in a different fracture model than was used for $RANK^{-/-}$ or $MMP\text{-}9^{-/-}$ mice; stabilized rather than un-stabilized fractures. Stabilized fracture models heal without a cartilage intermediate and are more analogous to membranous rather than endochondral ossification. Perhaps due to this model difference, bisphosphonates did not cause non-unions, but delayed remodeling of the woven bone callus.[167, 168]

In addition, in a study relevant to the work by Tanaka et al, osteopontin, which is expressed by OCLs and osteoblasts in bone calluses,[169, 170] has also been implicated in stimulating angiogenesis during fracture healing. Studies in $osteopontin^{-/-}$ mice have shown that these mice had decreased early stage angiogenesis and bone strength in healing calluses.[171]

1.2.4 Pathological Bone Angiogenesis

1.2.4.1 Angiogenesis in Bone Metastases

Angiogenesis has long been known to be important for formation of bone metastases in solid tumors. More recently angiogenesis has also been shown to be important for hematological tumor growth, including myeloma bone lesions where bone angiogenesis is clinically important.

Myeloma is a malignancy of plasma cells in which the malignant clone produces a monoclonal immunoglobulin and induces bone lesions.[172] It is thought to develop from

MGUS (monoclonal gammopathy of uncertain significance), a condition requiring no treatment in which a monoclonal immunoglobulin is detectable but the patients have no end organ damage.[172] The switch from a non-angiogenic to a pro-angiogenic phenotype in the bone marrow is thought to be important in the transition from MGUS to multiple myeloma.[173] Regulation of angiogenesis in myeloma results from signals from the myeloma cells superimposed on the normal angiogenesis regulatory mechanisms. Investigators have proposed paracrine loops between myeloma cells and bone marrow stromal cells to explain the enhanced angiogenesis in myeloma.[2] VEGF, bFGF and TGF-β derived from myeloma cells upregulate bone marrow stromal cell production of IL-6.[174-176] Adhesion of myeloma and bone marrow stromal cells likewise upregulated stromal cell IL-6.[176] IL-6 then increases production of VEGF by myeloma cells thus forming a loop.[174]

VEGF is a key angiogenic factor in myeloma *in vivo*, as illustrated by the ability of a VEGF inhibitor to block myeloma growth and angiogenesis and increase survival in a mouse model of myeloma.[177] However, the mechanisms responsible for the transition from a non-angiogenic phenotype in MGUS to a pro-angiogenic phenotype in myeloma are less clear. Asosingh et al reported that a switch from $CD45^+$ myeloma cells, which do not express VEGF, to $CD45^-$ myeloma cells, which do express VEGF, correlated with the angiogenic switch in a mouse model of myeloma.[178] Lack of CD45 expression was also correlated with progression from MGUS to myeloma and increased angiogenesis.[179] However, in another study the expression of VEGF, bFGF and their receptors did not differ between plasma cells from MGUS patients and myeloma patients, suggesting that other factors are responsible for the angiogenic switch.[180] The same paper also found that plasma cells from MGUS patients contained an

angiogenesis inhibitory activity.[180] A multitude of other factors have been proposed to contribute to myeloma angiogenesis including angiopoietin-1 and osteopontin.[146, 147]

1.2.4.2 Antiangiogenic Effects of Bisphosphonates

: An understanding of angiogenesis in bone metastases requires an understanding of the drugs most commonly used to treat them, nitrogen containing bisphosphonates (N-BPs), which have anti-tumor and anti-angiogenic effects. These drugs reduce the incidence and severity of skeletal complications, relieve metastatic bone pain, and improve patients' quality of life.[181] N-BPs are also effective in myeloma bone disease.[182] A major effect of N-BPs is to inhibit bone resorption through effects on the OCL. *In vitro* studies have suggested many possible mechanisms for the anti-resorptive effects of N-BPs, but the bulk of evidence is pointing towards disruption of the OCL cytoskeleton due to inhibition of farnesyl diphosphate synthase and resulting effects on prenylation of small GTPases in OCLs as the primary mechanism for the effects of N-BPs *in vivo*.[183, 184] Note, as discussed earlier, GTPases are involved in M-CSF and integrin induced OCL cytoskeletal organization.[50] The N-BPs alendronate and risedronate inhibited OCL resorption at ten fold lower concentrations than were required to induce apoptosis. Further, apoptosis inhibitors failed to inhibit resorption, thus arguing against a role for OCL apoptosis in N-BP inhibition of resorption.[185]

Due to the core structure of N-BPs, which is analogous to pyrophosphate, they are deposited in bone matrix, where they are released as the osteoclast resorbs bone and secretes acid. Thus, OCLs are likely exposed to higher concentrations of bisphosphonates *in vivo* than other cell types. Therefore, the cell specificity of bisphosphonates may be determined more by pharmacokinetics than by their molecular target. It has been estimated that OCLs are exposed to N-BP concentrations between 0.1 and 1 mM when resorbing bone.[186] Treatment of rabbits

with the N-BP risedronate inhibited the prenylation of Rab proteins in OCLs but not in other bone marrow cells, thus supporting the OCL as the target of N-BPs.[187] In a study using fluorescently labeled N-BPs *in vivo*, uptake was observed primarily in OCLs, with some uptake in a subpopulation of bone marrow cells and osteocytes.[188] Alternatively, cell culture experiments have suggested that cells close in proximity to OCLs can be exposed to high concentrations of N-BPs through transcytosis from OCLs.[189]

Bisphosphonates also have anti-tumor and anti-angiogenic effects. The mechanisms of these effects are under investigation. The N-BP zoledronic acid reduced angiogenesis and tumor burden in the 5T2 mouse myeloma model.[190] Zoledronate, alendronate and neridronate inhibited angiogenesis *in vitro* at concentrations ranging from 10 to 50 μM.[191-193] These concentrations are higher than the maximum serum zoledronic acid level achieved after infusion, so it unclear if a direct effect of zoledronate on endothelial cells can explain the anti-angiogenic effects of zoledronic acid.[194] There is one report of bisphosphonates inhibiting angiogenesis *in vivo* in a non-neoplastic situation outside of bone tissue – testosterone stimulated revascularization of the rat prostate after castration.[3] Bisphosphonates were shown to transiently accumulate in the prostate, so as with bone, their tissue specificity is likely regulated by pharmacokinetics.

N-BPs have been associated with a complication called osteonecrosis of the jaw (ONJ) with a reported cumulative incidence for cancer patients treated with intravenous N-BPs ranging between 1 and 11%.[195] The incidence in osteoporosis patients treated with bisphosphonates is many orders of magnitude lower. In ONJ, a section of the maxilla or mandible necroses and becomes exposed to the oral cavity. ONJ is painful and interferes with functioning. It is usually self-limited but can be have a prolonged course in a minority of patients. The mechanism by

which bisphosphonates may cause ONJ is unknown. A recent report from a phase III trial of the RANKL monoclonal antibody (denosumab) found a comparable number of cases of ONJ in patients treated with zoledronate and denosumab (Amgen website, August 3, 2009). This suggests that OCL inhibition in general, rather than specific properties of bisphosphonates, may cause ONJ. Bisphosphonate suppression of bone remodeling is one of the most popular hypotheses for ONJ pathogenesis.[196] Decreased angiogenesis due to bisphonates may also play a role in ONJ. Recently, ONJ was reported in patients treated with the anti-VEGF antibody bevacizumab.[197] Furthermore, a small retrospective study found more cases of ONJ in cancer patients treated with bisphosphonates and bevacizumab than bisphosphonates alone.[198]. ONJ is also linked to dental extraction or other dental surgery, and is associated with actinomyces infection. Because of their ability to inhibit remodeling of the callus of fixed fractures in long bones, bisphosphonates may also contribute to ONJ due to affects on bone healing after dental trauma, such as extraction.[167, 168] The conclusions of this dissertation offer potential insight into the pathogenesis of ONJ.

1.3 POTENTIAL MOLECULAR MECHANISMS OF OSTEOCLAST – STIMULATED ANGIOGENESIS

Macrophages, which share a close lineage relationship with osteoclasts, are well established as pro-angiogenic cells. Angiogenic factors produced by macrophages the regulation of their production provide a template for studies of the potential mechanisms utilized by OCLs to induce angiogenesis. In addition, production of several angiogenic factors has been previously reported in osteoclasts.[6]

1.3.1 Macrophages and Angiogenesis

1.3.1.1 Macrophage Angiogenic Factors

Macrophages express a wide array of angiogenic factors including ;VEGF, FGF-1, FGF-2, PDGF, TNF-α, angiopoietin 1, leptin, IL-6 and IL-8 .[199] [200] Human peripheral blood macrophages promoted endothelial cell proliferation through FGF-1, FGF-2 and PDGF.[7] VEGF was the primary angiogenic factor produced by hypoxic mouse peritoneal macrophages and RAW 264.7 cells in the mouse corneal angiogenesis assay.[8] Neutralizing antibodies to TNF-α inhibited mouse peritoneal macrophage induced angiogenesis in the chick chorioallantoic membrane, rat cornea and BCE capillary tube formation assays.[9] Furthermore, treatment of human peripheral blood macrophage conditioned media with antibodies to IL-8 or TNF-α or IL-8 antisense equally reduced rat corneal angiogenesis.[201] Because several different factors are required for macrophage stimulated angiogenesis, it is not improbable that several different osteoclast derived factors acting directly on endothelial cells will be required for OCLs to stimulate angiogenesis. Further the angiogenic phenotype of macrophages is greatly affected by their microenvironment. For example, conditioned media from activated macrophages stimulates angiogenesis, whereas conditioned media from resting macrophages inhibits endothelial cell proliferation.[202] Therefore, it will be important to determine the role activation of OCLs plays in OCL-stimulated angiogenesis.

In addition to these direct mechanisms, macrophages may stimulate angiogenesis indirectly; by inducing angiogenic factor secretion by another cell type or by activating a latent angiogenic factor. As an example of such an indirect mechanism, MMP-9 produced by macrophages was shown to be important for cervical cancer angiogenesis. The authors

hypothesized that MMP-9 exerted its pro-angiogenic effect through release of matrix bound VEGF and the resultant increased VEGFR2 binding.[13]

1.3.1.2 Tumor Associated Macrophages

Although macrophages might be expected to help the host eradicate tumors, the opposite occurs in most cases.[203] In greater than 80% of clinical studies, increased tumor associated macrophage (TAM) density is associated with a poor prognosis. In contrast, less than 10% of studies show association of TAMs with an improved prognosis.[204] It has been proposed that T_H2 type or anti-inflammatory cytokines such as IL-4, IL-10, TGF-β and IL-13 in the tumor microenvironment induce macrophages into an alternatively activated state, termed M2 macrophages, which lack anti-tumor activity.[205, 206] One of the major ways in which macrophages can stimulate tumor growth is through their angiogenic stimulatory capacity. For example, macrophages were associated with increased microvessel density in human breast tumor samples, and their production of MMP-9 was required for angiogenesis in a mouse cervical cancer model.[13]

Even though TAM's are a good illustration of the angiogenic potential of cells of the monocytic lineage (such as osteoclasts), it seems unlikely that angiogenesis induced by TAM's and osteoclasts will be regulated by identical mechanisms. For example, IL-4, IL-10 and IL-13, which play a role in the induction of the tumor enhancing phenotype of tumor associated macrophages, inhibit osteoclast formation in most experimental systems.[47, 48] However, an examination of how inflammatory cytokines and ischemic condtions, which do not inhibit osteoclast formation, and also regulate macrophage angiogenic factor production, should be useful in predicting ways that osteoclast-induced angiogenesis may be regulated.

1.3.1.3 Regulation of Macrophage Stimulated Angiogenesis By Ischemia

It has been over 25 years since macrophages were first shown to secrete soluble angiogenic factors in response to hypoxia.[207] Since then, there has been a flurry of activity investigating which angiogenic factors are induced by hypoxia in macrophages and the signaling mechanisms that control their production. Early on, lactate, which is associated with ischemic conditions due to anaerobic metabolism, was shown to increase the angiogenic stimulatory capacity of macrophages.[208] Macrophages accumulate in diseased tissues in poorly vascularized sites, thus illustrating their role in the response to ischemia.[199] Principle among the transcription factors shown to play a role in macrophage production of angiogenic factors is the HIF family of transcription factors.[209] Most data suggest that HIF-2α is more important than HIF-1α for the induction of angiogenic genes in macrophages exposed to hypoxia. Hypoxia induced HIF-2α protein to a much greater extent than HIF-1α protein in human monocyte derived macrophages.[210] Using an angiogenic factor cDNA array, White et al showed that overexpression of HIF-2α was much more effective than HIF-1α overexpression at increasing the transcription of pro-angiogenic genes in response to hypoxia.[200]

1.3.1.4 Regulation of Macrophage-Stimulated Angiogenesis by Inflammatory Cytokines and Bacterial Products

Macrophages are unlikely to be exposed to ischemia in the absence of biological factors. In addition to the effects of anti-inflammatory cytokines on tumor associated macrophages, inflammatory cytokines have also been shown to upregulate production of macrophage angiogenic factors. IFN-γ and LPS, well known macrophage activators, can induce angiogenic factor production in macrophages and increase their production of angiogenic factors in response to hypoxia.[199] The expression of some angiogenic factors stimulated by LPS involves platelet

activating factor (PAF) induction of NF-κB. PAF inhibitors and p65 (NF-κB subunit) antisense block NF-κB DNA binding, angiogenic factor production, and mouse matrigel plug angiogenesis induced by mouse peritoneal macrophages treated with LPS. Likewise in human monocytes, PAF antagonists or NF-κB inhibitors reduced LPS-stimulated endothelial cell sprout formation from microcarrier beads as well as monocyte production of VEGF, bFGF, TNF-α, IL-1α and IL-8.[211] LPS also stimulated VEGF release from murine macrophages by signaling through TLR4 and synergergizes with adenosing receptor A_{2A}.[212] TLRs 2, 7 and 9 also synergize with adenosine A_{2A} receptors.[213] M-CSF likewise stimulates secretion of VEGF in monocyte-derived macrophages, possibly through HIF-1α, as HIF-1α protein and DNA binding is increased by M-CSF treatment.[214] TGF-β, although usually classified as an anti-inflammatory cytokine, has also been shown to induce macrophage angiogenic factor production. TGF- β1 was required for head and neck cancer cell induction of VEGF and IL-8 in macrophages.[215] Overexpression of smad 3/4 or HIF-1α increased the induction of VEGF in mouse macrophages.[216]

1.3.2 Possible Osteoclast-Derived Angiogenic Factors

1.3.2.1 Osteopontin

Osteopontin (OPN) is released by myeloma cells [147] and osteoclasts [5] and was proposed to mediate OCL-stimulated angiogenesis. Experiments with $OPN^{-/-}$ mice demonstrated reduced angiogenesis in fracture healing experiments as well as reduced angiogenesis and osteoclastogenesis when bone chips were implanted subcutaneously in mice.[171, 217] OPN is expressed by OCLs, osteoblasts and endothelial cells and is a very abundant protein in bone that comprises 2% of the non-collagenous protein.[122, 218]

Osteopontin was proposed to stimulate angiogenesis by signaling through integrins on endothelial cells, principally αvβ3. Osteopontin prevents endothelial cell apoptosis through binding to αvβ3 and inducing NF-κB activation through a pathway that requires ras and src .[219] Osteopontin's prevention of endothelial cell apoptosis was further shown to involve stimulation of osteoprotegrin expression, which prevents TRAIL mediated endothelial cell apoptosis.[220] Osteopontin also stimulates adhesion and migration of endothelial cells.[221, 222] Osteopontin and VEGF cooperatively stimulate endothelial cell migration. Pretreatment of endothelial cells with VEGF increases cell migration in response to osteopontin and induces endothelial cell expression of osteopontin and αvβ3. VEGF also increases thrombin cleavage of osteopontin *in vivo*. Thrombin cleaved osteopontin stimulates endothelial cell migration better than full length osteopontin *in vitro*.[222] Osteopontin also has effects on OCLs and other cell types.

Osteopontin$^{-/-}$ mice were originally reported to have no changes in bone density, but to have increased OCL formation *in vitro*.[223] However, later studies have found a subtle increase in bone mass and increased OCL numbers *in vivo* as well as decreased OCL motility and resorption *in vitro*.[224] Thus, OPN seems to be required for the function of OCLs rather than their formation. This reduced functionality of OPN$^{-/-}$ OCLs was recently illustrated by the reduced size of their resorption space.[225] In addition to reducing the functionality of OCLs, OPN is required for the induction of bone loss in some pathological conditions. Osteopontin$^{-/-}$ mice lose less bone mass relative to controls with ovariectomy or PTH treatment.[226, 227] Furthermore, the increase in OCL number in response to PTH was blunted in OPN$^{-/-}$ mice. Implantation of breast cancer cells induced more bone loss in OPN$^{-/-}$ mice compared to wt, showing that OPN is not required for increased bone loss in all circumstances.[228]

1.3.2.2 IL-8 and Other CXC Chemokines

Different members of the CXC chemokine family can either stimulate or inhibit angiogenesis. CXC chemokines that have the sequence; glutamate, leucine, arginine immediately N-terminal to the first cysteine residue (ELR^+) are pro-angiogenic.[229] The pro-angiogenic effects of ELR^+ CXC chemokines are thought to be due to signaling through CXCR2.[230] CXC chemokines that lack the ELR motif are anti-angiogenic, and exert these effects primarily through isoforms of CXCR3.[231-234] ELR^+ CXC chemokines are secreted by several different cell types in bone marrow. Osteoclasts were reported to produce IL-8, but not ENA-78 (CXCL5), GRO-α (CXCL1), GRO-β (CXCL2), GRO-γ (CXCL3) PBP, CTAP-III, β-TG or NAP-2 (CXCL7).[121] In addition to CXCR2, IL-8 also stimulates angiogenesis through CXCR1. The relative contribution of CXCR1 and CXCR2 to IL-8 induced angiogenesis varies depending on the experimental system used, although it has been hypothesized that CXCR2 is more important.[235] CXCR2 blockade abolished angiogenesis induced by pancreatic cancer cells.[236] Blockade of CXCR1 or CXCR2 alone inhibited migration of human glioblastoma endothelial cells, and the combination almost completely inhibited their migration, which was due to autocrine IL-8.[237] IL-8 stimulates angiogenesis through stimulation of endothelial cell proliferation, apoptosis and production of MMPs.[238] These effects can also be mediated through endothelial cell autocrine IL-8.[239]

Like osteopontin, IL-8 production is increased by inflammatory mediators including IL-1, IL-6, TNF-α, and LPS, as well as reactive oxygen species and hypoxia.[235, 240, 241] IL-8 expression is regulated both transcriptionally and at the level of mRNA stability by pathways involving JNK, IKK and p38 kinases. The IL-8 promoter has at least 3 transcription factor binding sites; AP-1, NF-κB, and C-EBP/NF-IL-6.[235] Initial studies on hypoxic regulation of

IL-8 expression did not detect a hypoxia –responsive element in the IL-8 promoter and found that IL-8 hypoxic upregulation was primarily due to AP-1.[241] More recently, studies have shown that the IL-8 promoter does contain a site that can serve as a hypoxia-responsive element and that HIF-1α contributes to IL-8 regulation.[242] IL-8 promotes OCL formation both by direct effects on the OCL and through upregulation of RANKL in stromal cells.[243, 244]

1.3.2.3 MMP-9

OCLs may also stimulate angiogenesis by release of proteinases, which can stimulated angiogenesis in many ways including; basement membrane degradation, less pericyte attachement, increased integrin signaling, or release of angiogenic stimulators (or inhibitors) from matrix.[245] Such an enzymatic mechanism for osteoclast-stimulated angiogenesis may be more feasible than osteoclast-secreted angiogenic factors acting directly on endothelial cells, because OCLs are rare cells in bone. Thus, proteinases could multiply the pro-angiogenic effects of OCLs. An obvious candidate for this mechanism of action is MMP-9. A possible role for OCL-derived MMP-9 in angiogenesis in development or fracture healing was briefly described earlier. MMP-9 is a matrix proteinase, classically known to degrade denatured collagen (gelatin), but which also has other substrate specificities.[245] Early studies showed that osteoclasts express high levels of MMP-9. It was originally thought that MMP-9 was specifically expressed by osteoclasts or committed osteoclast precursors in bone.[246, 247] However, MMP-9 can also be expressed by other cell types such as hypertrophic chrondrocytes or osteoblasts during development, or macrophages and neutrophils expecially during fracture healing.[164, 248, 249]

Observations of MMP-9$^{-/-}$ mice suggested that osteoclasts may play a role in angiogenesis.[14] Yet, this possibility has remained unexplored. MMP-9$^{-/-}$ mice are viable but

show a bone phenotype consisting of delayed endochondral ossification and delayed blood vessel invasion into the metaphyseal growth plate center. In this same report, the authors reported that op/op and c-fos$^{-/-}$ osteopetrotic mice showed a milder growth plate defect than the MMP-9$^{-/-}$ mice but did not show the data. The initial study of the MMP-9$^{-/-}$ mouse found a similar pattern of expression of MMP-9 and the OCL marker TRAP in growth plates, but some cells at the chondro-osseous expressed MMP-9 but not TRAP or the endothelial cell marker CD31.[14] A later study failed to show a defect in endochondral ossification in the same osteopetrotic (c-fos$^{-/-}$ and op/op) mice as well as in clodronate treated mice.[4] However, this report examined mouse tail vertebrae, rather than the growth plate of long bones that were examined in studies of MMP-9$^{-/-}$ mice. Differences in the relative importance of the OCL to angiogenesis at various angiogenic sites may exist. In agreement with this, the MMP-9$^{-/-}$ mouse has no ossification or vessel invasion defect in the epiphyses of long bones.

Several investigations have reported that MMP-9 stimulates angiogenesis through activation or release of growth factors. MMP-9 was first implicated as the angiogenic switch in a mouse pancreatic cancer model in which MMP-9 released matrix (heparan) associated VEGF and increased VEGF receptor occupancy. The MMP-9 expressing cells in this study were not tumor cells but rather appeared to be infiltrating immune cells.[12] Likewise, infiltrating zoledronate-sensitive macrophages were implicated in cervical cancer angiogenesis by way of VEGF release.[13] MMP-9 release of matrix bound VEGF was suggested to be important for angiogenesis in the long bone growth plate.[161] MMP-9 can also to act upon other pro and anti-angiogenic factors. MMP-9 increases the activity of the pro-angiogenic molecule IL-8 on neutrophil activation, although angiogenesis was not examined.[250] Furthermore, the pro-angiogenic molecule GRO-α as well as the angiogenesis inhibitor PF-4 were degraded and

inactivated by MMP-9.[250] In addition, MMP-9 activates TGF-β, which stimulates angiogenesis under most circumstances.[251, 252] Osteoclasts have long been hypothesized to release TGF- β from bone matrix. MMP-9 and MMP-2 can activate TGF- β through cleavage of latent TGF- β binding protein – 1.[11] Thus, OCL-derived MMP-9 may increase the activity of TGF-β released from bone matrix by OCLs.

MMP-9 affects OCL migration, which may be important for their ability to stimulate angiogenesis. As discussed earlier, MMP-9 release of matrix bound VEGF is important for OCL and vessel invasion into the primary ossification center.[161] In this study, resorption of E17 metatarsal explants but not more mature bones was inhibited by lack of MMP-9. This result was explanined by the MMP-$9^{-/-}$ migratory defect rather than by an effect on solubilization of calcified matrix. At this stage, OCLs must invade from the periosteum in order to resorb calcified matrix. In agreement with these data, an *in vitro* study found that lack of MMP-9 inhibited OCL migration trough matrigel to underlying bone slices, and resulted in reduced resorption of due to the migratory effects.[253] Furthermore, MMP-9 is important for migration of macrophage cell lines capable of forming OCLs.[254, 255] Because of this importance for migration, OCL-derived MMP-9 may stimulate angiogenesis indirectly, by increasing the number of OCL at the angiogenic site. **Therefore, osteoclast-derived MMP-9 may stimulate angiogenesis by at least three different mechanisms; activation of VEGF or TGF-β, or affecting OCL recruitment to the angiogenic site**.

1.3.2.4 Other Proteinases

In addition to MMP-9, several families of proteinases; MMPs, ADAMs and serine proteinases are involved in angiogenesis, many of which are expressed by OCLs (Reviewed in [256]). Many of the well studied angiogenic MMPs are expressed by OCLs, including MMP-9,

MMP-7, MMP-2 and MT1-MMP.[248, 257] Of note, MT1-MMP (MMP-14)$^{-/-}$ mice have a similar, but more severe, phenotype than MMP-9$^{-/-}$ mice, including defective endochondral ossification and vessel invasion, and inhibition of both axial and craniofacial skeletal growth.[258] A recent report showed that MMP-7 was predominantly expressed by OCLs and important for growth of breast cancer bone metastases.[257] However, angiogenesis was not analyzed in this report. MMP-7 may stimulate angiogenesis by several possible mechanisms, including stimulating endothelial cell migration or releasing EGF or VEGF from matrix.[245, 259, 260]

The ADAM family of proteinases is similar in structure to the MMPs and also affect angiogenesis by similar general mechanisms. Many ADAMs, including ADAMs 8, 9, 10, 15, 17 and 28, are expressed by OCLs.[261] ADAM-17 (TACE) is especially well studied in angiogenesis due to it's ability to release membrane bound TNF-α.[256] Of the serine proteinases, plasmin and its regulators u-PA, t-PA and PAIs are of primary importance to angiogenesis. Plasmin is best known for fibrinolysis, but also affects angiogenesis by activating other proteinases.[256] Activation of proteinases relevant to angiogenesis involves complex pathways with much cross talk between various kinds of proteinases. A well known cascade of proteinase activation controls the activity of MT1-MMP (MMP-14), MMP-2, MMP-13 and MMP-9. MT1-MMP is activated by the serine proteinases furin and PC6.[262] It in turn activates pro-MMP-2 and pro-MMP-13.[263, 264] MMP-2 activity can be inhibited by β1 and αvβ3 integrin ligation.[265] Both MMP-2 and MMP-13 are capable of activating MMP-9.[266, 267]

1.3.2.5 TGF-β

A particularly well-studied molecule that may be released by OCLs from calcified bone matrix, resulting in angiogenesis stimulation, is TGF-β. Bone matrix is a rich source of growth factors and contains factors such as PDGF, aFGF, bFGF and TGF-β.[268, 269] TGF-β has received an enormous amount of attention in the past two decades, as the key component in the "vicious cycle" hypothesis in which metastatic tumor cells stimulate OCLs, that release TGF-β from bone matrix to further stimulate tumor growth.[109] OCL-released TGF-β may act on vessels directly, primarily on pericytes and mural cells as described above.[96] Alternatively, TGF-β may stimulate angiogenesis through induced angiogenic factor expression in other cells. TGF-β as well as bone morphogenetic proteins have been shown to induce VEGF expression in osteoblasts.[108, 111] The role of osteoblastic VEGF in angiogenesis in bone is well demonstrated.[15]

Although the concept of osteoclastic release of TGF-β from calcified matrix has become dogma in bone biology and inspired promising experimental treatment approaches, the biochemical evidence that TGF-β comes directly from calcified matrix is limited.[10, 11, 270] Regardless of the exact source, TGF- β is associated with increased osteoclastogenesis and has important roles for bone biology.[109]

The literature review above provides the necessary background information and rationale for the following studies. Although some have described situations where angiogenesis does not require OCLs, we have identified models where OCLs do play a role, and are the first to show that OCLs are important for angiogenesis *in vivo*. We are confident that future studies will describe additional ways in which OCLs contribute to angiogenesis. The data in this dissertation

firstly demonstrates that OCLs can play a role in angiogenesis and then show that MMP-9 affects OCL-stimulated angiogenesis primarily through allowing OCLs to migrate to the angiogenic site. These studies allowed us to develop the following model of OCL-stimulated angiogenesis angiogenesis (Figure 1.5).

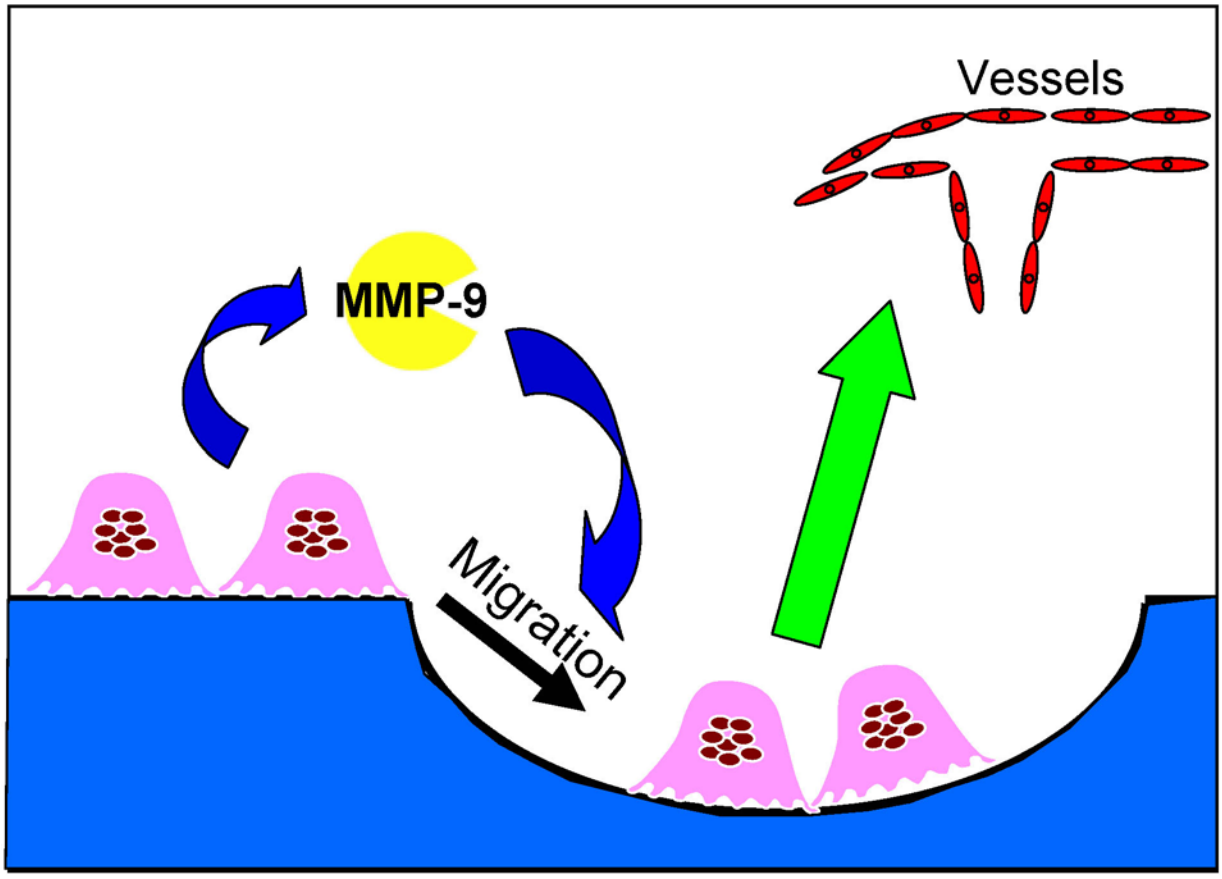


Figure 1.5. Model of osteoclast-stimulated angiogenesis

2.0 OSTEOCLASTS CONTRIBUTE TO ANGIOGENESIS

2.1 SUMMARY

Osteoclastogenesis is correlated with angiogenesis in biological processes such as development, fracture healing and inflammatory and malignant bone diseases. However it is unclear if OCLs play a causal role in angiogenesis. Other investigators previous or concurrent with these studies reported conflicting data on a requirement for OCLs in angiogenesis. To determine if OCLs are important for angiogenesis, we examined their contribution to the process in three model systems. Firstly, we examined the ability of conditioned media from human bone marrow OCLs to stimulate angiogenesis in the HUVEC / fibroblast co-culture angiogenesis assay. To determine if OCLs stimulated angiogenesis in a bone microenvironment *in vitro*, we employed the fetal mouse metatarsal angiogenesis assay. This assay employs bone explants from fetal mice, which contain bone cell types including OCLs, osteoblasts, chondrocytes, fibroblasts and endothelial cells, to permit testing OCL modulators for their effects on angiogenesis. We examined the effect on OCL-stimulated angiogenesis *in vivo* after 5 day RANKL or PTHrP (1-34) treatment of mice by histological evaluation of fixed frozen sections stained for the OCL marker TRAP and immunohistochemical staining of vessels (CD31) in calvaria. Human bone marrow OCL conditioned media stimulated *in vitro* angiogenesis at a comparable level to other bone marrow cell types known to stimulate angiogenesis. OCL inhibition with osteoprotegerin

decreased angiogenesis and OCL formation and activity in parallel in metatarsal explants. Conversely, continuous PTHrP (1-34) increased both area covered by endothelial cells and osteoclastic resorption (CTX) in metatarsal explants. This ability of PTHrP to stimulate metatarsal angiogenesis disappeared with osteoprotegerin treatment, and PTHrP did not induce angiogenesis in metatarsals from OCL deficient op/op mice, thus showing that the angiogenic effect of PTHrP requires OCLs. Furthermore, *in vivo* studies demonstrated that OCL stimulation with 5 days of RANKL or PTHrP treatment increased vessel density in mouse calvaria. Total CD31+ vessel area was dramatically increased between the bone tables in PTHrP compared to vehicle treated animals, with most of the effect due to vessel number. RANKL increased vessel density and area in the outer table of calvaria *in vivo*. Thus, by demonstrating OCL stimulation of angiogenesis in two *in vitro* models

2.2 INTRODUCTION

Both osteoclastogenesis and angiogenesis are enhanced in pathological conditions, such as multiple myeloma, bone metastases and rheumatoid arthritis, which are associated with locally increased inflammatory cytokines.[2, 137] Osteoclasts (OCLs) and blood vessels are closely associated, with a vessel present at every bone remodeling compartment.[107] However, few studies have examined if and how OCLs may play a role in angiogenesis. OCL conditioned media has been reported to be angiogenic *in vitro* and this angiogenic activity was attributed to secretion of osteopontin by OCLs, which was increased when OCLs were co-cultured with multiple myeloma cells.[5, 150] However, other investigators reported that OCLs were not

required for angiogenesis. Further, osteopetrotic (lacking OCLs) genotypes (op/op or c-fos $^{-/-}$) or clodronate treatment of wild type mice did not inhibit developmental vessel invasion into mouse caudal vertebrae.[4] Likewise, op/op mice have normal levels of vessel invasion in their epiphyses.[149] Therefore, we chose to investigate whether OCLs are important for angiogenesis

2.3 MATERIALS AND METHODS

Materials: Monoclonal rat anti-mouse CD31 was purchased from BD-Pharmingen (#550274). Vector Laboratories supplied biotinylated rabbit anti-rat (mouse adsorbed) and SA-HRP. The RatLaps C-terminal type I collagen telopeptide (CTX) assay was obtained from Nordic Biosciences / Immunodiagnostic Systems, and the CryoJane sectioning aid and adhesive slides from Instrumedics – Leica. Human *in vitro* angiogenesis assays were purchased from TCS Cellworks. rh OPG-Fc, rh RANKL and rh M-CSF were from R&D Systems. Recombinant mouse RANKL-GST was generously provided by Dr. F. Patrick Ross (Washington Univ, St. Louis). h PTHrP (1-34) was purchased from American Peptide. The 23c6 monoclonal antibody, which identifies αvβ3 integrin, cell culture supernatant was provided by Dr. Michael Horton, University College, London, UK. The BCA protein assay was from Pierce. ImmunoHistoMount aqueous mounting media and other chemicals or supplies were purchased from Santa Cruz, Sigma-Aldrich, Fisher Scientific, Electron Microscopy Sciences or Gibco. C57BL/6 mice were from Harlan.

Human OCL culture and analysis of angiogenic activity of conditioned media in vitro: After obtaining informed consent, bone marrow aspirates were collected in heparinized syringes from normal donors. These studies were approved by the University of Pittsburgh Institutional Review Board. Marrow mononuclear cells were separated by density sedimentation on ficoll-hypaque. 10^7 cells were incubated overnight in 10% FCS αMEM in 10 cm culture dishes. Non-adherent cells were removed by gentle washing. Adherent cells were cultured in Dexter-type cultures for 21 days in 10% FCS IMDM with twice weekly media changes to obtain marrow stromal cells.[272] For osteoclast or macrophage culture, non-adherent cells were diluted to 10^6 / mL in α-MEM with 20% horse serum, 10 ng/mL rh M-CSF with or without 50 ng/mL rh RANKL and seeded at 30 mL per T-75 flask. Cultures were maintained for 3 weeks with twice weekly changes of half the media volume containing a full complement of cytokines, as previously described.[19] Osteoclast cultures were trypsinized for 5 minutes at 37°C to remove contaminating cell types, then rested for 24 hours, followed by RNA or conditioned media collection. Serum free conditioned media was collected by washing the cultures three times with α-MEM, adding back 7.5 mL of α-MEM per flask and then incubating the cells for 24 hours at 37°C. Cultures were fixed and stained for $\alpha_v\beta_3$ integrin with the 23c6 monoclonal antibody that identifies OCLs and Vector ABC-AP kit. Conditioned media or control media was concentrated 10 fold on Amicon 3 KD cut-off concentration columns that were pre-blocked with 1% BSA, then diluted back so that at final concentration CM from 30,000 OCLs per ml was added to the TCS Cellworks *in vitro* angiogenesis assay. Co-cultures of human umbilical vein endothelial cells (HUVECs) and fibroblasts was performed per manufacturer's protocol.[273] CM was diluted 1:2 to provided assay media. After 11 days, cultures were stained for CD31.

Cultures were photographed with an Olympus multimode dissecting microscope and quantified for total $CD31^+$ area with ImageJ and for angiogenic tube formation with Metamorph software.

Fetal Mouse Metatarsal Angiogenesis Assay: The assay was conducted as originally described with minor modifications.[17] Briefly, embryos were harvested from CB6 F1 x CD-1 (outbred), C57BL/6 WT or C57BL/6 MMP-$9^{-/-}$ pregnant female mice that were sacrificed with an excess of isofluorane anesthesia at 17.5 dpc. The middle three metatarsals were dissected from each hind leg and cultured in 24 well plates containing 10% fetal calf serum α–mimimal essential media with the indicated treatments for 15 days. At least 10 bones were used per group. Media volume was maintained at 150 µl for the first three days and 250 µl subsequently. Media was replaced every three days and spent media was stored at -80°C for measurement of CTX (RatLaps™, Nordic Biosciences). Media from freeze-thawed bones was used as a blank for CTX measurements. Explants were then stained for CD31 as follows: Bones were fixed for 15 minutes with zinc macrodex formalin, washed twice with PBS and blocked overnight at 4°C in PBS containing 2% rabbit serum, 0.1% triton X-100, 0.05% tween-20, 1% BSA, 0.1% gelatin and 0.05% sodium azide. All PBS buffers were at pH 7.2. The primary antibody was applied at a dilution of 1:50 in PBS plus 1% BSA and 0.1% gelatin. Secondary antibody and SA-HRP (Vector Laboratories) were applied in PBS at 1:100 or 1:250 dilution respectively, and explants were stained with AEC-HRP substrate. Images were acquired with an Olympus Multimode dissecting microscope and were quantified either with the Metamorph™ angiogenesis tube formation application for tube area, length and branches, or with ImageJ for total $CD31^+$ area and corrected for the area of bones stained with control IgG in place of primary antibody.

TRAP activity was extracted from metatarsal explants that were fixed and stained for CD31 and stored dry. Bones were rehydrated in water, removed and homogenized with a 2 mL ground glass homogenizer in 120 µl NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM tris pH 8), then rotated at 4^{o}C overnight and cleared by centrifugation. To assay TRAP activity, 35 µl of homogenate was added to 200 µl TRAP substrate (50 mM sodium acetate pH 5, 25 mM sodium tartrate, 0.4 mM $MnCl_2$, 0.4% N,N,-dimethylformamide, 0.2 mg/mL fast red violet, 0.5 mg/mL napthol AS-MX phosphate), then incubated 3 hours at 37^{o}C and the absorbance read at 540 nm. Results were corrected for total protein, which was assayed by adding 25 µl homogenate to 250 µl BCA assay reagent, incubating 2 hrs at 37^{o}C and reading the absorbance at 562 nm.

Histology and Immunohistochemistry: Histological analyses were performed in fixed-frozen decalcified tissues from mice. Bones were fixed overnight in 2% paraformaldehyde, decalcified for 4 days in 10% EDTA pH 7.4, imbedded in 30% sucrose in PBS overnight and snap frozen in OCT by immersion in LN_2 cooled isopentane. Seven µm sections were cut on a cryostat equipped with a CryoJane tape-transfer system. Slides were stored frozen until use, and then thawed and washed 3 times in PBS. For immunohistochemistry, slides were peroxidase quenched in 0.3% H_2O_2 in MeOH, washed 3x for 5 minutes in PBST (0.05% Tween-20), then blocked for 30 minutes in 5% serum from the same species from which the secondary antibody was derived. Primary antibodies were applied overnight at 4^{o}C in PBS at the following dilutions; anti-CD31 (1:100), anti-MMP-9 (1:4000), followed by washes in PBST, and then biotinylated secondary antibodies (anti-rat 1:100, anti-rabbit 1:2500 dilutions), SA-HRP (1:250 dilution) and DAB peroxidase substrate were added. TRAP histochemistry was performed with a substrate

solution containing 50 mM sodium acetate pH 5, 25 mM sodium tartrate, 0.4 mM $MnCl_2$, 0.4% N,N,-dimethylformamide, 0.2 mg/mL fast red violet, 0.5 mg/mL napthol AS-BI phosphate as described previously.[274] Slides were coverslipped in aqueous mounting media.

In vivo studies: Six (2 male and 4 female) C57BL/6 7 week old mice per group were injected with m-RANKL-GST (1 mg/kg in 50 - 65 µl PBS) subcutaneously over the calvaria daily for 5 days under light isoflurane anesthesia. Similarly, for studies involving PTHrP, six 5 week old male C57BL/6 were treated with 2 µg h PTHrP (1-34) in 100 µl 1% BSA-PBS pH 5.2 systemically by subcutaneous injection 4 times a day for 5 days.[51, 275] Control mice were injected with vehicle. After 5 days, blood was collected by retro-orbital puncture and mice were sacrificed. Calvaria were sectioned in a coronal orientation anterior to the junction of the sagittal and coronal sutures, and stained for CD31, TRAP or MMP-9. All quantitative histological analyses were performed by a blinded observer. For PTHrP treated mice, $CD31^+$ vessels were quantified in the inner table adjacent to the midline of 10x objective images by selecting the DAB signal with a hue saturation intensity filter (Fovea Pro software, Reindeer Graphics) selected to limit to true signal from 45 to 90° area running within Adobe Photoshop 7. For PTHrP treated mice between bone tables (parasagittal spaces), or RANKL treated mice in the outer table, $CD31^+$ vessels were quantified by counting vessel numbers and using a grid map projected with ImagePro software to determine total area covered by vessels (including lumens). Average vessel size was calculated from total area and vessel density. OCLs were quantified in PTHrP treated bones by calculating $TRAP^+$ area as for DAB using Fovea Pro as above selecting between 45-89°. OCLs were quantified in RANKL treated calvaria calculating the $TRAP^+$ (resorptive) surface of the sub-periosteal surface of the calvarial outer table by grid map

counting, as well as $TRAP^+$ area. All animal protocols were approved by the IACUC of VA Pittsburgh Healthcare System, University of Pittsburgh, or Virginia Commonwealth University.

Statistical Analyses: The unpaired Student's t-tests or one way ANOVA with LSD procedure were used for analyzing 2 or multiple groups respectively. To analyze correlation, the Pearson correlation coefficient was calculated by linear regression, and the 1 sample F-test for a correlation coefficient was used to test for significance. Two-tailed analyses were performed with SPSS software. Significance was set at $\alpha = 0.05$.

2.4 RESULTS

2.4.1 OCL Conditioned Media Stimulates Angiogenesis *in vitro* to a Similar Extent as Other Bone Marrow Angiogenic Cell Types

We developed a model system where the angiogenic activity of OCL conditioned media (CM) could be assayed with co-cultures of HUVECs and fibroblasts. Several initial dose finding studies determined that OCL CM had to be concentrated to observe an effect. As shown in Figure 2.1 (A), the angiogenic effect of OCL CM was dose responsive but plateaued. Based on these dose response studies, we concentrated CM adequately so that the media in the angiogenesis assay contained the secretions of 30,000 OCLs per ml. Because of the need to concentrate conditioned media and because of interfering effects of serum used in OCL cultures, CM was collected under serum free conditions.

To compare the angiogenic capacity of OCLs to other bone marrow cell types known to stimulate angiogenesis, we compared the activity of OCL, macrophage or bone marrow stromal cell conditioned media collected from equivalent numbers of cells. The angiogenic capacity of OCLs was similar to these other cell types (Figure 2.1 B).

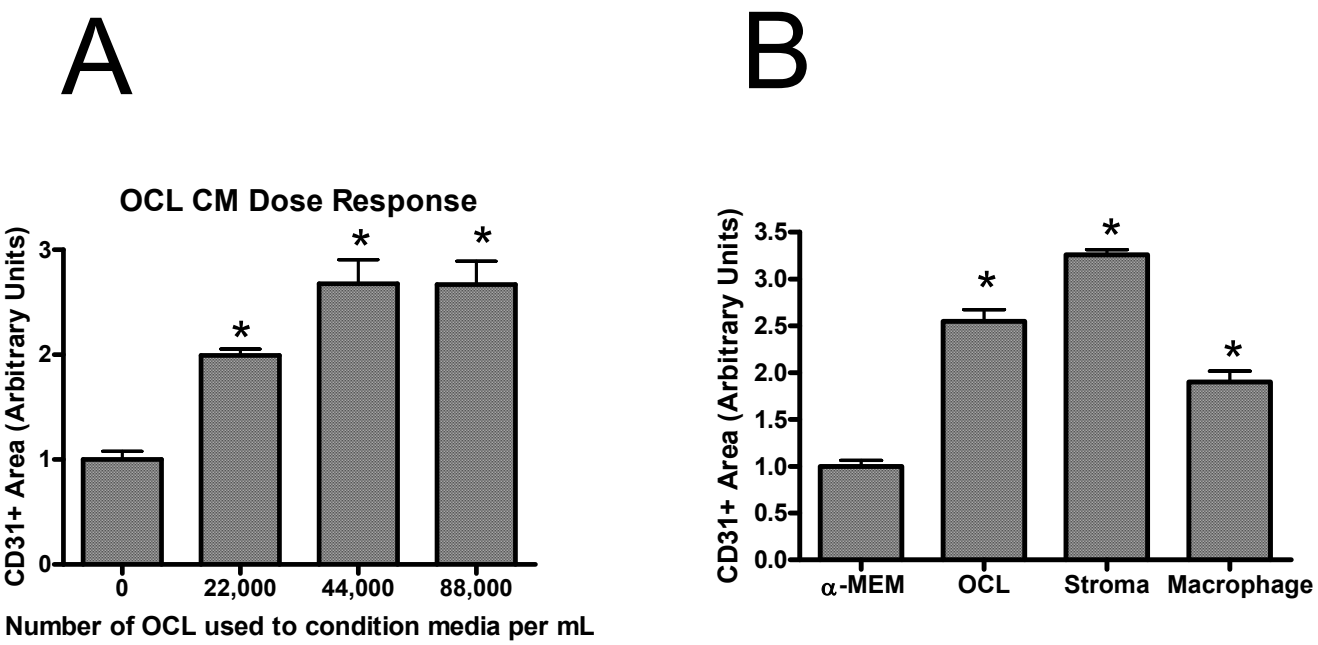
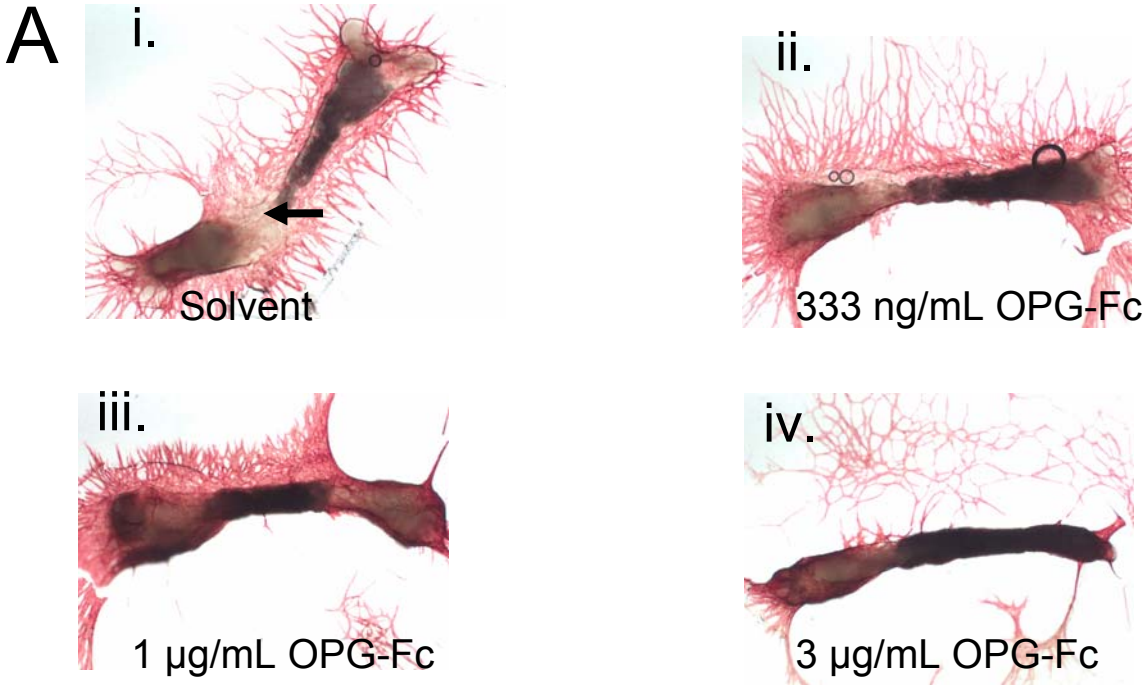


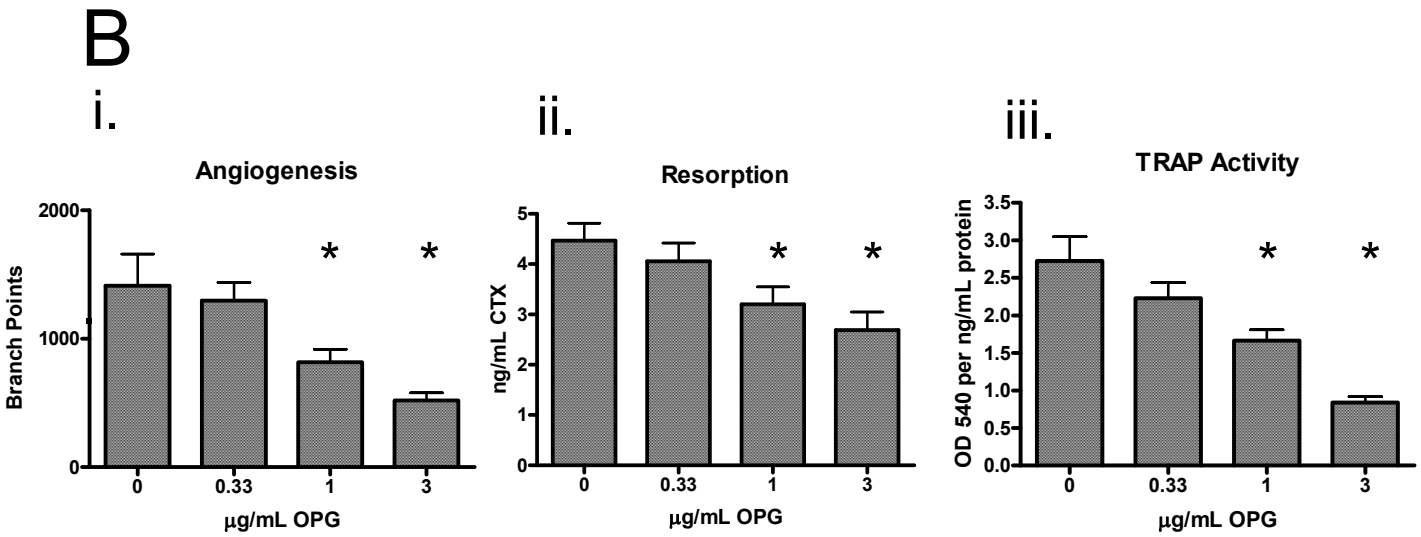
Figure 2.1. Osteoclast conditioned media stimulates angiogenesis *in vitro*.

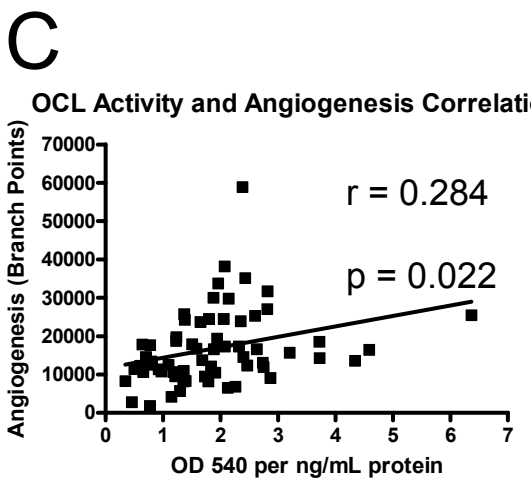
(A) OCL CM stimulates angiogenesis in the HUVEC / fibroblast co-culture angiogenesis assay. Serum free conditioned media was collected from human BM OCL cultures and concentrated 5.6 fold, then diluted out to yield the working concentrations of products of indicated number of OCL / ml assay media. (B) OCL CM stimulates angiogenesis by comparable amounts to other angiogenic bone marrow cell types on a per-cell basis. Conditoned media was concentrated so that products of 30,000 OCL per ml and products of equivalent total cell numbers for each cell type were added to the HUVEC / fibroblast co-culture angiogenesis assay.

2.4.2 OCL inhibition decreases angiogenesis in metatarsal explants

After having shown that OCL CM is angiogenic, we wanted to determine if OCLs can stimulate angiogenesis under more physiological circumstances. To determine if OCLs also contribute to angiogenesis in a more physiologic model for bone than purified cell cultures, we determined the effects of modulating OCL number and activity on angiogenesis in the fetal mouse metatarsal assay. In this assay, metatarsals from E17.5 mice are cultured *in vitro* in the presence or absence of cytokines or hormones. At this developmental stage, the primary ossification center is formed but not yet invaded by OCL precursors, which are in the periosteum. Endothelial cells form tubes in a mixed cellular outgrowth during culture.[17] This assay has been used to analyze the effects of osteoblast specific gene knockouts on angiogenesis.[15] As shown in Figure 2.2 A, inhibition of OCL formation with osteoprotegerin (OPG) reduced angiogenesis in a dose-dependent manner, as measured by staining endothelial cells with CD31 and quantitative image analysis of angiogenic tube formation. To verify that OPG inhibited OCL formation and activity, we measured type I collagen (bone collagen) C-terminal telopeptide (CTX) levels in the conditioned media (CM) or activity of TRAP (OCL marker) extracted from the bone explants treated with OPG (Figure 2.2 B). There was a parallel decrease in angiogenesis, CTX concentration, and TRAP activity. To verify that OPG was not toxic to endothelial cells, we treated the HUVEC / fibroblast co-culture angiogenesis assay, which does not contain OCLs, with equivalent doses of OPG and observed a slight increase in angiogenesis (2.2 D). rather than any decrease due to possible toxitity. Direct stimulatory effects of OPG on endothelial cell have been previously observed.[135] Therefore, the metatarsal studies may have slightly underestimated the importance for OCLs to angiogenesis.


A
i.
Solvent
ii.
333 ng/mL OPG-Fc
iii.
1 µg/mL OPG-Fc
iv.
3 µg/mL OPG-Fc


B
i.
Angiogenesis
Branch Points
2000
1000
0
0
0.33
1
3
µg/mL OPG
ii.
Resorption
ng/mL CTX
5
4
3
2
1
0
0
0.33
1
3
µg/mL OPG
iii.
TRAP Activity
OD 540 per ng/mL protein
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.33
1
3
µg/mL OPG


C
OCL Activity and Angiogenesis Correlation
Angiogenesis (Branch Points)
70000
60000
50000
40000
30000
20000
10000
0
r = 0.284
p = 0.022
0
1
2
3
4
5
6
7
OD 540 per ng/mL protein

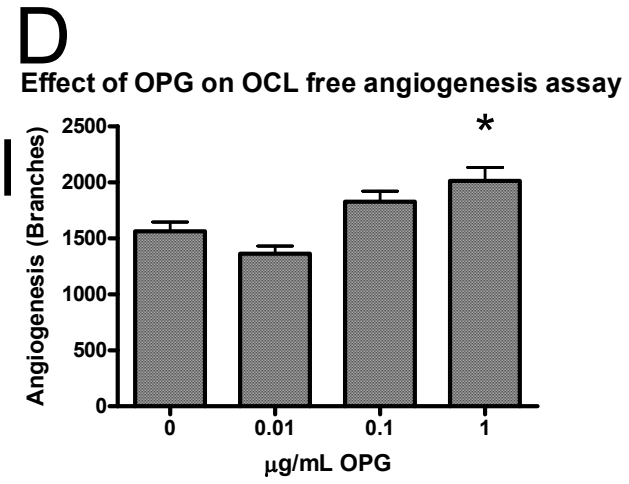

D
Effect of OPG on OCL free angiogenesis assay
Angiogenesis (Branches)
2500
2000
1500
1000
500
0
0
0.01
0.1
1
µg/mL OPG

Figure 2.2. Osteoclast inhibition with osteoprotegerin decreases angiogenesis in fetal metatarsal explants.

(A) Metatarsal explants stained for endothelial cells (Red, CD31). 17.5 ED outbred fetal mouse metatarsals were cultured with indicated treatments for 15 days before fixation: (i) control, (ii) 333 ng/mL, (iii) 1 μg/mL, (iv) 3 μ/mL rh OPG-Fc. Arrow: area of osteoclastic resorption which is prominent in the control bones and decreases with increasing OPG. (B) Quantitation of angiogenic outgrowth and OCL number and activity in metatarsal explants. (i) Number of branches and other angiogenesis tube formation parameters quantified at end of the assay period (15 days). (ii) CTX (RatLaps) assayed from metatarsal explant conditioned media collected from days 7 to 9 of culture. (iii) TRAP activity extracted by homogenization of bones after 15 days of culture and assayed by color development using a TRAP substrate. Mean ± SEM. *: $p < 0.05$. Images aquired as whole mounts in water with an Olympus IX71 microscope with a UPlanFLN objective, NA of 0.13 and a Spot RTKE camera with Spot Advanced software. Original magnification x 4. (C) Correlation of OCL activity and angiogenesis. The TRAP activity extracted from bone explants and angiogenesis (branch points) was correlated for all samples treated with varying concentrations of OPG-Fc, and analyzed by linear regression. r: Pearson correlation coefficient, p: significance by 1 sample F-test for linear regression. (D) Lack of anti-angiogenic effect in the OCL free HUVEC / fibroblast co-culture angiogenesis assay. The assay was treated with the indicated concentrations of rh OPG-Fc and quantified with Metamorph software for angiogenic tube formation. Mean ± SEM. *: $p < 0.05$.

2.4.3 Osteoclast Stimulation Increases Angiogenesis in Metatarsal Explants

Having shown that OCL inhibition in metatarsal explants decreased angiogenesis, we next investigated if angiogenesis was increased by OCL stimulation. As shown in Figure 2.3 A, stimulation of OCL formation with PTHrP, which stimulates osteoclastogenesis primarily through increased RANKL expression on osteoblasts, increased the area of $CD31^+$ endothelial cells in metatarsal explant cultures. Because PTHrP can have direct effects on osteoblast differentiation or survival, we also treated the explants with OPG to determine if the angiogenic effect of PTHrP required OCLs. PTHrP failed to stimulate angiogenesis in the presence of OPG (Figure 2.3 A (iv)). OCL stimulation and inhibition did not simply have opposite effects on explant angiogenesis, but rather had differing effects on the morphology of the endothelial cell outgrowth. As shown in figure 2.3 B (i), PTHrP increased $CD31^+$ area 1.5 fold due to increased density of endothelial cells adjacent to the bone. However, parameters of endothelial tube formation, such as number of branch points, which were inhibited by OPG, were not increased by PTHrP treatment (Figure 2.3 B (ii)). The reasons for these contrasting effects on endothelial morphology are under investigation.

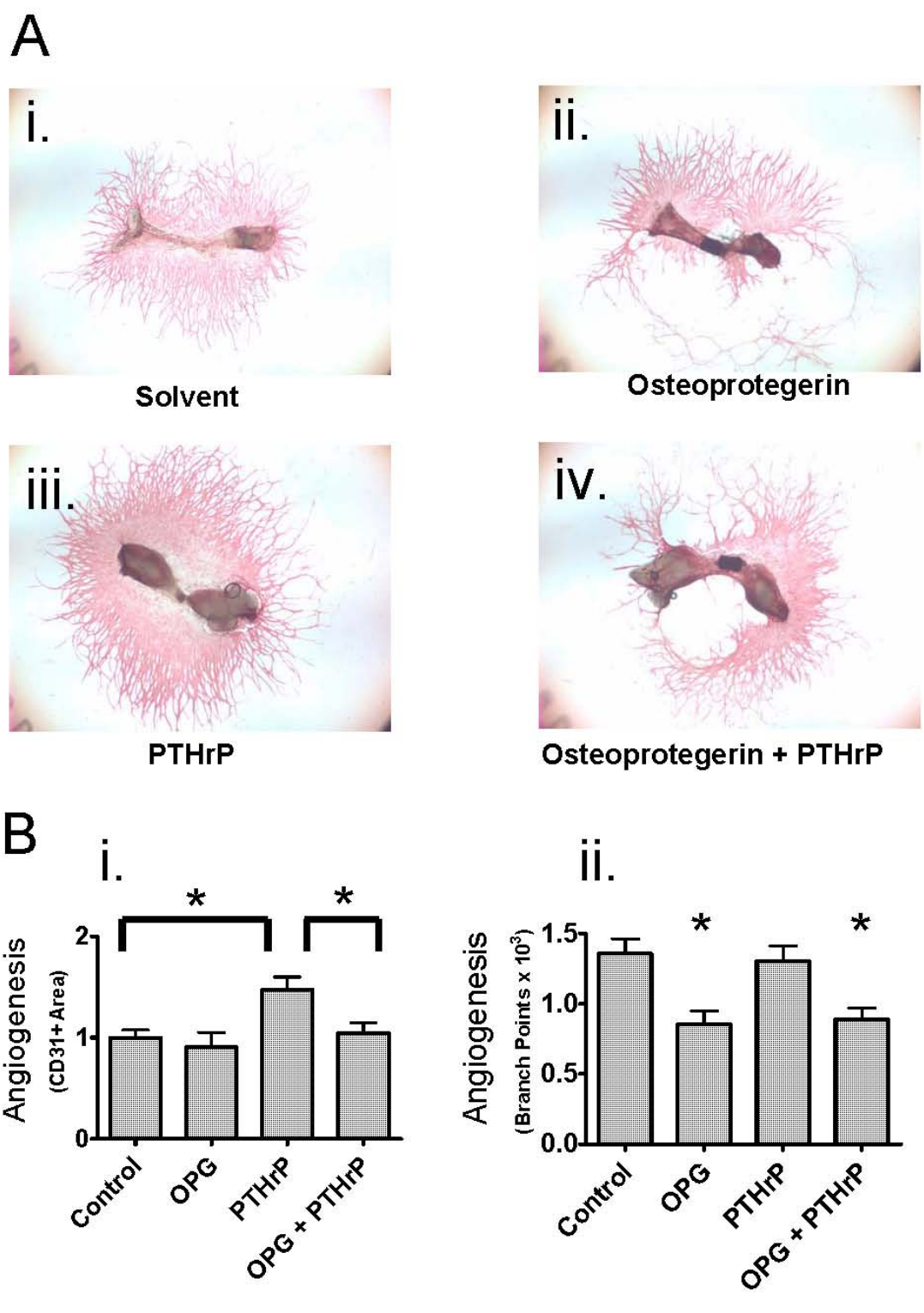

A
i.
Solvent
ii.
Osteoprotegerin
iii.
PTHrP
iv.
Osteoprotegerin + PTHrP
B
i.
Angiogenesis
(CD31+ Area)
2
1
0
*
*
Control
OPG
PTHrP
OPG + PTHrP
ii.
Angiogenesis
(Branch Points x 10³)
1.5
1.0
0.5
0.0
*
*
Control
OPG
PTHrP
OPG + PTHrP

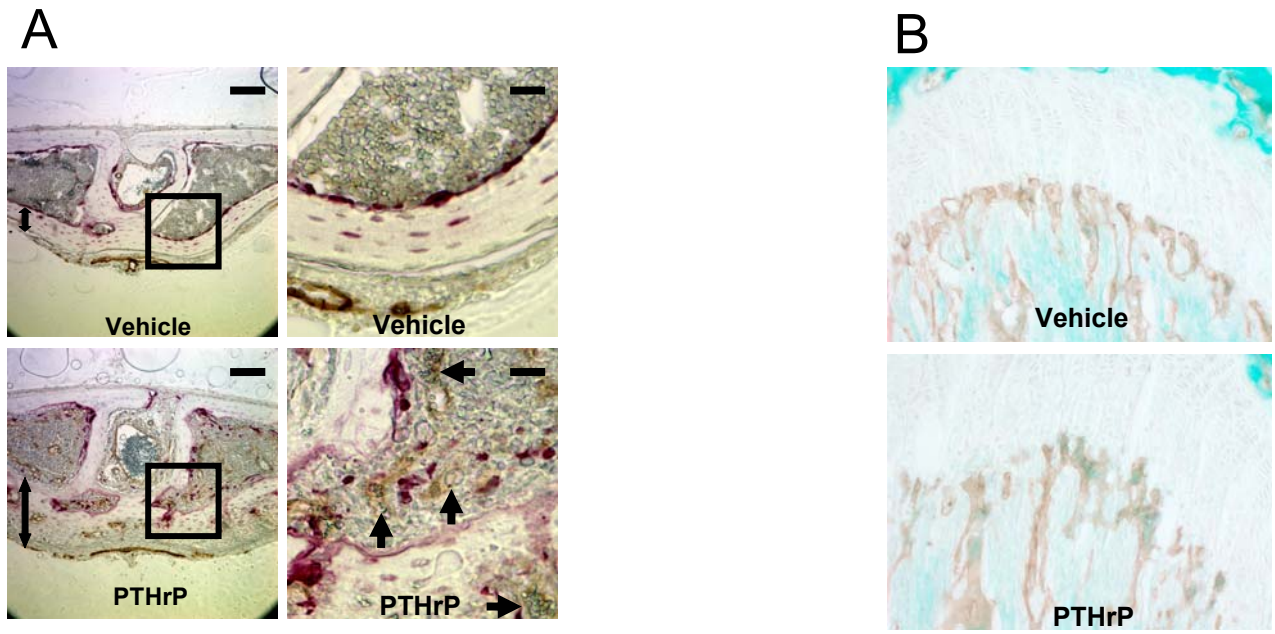
Figure 2.3. Osteoclast stimulation increases angiogenesis in fetal metatarsal explants

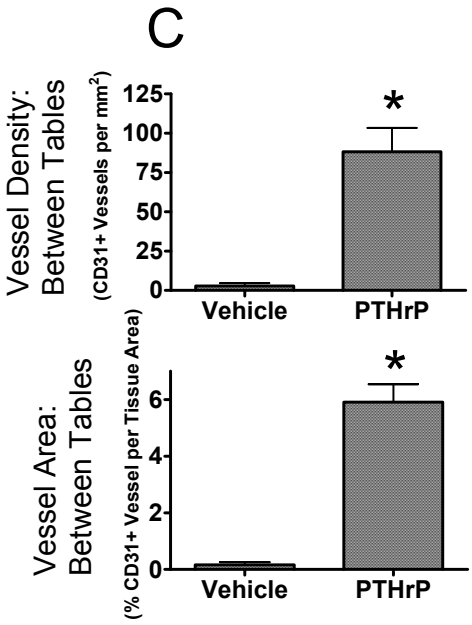
(A) PTHrP stimulates angiogenic outgrowth from metatarsal explants by a mechanism requiring osteoclasts. Whole mount fetal metatarsal explants from outbred mice cultured for 15 days with vehicles (i), 3 μg/mL rh OPG-Fc (ii), 100 nM h PTHrP (1-34) (iii) or OPG + PTHrP (iv), fixed and stained for CD31 (Red). Images aquired as whole mounts in water with an Olympus IX71 microscope with a Plan N 2x objective, NA of 0.06 and a Spot RTKE camera with Spot Advanced software. Original magnification x 2. (B) Quantification of angiogenic response to PTHrP and OPG. Angiogenesis was quantified by measurements of total CD31+ area (i) or angiogenic tube formation (branches) (ii). Mean ± SEM. *: $p < 0.05$. Similar results were seen in 2 experiments. Results represent one representative experiment of 2 performed.

2.4.4 OCL Stimulation with PTHrP or RANKL Increases Angiogenesis *in vivo* in Mouse Calvaria

We next determined if OCL stimulation increased angiogenesis in mice treated with PTHrP. Therefore, mice were treated with PTHrP every 6 hours subcutaneously for 5 days and then analyzed for OCLs and vessels histologically. This protocol has been used previously to dramatically induce OCL formation in calvaria and by frequent dosing is designed to minimize possible osteoblastic (anabolic) effects of PTHrP.[275] OCLs and blood vessels, labeled for the endothelial cell marker CD31, were dramatically increased between the bone tables in calvaria following PTHrP treatment (Figure 2.4A). OCL stimulation with PTHrP was less dramatic in femorae. The pattern of vessels in the metaphysis adjacent to the growth plate was changed, but there were no significant differences in vessel area (Figure 2.4B). The dramatic increase in total CD31+ vessel area normalized to tissue area was primarily due to increased vessel number rather average size of each vessel (Figure 2.4C). Average vessel size was not significantly increased

(data not shown). Cells of the reticuloendothelial system, such as those found in sinusoids of the bone marrow or liver do not express CD31.[104] Thus, these data reflect changes in endothelial cells of capillaries and arterioles rather than the venous sinusoids. PTHrP treatment was also characterized by an anabolic response within the calvarial inner table and associated tissue, resembling the response seen in hyperparathyroidism termed "osteitis fibrosa cystica." The thickness of the inner table was increased 4 fold with PTHrP treatment (Figure 2.4D). $CD31^+$ vessels in the inner table increased in proportion with tissue area in PTHrP treated bones. Thus $CD31^+$ vessels in the inner table were not increased when normalized for tissue area (data not shown). As an illustration of the osteoclastic response to PTHrP, OCLs, as measured by $TRAP^+$ area within the whole section, were increased with PTHrP treatment (Figure 2.4E).


A
Vehicle
Vehicle
PTHrP
PTHrP
B
Vehicle
PTHrP


C
Vessel Density: Between Tables
(CD31+ Vessels per mm2)
125
100
75
50
25
0
*
Vehicle
PTHrP
Vessel Area: Between Tables
(% CD31+ Vessel per Tissue Area)
6
4
2
0
*
Vehicle
PTHrP

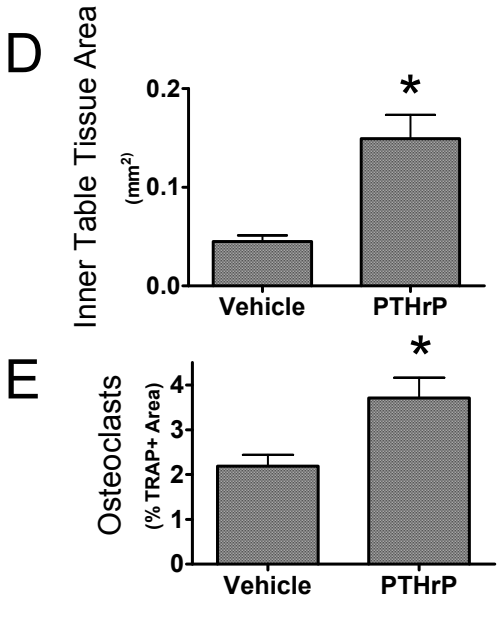

D
Inner Table Tissue Area
(mm2)
0.2
0.1
0.0
*
Vehicle
PTHrP
E
Osteoclasts
(% TRAP+ Area)
4
3
2
1
0
*
Vehicle
PTHrP

Figure 2.4. PTHrP stimulates angiogenesis *in vivo*

(A) Stimulation of OCL activity by PTHrP increases angiogenesis in mouse calvaria *in vivo*. Coronal sections of calvaria from mice treated with h PTHrP (1-34) every 6 hours for 5 days, labeled for CD31 (brown) and TRAP (red), and lightly counterstained with hematoxylin. Prominent CD31+ vessels are indicated by arrows. The inner table thickness is indicated by a 2 headed arrow. (B) Effects of PTHrP on vessels at femoral growth plate. Images are oriented with the growth plate at the top and metaphysis at the bottom. Sections are labeled for CD31 (brown) and counterstained (methyl green) (C) PTHrP increases CD31+ vessel density between calvarial bone tables. Total vessel area and vessel density quantified by grid map counting by a blinded observer of the para-sagittal areas of CD31 stained calvarial sections. (D) PTHrP increases thickness of inner table. Total tissue area of the inner table was quantified by image analysis by a blinded observer. (E) PTHrP induces OCL formation in calvaria. Total TRAP+ area as a percentage of tissue area was quantified by image analysis by a blinded observer. Mean ± SEM. *: $p < 0.05$ compared to vehicle. Results represent one representative experiment of 2 performed.

Because PTHrP can affect osteoblasts and other cells in addition to OCLs, we next studied the effect of RANKL on angiogenesis since RANKL does not affect osteoblasts, (reviewed in[63]). We stimulated OCLs by supra-calvarial injection of RANKL to determine if OCL stimulation increased angiogenesis. We observed dramatic bone resorption and OCL formation in the outer table with RANKL treatment (Figure 2.5 A). The resorbed area was replaced by non-calcified tissue resembling osteitis fibrosa and containing dense vessel invasion. Both vessel area and vessel size were increased by OCL stimulation (Figure 2.5 B). As expected, RANKL increased OCL formation as measured by $TRAP^+$ area and resorptive surface (Figure 2.5 C).

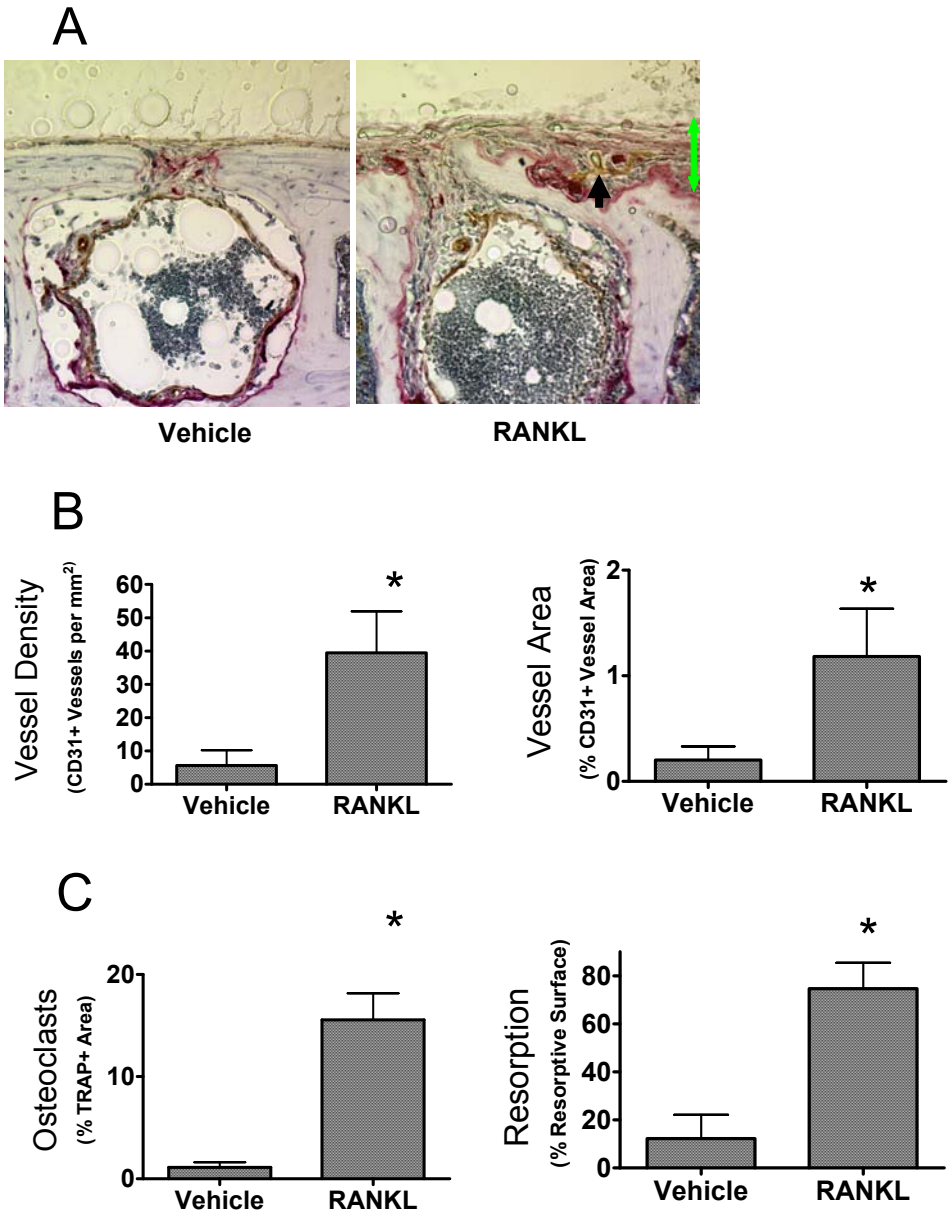

A
Vehicle
RANKL
B
Vessel Density
(CD31+ Vessels per mm2)
60
50
40
30
20
10
0
*
Vehicle
RANKL
Vessel Area
(% CD31+ Vessel Area)
2
1
0
*
Vehicle
RANKL
C
Osteoclasts
(% TRAP+ Area)
20
10
0
*
Vehicle
RANKL
Resorption
(% Resorptive Surface)
80
60
40
20
0
*
Vehicle
RANKL

Figure 2.5. RANKL stimulates angiogenesis *in vivo*

(A) Supra-calvarial RANKL increases angiogenesis and OCL formation in calvaria of mice. Images of the outer table of calvarial sections stained for TRAP (red) and CD31 (brown) and lightly counterstained with hematoxylin. Arrow: $CD31^+$ vessel in remodeling bone. Green two headed arrow indicates the thickness of RANKL induced bone remodeling. Outer surface is at the top of the image. Auto contrast and auto color adjustment was performed with Adobe Photoshop software. (B) Quantification of RANKL induced vessel response by calculation of $CD31^+$ vessel density or area in the outer table of 10x objective images taken at the calvarial midline. (C) Total percent $TRAP^+$ area or the calvarial outer table or resorptive surface of the outer table sub-periosteal surface. Mean ± SEM. *: $p < 0.05$

2.5 CONCLUSIONS

In these studies we demonstrated that OCLs stimulated angiogenesis *in vitro*, in bone explants and *in vivo* in calvaria. We showed that OCL conditioned media is angiogenic, and of comparable activity to other bone marrow cell types known to stimulate angiogenesis. We show that stimulating or blocking OCL formation and activity in bone organ cultures results in parallel changes in angiogenesis, and stimulating osteoclastogenesis *in vivo* increases angiogenesis. Prior to our studies, the role of OCLs in angiogenesis was unclear. Prior to, or concurrent with our studies, other groups have reported angiogenic activity of OCL CM but have not demonstrated an *in vivo* role of OCLs in angiogenesis.[5, 150]

The limited literature looking at a possible role for OCL stimulated angiogenesis *in vivo* is contradictory. Vu et al reported in their discussion section that osteopetrotic (c-fos $^{-/-}$ and op/op) mice have delayed blood vessel invasion into the primary ossification center and growth

plate *in vivo*.[14] However, other investigators found that OCLs do not play a role in angiogenesis and reported that lack of OCLs did not affect blood vessel invasion into the epiphyses of tibiae or mouse tail vertebrae.[4, 149] These results suggest that OCLs are likely to be less important for angiogenesis in vertebrae and long bone epiphyses. Differences in the role of other bone cell types on angiogenesis at different bone anatomic sites have been reported, and thus the contribution of OCLs to angiogenesis may differ at different anatomic sites. For example, osteoblast contributions to angiogenesis are more important in long bones than calvaria.[15]

The studies in this chapter show an important role for the OCL in angiogenesis stimulation. We showed that OCL conditioned media has angiogenic activity *in vitro*. This data demonstrates that OCLs secrete a factor(s) which can act directly on endothelial cells. The studies in this chapter do not show whether such a direct effect of OCLs on angiogenesis is their primary mechanism for angiogenesis stimulation *in vivo*. More complex mechanisms may be at play. OCL-stimulated angiogenesis may result from release of angiogenic factors from matrix. Alternatively, a linked osteoblastic response may also be required for OCL-stimulated angiogenesis. In support of a coupled proliferative response of osteoblasts or other cells playing a role in OCL-stimulated angiogenesis, OCL stimulation by PTHrP or RANKL *in vivo* was indeed accompanied by osteoblastic and "osteitis fibrosa" responses. Since osteoblast derived VEGF is clearly important for angiogenesis, osteoblasts and OCLs may cooperate to stimulate angiogenesis in bone.

Because PTHrP can also induce proliferation of osteoblasts as well as OCLs, we can not rule out that osteoblastic effects of PTHrP may have contributed to its angiogenic effects *in vivo*.[63] However, because RANKL, which has no osteoblastic effects, also simulated

angiogenesis, we are confident in reporting that OCLs stimulate angiogenesis *in vivo*. We showed in the metatarsal system that any possible angiogenic effects of PTHrP due to its direct effects on osteoblasts require OCLs, because the angiogenic effects or PTHrP were blocked by the OCL inhibitor osteoprotegerin (OPG). We attempted to perform the analogous experiment *in vivo* but were unable to obtain sufficient quantities of OPG that was active in mice.

It is possible that OPG, which we used to modulate OCL activity, may also affect endothelial cells. Several studies have reported possible direct stimulatory effects of OPG on endothelial cells, suggesting that we may have underestimated the contribution of OCLs to angiogenesis in our experiments using OPG.[67-69] Further, OPG can stimulate angiogenesis or endothelial cell survival in cell culture or aortic ring explants.[135, 220] In agreement with these findings, when equivalent concentrations of OPG to those used to inhibit metatarsal angiogenesis were tested in the HUVEC / fibroblast co-culture angiogenesis assay, which lacks OCLs, we observed a slight stimulation rather than any inhibition of angiogenesis in the HUVEC cultures. It is unclear if PTHrP can directly inhibit or stimulate endothelial cells. Two studies reported PTHrP inhibited and one reported PTHrP stimulated endothelial cells.[67-69] Like PTHrP, it is unclear if RANKL has possible direct effects on endothelial cells.[132, 135]

In the studies described in the following chapter, we investigated the mechanisms required for OCLs to stimulate angiogenesis and found that MMP-9 is required for OCL stimulated angiogenesis in metatarsal explants and *in vivo*.

3.0 OSTEOCLAST-STIMULATED ANGIOGENESIS REQUIRES MMP-9

3.1 SUMMARY

The studies in this chapter investigate the molecular mechanisms underlying OCL-stimulated angiogenesis. We first reviewed the literature and studied the expression of angiogenic factors by OCLs to determine which molecules may be used by OCLs to stimulate angiogenesis. Three factors we investigated, IL-8, MCP-1 and osteopontin, were not important for OCL-stimulated angiogenesis in the HUVEC / fibroblast *in vitro* angiogenesis assay. Most of the studies in this chapter investigate whether MMP-9 is important for OCL stimulated angiogenesis. MMP-9 was a logical choice for the mediator of OCL-stimulated angiogenesis for several reasons. It is expressed highly by OCLs and was expressed by OCLs almost 100 fold higher than any other angiogenic factor on the SA Biosciences human angiogenesis Q-PCR array. Importantly, it is primarily expressed by OCLs in bone. It is known to stimulate angiogenesis by release of matrix bound VEGF. We studied the role of MMP-9 in OCL-stimulated angiogenesis using all three of the model systems described in chapter 2. Lack of MMP-9 reduced OCL stimulated angiogenesis in metatarsal explants as shown by loss of the pro-angiogenic response to PTHrP. Likewise, lack of MMP-9 also reduced OCL-stimulated angiogenesis in calvaria *in vivo* as shown by a blunted angiogenic response to RANKL. Interestingly, PTHrP or RANKL stimulated osteoclasts less in MMP-9$^{-/-}$ than WT metatarsal

explants and calvaria *in vivo*. However, the number of vessels per OCL was not reduced by lack of MMP-9 *in vivo* and lack of MMP-9 did not decrease OCL formation from bone marrow cells *in vitro*. A neutralizing antibody to MMP-9 did not block the pro-angiogenic activity of MMP-9 conditioned media. From these data we concluded that MMP-9 is required for OCL-stimulated angiogenesis primarily due to its known effects on OCL migration.

3.2 INTRODUCTION

A search of the literature showed that OCLs secrete many different angiogenic factors, which can act directly on endothelial cells, including VEGF, IL-8, osteopontin, bFGF, PDGF, Angiopoietin-1 and Angiopoietin-2.[6, 119-123] Furthermore, during the course of our studies, Tanaka et al reported that OCLs stimulate angiogenesis *in vitro* due to secretion of osteopontin.[5] All of these molecules would be expected to stimulate angiogenesis by acting directly on endothelial cells.

However, there are many ways in which OCLs could stimulate angiogenesis more indirectly. They could release angiogenic factors from calcified or non-calcified matrix. For example, osteoclastic resorption is theorized to release active TGF-β from bone matrix.[10] This observation has led to a large body of literature on the "viscious cycle" of cancer bone metastasis.[109] Secondly, increased OCL number and activity could lead to proliferation or increased angiogenic factor expression in another cell type which would then secrete the direct stimulators of angiogenesis. For example, in most physiological situations, increases in OCL number are coupled to increased osteoblastogenesis. The ability of osteoblasts to stimulate angiogenesis is well known.[15]

Early studies in MMP-9$^{-/-}$ mice suggested that OCLs may stimulate angiogenesis by secretion of MMP-9, but the hypothesis has not been further pursued. MMP-9$^{-/-}$ mice display delayed endochondral ossification and vessel invasion into the primary ossification center, accompanied by lengthened growth plates. However, this phenotype resolves, and adults have normal appearing bones, which are slightly shorter than in WT animals.[14] These investigators soon delineated the mechanism for the effects of MMP-9 on OCL invasion and reported that MMP-9 release of matrix bound VEGF is important for OCL recruitment to the long bone growth plate. MMP-9 decreased resorption in E17 mouse metatarsal explants, but not in more mature bones. These effects were due to the effects of MMP-9 on invasion or migration rather than matrix solubilization.[161] Likewise, others reported MMP-9 is important for OCL or OCL precursor migration *in vitro*.[253-255] Similarly, MMP-9 delays OCL and vessel invasion into long bone fracture calluses.[164] In bone, MMP-9 is predominantly expressed by OCLs and committed OCL precursors, but can be expressed by other cell types such as osteoblasts and hypertrophic chondrocytes during development, and macrophages and neutrophils during fracture repair.[14, 164, 248, 276] Therefore, OCLs are likely responsible for most of the effects of MMP-9 in bone.

Several studies have reported that MMP-9 stimulates angiogenesis through activation or release of growth factors. MMP-9 was first implicated as the angiogenic switch in a mouse pancreatic cancer model in which MMP-9 released matrix (heparan) associated VEGF and increased VEGF signaling.[12] Likewise, MMP-9 expression and resultant VEGF activation by macrophages was required for angiogenesis in a cervical cancer model.[13]

We studied the mechanisms of OCL-stimulated angiogenesis by first studying OCL expression of angiogenic factors, then testing likely candidates for their importance to OCL

mediated angiogenesis. We found that MMP-9 is important for OCL stimulated angiogenesis in metatarsal explants and *in vivo* primarily due to its effect on OCL migration.

3.3 MATERIALS AND METHODS

Materials: Primary antibodies: Polyclonal rabbit anti-mouse MMP-9 was purchased from Abcam (#ab38898). The 11b5 mouse monoclonal IgM anti-VEGF-A in complex with VEGFR-1 or R-2 antibody was obtained from East Coast Bio (#CD302). Secondary antibodies: Vector Laboratories supplied biotinylated goat anti-rabbit secondary antibodies and SA-HRP. Jackson ImmunoResearch supplied biotinylated goat anti-mouse IgM μ chain and goat movalent Fab anti-mouse μ chain (for blocking endogenous IgM). Mouse anti-human MMP-9 neutralizing antibody clone # 6-6B (cat # IM09L) was provided by Calbiochem. Recombinant human osteopontin rh IL-8, rh MCP-1, rh $VEGF_{165}$, mouse monoclonal anti human IL-8 neutralizing antibody (clone #6217) and "Quantikine" ELISA assays for human osteopontin, MCP-1 and IL-8 were from R&D Systems. RNA Bee (phenol chloroform) RNA purification reagent was from Tel-Test. cDNA synthesis and PCR reagents (Superscript II reverse transcriptase, oligo dT, first strand buffer, DTT, Taq DNA polymerase, PCR buffer, dNTPs, and $MgCl_2$) were from Invitrogen. Human angiogenesis quantitative real-time PCR array and RT^2 cDNA synthesis kit were from SA Biosciences. RNeasy RNA purification kit was from Qiagen. MMP-$9^{-/-}$ mice were generated as described and backcrossed to C57BL/6 mice for 10 generations.[14] Timed pregnant C57BL/6 mice were from The Jackson Laboratory.

Analysis of Human OCL angiogenic factor expression by reverse transcriptase PCR: Human bone marrow OCLs were cultured as described in chapter 2. Total RNA was purified using RNA Bee (phenol chloroform extraction). 1 μg total RNA was reverse transcribed using Superscript II reverse transcriptase. PCR was performed on a Applied Biosystems GeneAmp 2700 thermocycler with the following program: initial incubation for 5 minutes at 95°C followed by 35 to 40 cycles of denaturing at 95°C for 1 minute, indicated annealing temperature for 1 minute, and extension at 72°C for 1 minute followed by a final extension for 10 minutes at 72°C. Products were visualized on 2% agarose gels stained by soaking in 1 μg/ml ethidium bromide in TAE buffer. PCR for β-actin was performed to verify successful cDNA synthesis and confirm negative results.

Table 3.1. Primers used for analysis of human OCL angiogenic factor expression by reverse transcriptase PCR

Gene	Sequence	Size (bp)	Annealing Temperature
VEGFA	Sense 5' gaaaccatgaactttctgc Antisense 5' cgcctcggcttgtca	470, 602, 674 (3 splice isoforms)	50°C
FGF2	Sense 5' agcggctgtactgcaaaaac Antisense 5' cccaggtcctgttttggat	338	57°C
ANGPT1	Sense 5' aaatggaaggaaaacacaaggaa Antisense 5' atctgcacagtctctaaatggt	263	58°C
ANGPT2	Sense 5' ggatctggggagagaggaac Antisense 5' ctctgcaccgagtcatcgta	535	60°C
ANGPT4	Sense 5' cccagatgccagagaccttt Antisense 5' cacctgctcacctgccatta	368	60°C
IL8	Sense 5' cgatgtcagtgcataaagaca Antisense 5' tgaattctcagccctcttcaaaaa	201	57°C

Human OCL culture angiogenic factor array analysis: For comparison of the profile of angiogenic factors expressed in human OCL or macrophage culture, OCLs were cultured as described in chapter 2 with the following modifications: non-adherent cells were diluted to 2 x 10^6 / ml in α-MEM with 20% horse serum, 10 ng/ml rh M-CSF with or without 50 ng/ml rh RANKL and seeded 0.4 ml per well of 48 well plates. Cultures were maintained for 17 days with twice weekly changes of half the media volume containing a full complement of cytokines, as previously described.[19] Expression of angiogenic factors was determined in OCL (RANKL + M-CSF treated) vs control cultures cultured with M-CSF only using the SA biosciences real time Q-PCR human angiogenic factor array. For replicates of the profile of angiogenic factors expressed in human purified OCLs, cultures were performed as described in chapter 2.

RNA (primarily mRNA) was purified with Qiagen RNeasy kit and reverse transcribed with the SA biosciences RT^2 first strand kit. Real time PCR was performed on a BioRad I-Cycler with provided primers and SYBR green. Ct values were calculated by the default settings of the I-Cycler software. Genomic DNA controls were negative. Data was analyzed by the ΔΔCt method compared to the average Ct value of the 4 or 5 housekeeping genes. Expression of each gene relative to housekeeping genes was calculated as $2^{-\Delta Ct}$. Genes that had Ct values of higher than 35 cycles were considered not expressed and not analyzed. The following genes were included on the array:

Table 3.2. Genes analyzed on the SA Biosciences human angiogenesis array.

Group	Gene Symbol	Description	Aliases
Growth factors and receptors	ANGPT1	Angiopoietin-1	
	ANGPT2	Angiopoietin-2	
	TYMP	Thymidine phosphorylase	ECGF1, PDECGF1
	ANPEP	Alanyl aminopeptidase	APN, CD13
	EREG	Epiregulin	
	FGF1	Fibroblast growth factor 1	
	FGF2	Fibroblast growth factor 2	Basic FGF
	FIGF	c-fos induced growth factor	VEGF-D
	FLT1	Fms-like tyrosine kinase 1	VEGF-R1
	JAG1	Jagged 1	
	KDR	Kinase insert domain receptor	Flk-1, VEGF-R2
	LAMA5	Laminin alpha 5	
	NRP1	Neuropilin-1	
	NRP2	Neuropilin-2	
	PGF	Placental growth factor	PlGF, PLGF-2
	PLXDC1	Plexin domain containing 1	
	STAB1	Stabilin-1	
	VEGFA	Vascular endothelial growth factor	VEGF, VPF
	VEGFC	Vascular endothelial growth factor C	
	S1PR1	Sphingosine-1-phosphate receptor-1	EDG-1
	EFNA-1	Ephrin-A1	
	EFNA-3	Ephrin-A3	
	EFNB2	Ephrin-B2	
	EPHB4	EPH receptor B4	Ephrin receptor EphB4
	EGF	Epidermal growth factor	
	FGFR3	Fibroblast growth factor receptor 3	
	HGF	Hepatocyte growth factor	
	IGF1	Insulin like growth factor 1	
	PDGFA	Platlet derived growth factor, alpha polypeptide	PDGF A-chain
	TEK	TEK tyrpsine kinase, endothelial	Tie-2
	TGFA	Transforming growth factor alpha	
	TGFB1	Transforming growth factor beta 1	TGF-β1
	TGFB2	Transforming growth factor	

		beta 2	
	TGFBR1	Transforming growth factor beta, receptor 1	
Adhesion Molecules	ANGPTL3	Angiopoietin-like 3	ANGPT5
	BAI1	Brain specific angiogenesis inhibitor-1	
	COL4A3	Collagen type 4, alpha 3	Tumstatin
	CDH5	Cadherin 5, type 2	VE-Cadherin
	COL18A1	Collagen, type XVIII, alpha 1	Endostatin
	ENG	Endoglin	
	ITGAV	Alpha v integrin	
	ITGB3	Beta 3 integrin	
	THBS1	Thrombospondin-1	
	THBS2	Thrombospondin-2	
Proteinases, Proteinase Inhibitors and other Matrix proteins	ANGPTL4	Angiopoietin-like 4	ANGPTL2/ARP4
	PECAM1	Platelet/endothelial cell adhesion molecule (CD31 antigen)	CD31
	PF4	Platelet factor 4	CXCL4/SCYB4
	PROK2	Prokineticin 2	BV8/KAL4
	Gene Symbol	Description	Aliases
Proteinases, Proteinase Inhibitors and other Matrix proteins (continued)	SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	PEDF
	TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	B94
	HPSE	Heparanase	HPA/HPR1
	LECT1	Leukocyte cell derived chemotaxin 1	BRICD3/CHM-I
	LEP	Leptin	OB/OBS
	MMP2	Matrix metallopeptidase 2	Gelatinase A
	MMP9	Matrix metallopeptidase 9	Gelatinase B
	PLAU	Plasminogen activator, urokinase	uPA
	PLG	Plasminogen	
	TIMP1	TIMP metallopeptidase inhibitor 1	
	TIMP2	TIMP metallopeptidase inhibitor 2	
	TIMP3	TIMP metallopeptidase inhibitor 3	
Cytokines and Chemokines	CCL11	Chemokine (C-C motif) ligand 11	SCYA11

	CCL2	Chemokine (C-C motif) ligand 2	GDCF-2/GDCF-2
	CXCL1	Chemokine (C-X-C motif) ligand 1	GRO1/GROa
	CXCL10	Chemokine (C-X-C motif) ligand 10	C7/IFI10
	CXCL3	Chemokine (C-X-C motif) ligand 3	CINC-2b/GRO3
	CXCL5	Chemokine (C-X-C motif) ligand 5	ENA-78/SCYB5
	CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CKA-3/GCP-2
	CXCL9	Chemokine (C-X-C motif) ligand 9	CMK/Humig
	IFNA1	Interferon, alpha 1	IFL/IFN
	IFNB1	Interferon, beta 1, fibroblast	IFB/IFF
	IFNG	Interferon, gamma	IFG/IFI
	IL6	Interleukin 6	BSF2/HGF
	IL8	Interleukin 8	3-10C/AMCF-I
	MDK	Midkine	MK/NEGF2
	TNF	Tumor necrosis factor	DIF/TNF-alpha
Transcription factors and others	HAND2	Heart and neural crest derivatives expressed 2	DHAND2/Hed
	SPHK1	Sphingosine kinase 1	SPHK
	AKT1	V-akt	AKT/PKB
	HIF1A	Hypoxia-inducible factor 1, alpha subunit	HIF-1alpha
	ID1	Inhibitor of DNA binding 1	ID
	ID3	Inhibitor of DNA binding 3	HEIR-1
	NOTCH4	Notch homolog 4 (Drosophila)	INT3/NOTCH3
	PTGS1	Prostaglandin-endoperoxide synthase 1	COX1/COX3
Housekeeping Genes	B2M	Beta-2-microglobulin	B2M
	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT
	RPL13A	Ribosomal protein L13a	RPL13A
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD
	ACTB	Actin, beta	PS1TP5BP1

Fetal Mouse Metatarsal Angiogenesis Assay: General assay procedures are described in chapter 2. Images of CD31 stained explants were acquired with an Olympus Multimode dissecting microscope and were quantified either with ImageJ for total $CD31^+$ area and corrected for the area of bones stained with control IgG in place of primary antibody. For comparing the angiogenic and osteoclastic response of MMP-9 $^{-/-}$ vs. wt metatarsals treated with PTHrP, 7 litters of C57BL/6 and 6 litters of C57BL/6 MMP-9 $^{-/-}$ were treated with 100 nM PTHrP or solvent separately. The mean fold increase in $CD31^+$ area with PTHrP treatment was calculated for each litter. The effect of PTHrP treatment on each genotype was compared by calculating the mean increases (fold control) per litter. This analysis was necessitated by the large variability in angiogenesis that was seen among litters in solvent treated explants.

Histology and Immunohistochemistry: Labeling of CD31 and TRAP in fixed frozen sections was performed as described in chapter 2. For 11b5 labeling, blocking was accomplished with 10% goat serum, 1% horse serum (HS) PBS, followed by PBST washes x2. Blocking of endogenous IgM was accomplished with Fab anti-μ 0.1 mg/mL in PBS, followed by primary antibody (culture supernatant 1:10 in 1% HS-PBS), 1% HS-PBS washes x5, goat anti-μ in 1% HS-PBS, PBS washes x5 and SA-HRP and substrate as above.

In vivo studies: MMP-$9^{-/-}$ C57BL/6 mice were injected with m RANKL-GST along side the WT mice described in chapter 2. Data from WT mice is reported again in this chapter and compared to $MMP^{-/-}$ mice.

In vitro angiogenesis assay: Human OCL conditioned media was collected and the TCS CellWorks *in vitro* angiogenesis assay was performed as in chapter 2. For MMP-9 neutralization, neutralizing antibody clone # 6-6B or moue IgG control were diluted to a final concentration of 3 μg/mL and pre-incubated for 1 hour at 37^{o}C. This concentration is 3 fold higher than the concentration reported to inhibit MMP-9 activity in bioassays.[277]

Statistical Analyses: The unpaired Student's t-test or one way ANOVA with LSD procedure were used for analyzing 2 or multiple groups respectively. The ratio t-test (paired t-test on logarithms of vehicle vs treated samples) was used for analysis fold control data. Two-tailed analyses were performed with SPSS software. Significance was set at $\alpha = 0.05$.

3.4 RESULTS

3.4.1 Analysis of OCL Angiogenic Factor Expression

We analyzed gene expression of angiogenic factors in human OCL cultures by reverse transcriptase PCR and the SA Biosciences quantitative real time PCR human angiogenesis array. We took a candidate factor approach in the reverse transcriptase PCR studies. The results of these studies are shown in Figure 3.1. Purified human OCL cultures expresses angiopoietin-1, angiopoietin-4, and FGF-2, but did not express VEGF or angiopoietin-2.

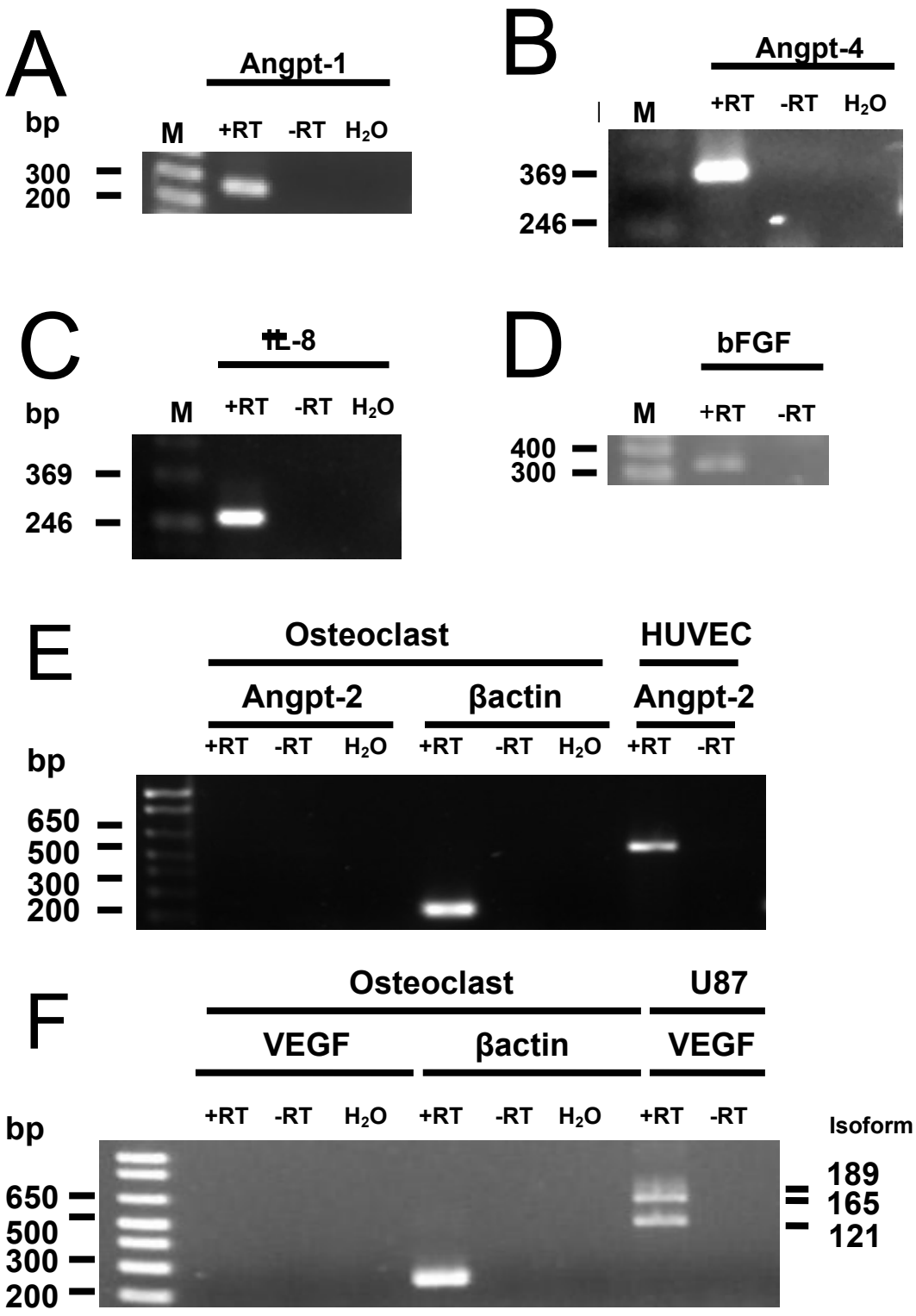

A
Angpt-1
bp
M
+RT
-RT
H2O
300
200
B
Angpt-4
M
+RT
-RT
H2O
369
246
C
IL-8
bp
M
+RT
-RT
H2O
369
246
D
bFGF
M
+RT
-RT
400
300
E
Osteoclast
HUVEC
Angpt-2
βactin
Angpt-2
+RT
-RT
H2O
+RT
-RT
H2O
+RT
-RT
bp
650
500
300
200
F
Osteoclast
U87
VEGF
βactin
VEGF
+RT
-RT
H2O
+RT
-RT
H2O
+RT
-RT
bp
650
500
300
200
Isoform
189
165
121

Figure 3.1. Analysis of osteoclast angiogenic factor expression by reverse transcriptase PCR

RNA collected from human bone marrow OCL cultures or control cell types was analyzed by reverse transcriptase PCR

To analyze angiogenic factor gene expression of human OCLs on a wider scale, we used the SA Biosciences human angiogenesis real Q-PCR array. To determine the angiogenic factors expressed by OCLs, the level of angiogenic factor expression in two independent purified human OCL cultures was analyzed. The 15 most highly expressed angiogenic factors in both cultures are reported in table 3.3. The following genes were in the top 15 in both groups.: MMP-9, TIMP1, TIMP2, MCP-1, ANPEP, HIF1-α, αv integrin, NRP1, NRP2 and IL-8.

Table 3.3. 15 most highly expressed angiogenic factors (or inhibitors) in 2 purified human OCL cultures.

Expression is reported relative to the 4 housekeeping genes: HPRT1, RPL13A, GAPDH and ACTB.

Culture # 1		**Culture # 2**	
Gene	**Relative Expression**	**Gene**	**Relative Expression**
MMP9	19.69831	MMP9	15.45498
TIMP2	0.870551	TIMP1	1.931873
ANPEP	0.659754	CCL2 (MCP-1)	0.68302
CCL2 (MCP-1)	0.466516	TIMP2	0.392292
HIF-1a	0.435275	IL-8	0.34151
alphaV integrin	0.307786	ANPEP	0.31864
Endoglin	0.267943	AlphaV integrin	0.31864
TIMP1	0.217638	CXCL9 (MIG)	0.241484
uPA	0.176777	TNF-a	0.129408
TGFB1	0.164938	HIF-1a	0.098073
NRP1	0.143587	NRP2	0.056328
NRP2	0.076947	NRP1	0.03983
IL-8	0.058315	CXCL10	0.03983
CXCL5 (ENA-78)	0.044194	Beta3 Integrin	0.03983
ECGF1	0.044194	CXCL1 (GRO-alpha)	0.037163

Alternatively, to analyze angiogenic factors induced by OCL differentiation, we compared angiogenic factor expression in human bone marrow non-adherent cell OCL cultures (cultured with RANKL and M-CSF) compared with cells cultured with M-CSF alone containing mostly macrophages. Genes upregulated at least 4 fold in OCL relative to control cultures are reported in Table 3.4. Expression of angiopoietin-2 was not observed by conventional reverse transcriptase PCR but was detected on the array at a very low level.

Table 3.4. Angiogenic Factors Upregulated by OCL differentiation

***** Expression of angiogenic factors relative to 5 housekeeping genes: B2M, HPRT1, RPL13A, GAPDH and ACTB in OCLs cultured from human bone marrow adherent cells treated with RANKL and M-CSF was assayed with the SA Biosciences human angiogenesis Q-PCR array.

† The fold expression in human bone marrow OCL cutltures relative to human bone marrow adherent cells cultured with M-CSF only was calculated normalized to housekeeping genes. Only factors upregulated at least 4 fold are reported.

Angiogenic factor	OCL expression relative to 5 housekeeping genes*	Fold upregulation compared to M-CSF treated cultures†
MMP-9	47	7.1
ANPEP (APN, CD13)	0.48	4.1
β3 integrin	0.069	28.2
MMP-2	0.069	7.1
Neuropilin-2	0.060	5.7
Sphingosine Kinase 1	0.020	11
CXCL5 (ENA-78)	0.012	8.1
Notch-4	0.0061	5.0
COL18A1 (endostatin)	0.0026	8.1
Angiopoietin-2	0.00020	4.1

As shown in Table 3.3 and 3.4, MMP-9 was much more highly expressed in OCLs than the other genes analyzed. MMP-9 was the most highly expressed pro-angiogenic factor by human OCLs at the mRNA level and was expressed approximately an order of magnitude more highly than all other angiogenic factors examined. It was also upregulated over 7 fold compared to control cultures of primarily macrophages treated only with M-CSF.

3.4.2 MMP-9 is Required for OCL Stimulation of Angiogenesis in Metatarsal Explants

Because MMP-9 can be pro-angiogenic, is expressed highly by OCLs, and its null allele delays blood vessel invasion of the growth plate, we next determined if OCLs stimulate angiogenesis in part by secretion of MMP-9.

We tested the capacity of PTHrP to stimulate angiogenesis in metatarsal explants from MMP-$9^{-/-}$ or WT C57BL/6 mice by treating metatarsals from 7 WT and 6 MMP-$9^{-/-}$ C57BL/6 litters of mouse embryos with 100 nM PTHrP (1-34) or vehicle and comparing the ability of PTHrP to stimulate angiogenesis as measured by $CD31^{+}$ area. Because of the large variability in the level of basal angiogenesis and resorption among litters, angiogenesis and OCL activity data were analyzed in terms of fold control for each litter. As indicated by the positive (ratio greater than unity) effects of PTHrP vs. vehicle, PTHrP increased angiogenesis in WT but not in MMP-$9^{-/-}$ metatarsal explants, and the angiogenic effects differed between the genotypes (Figure 3.2A, 3.2B).

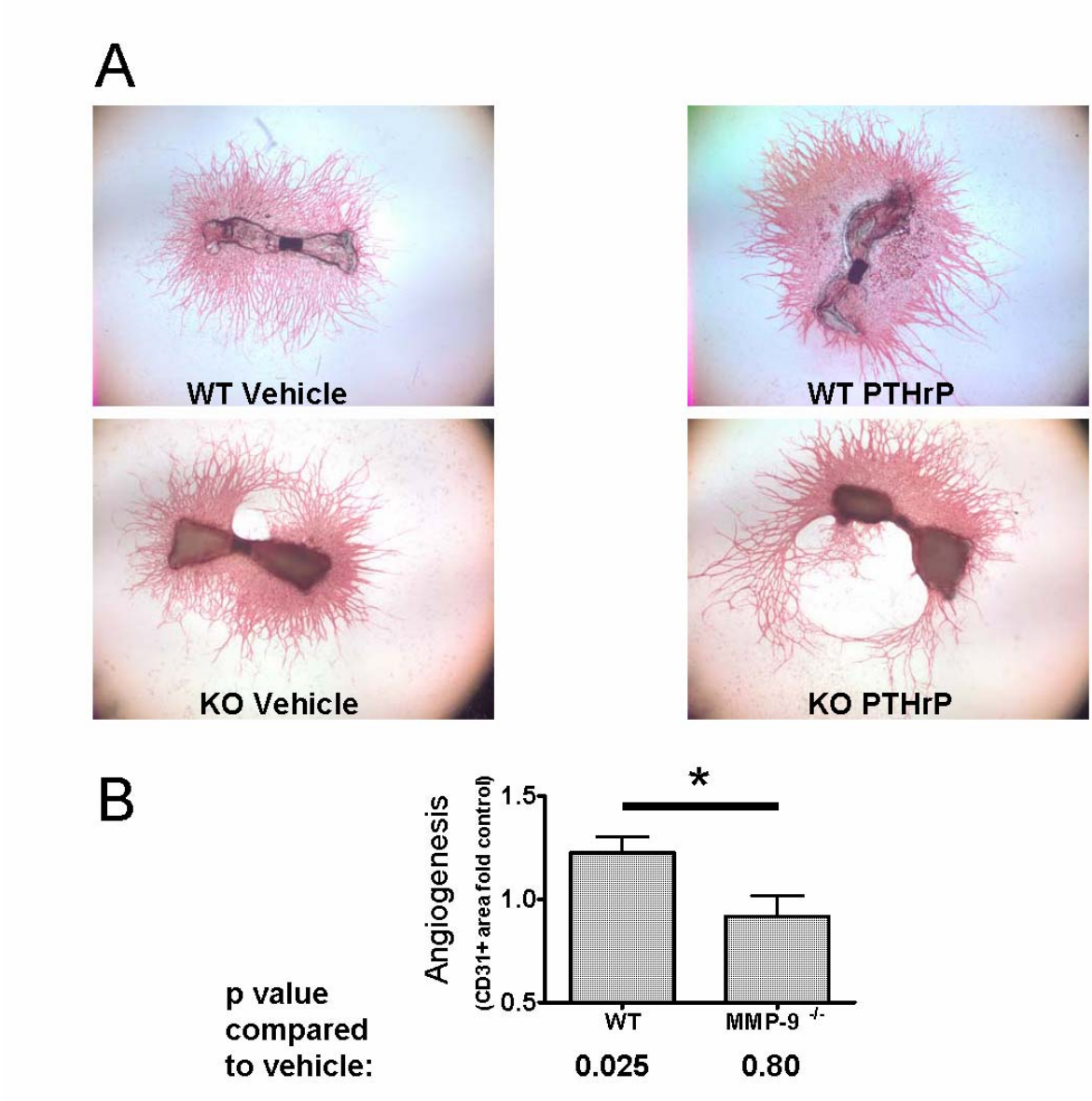


Figure 3.2. MMP-9 is required for OCL-stimulated angiogenesis in metatarsal explants.

PTHrP-induced metatarsal angiogenesis is blunted in MMP-9$^{-/-}$ explants. Sample 2x original magnification images of WT or MMP-9 $^{-/-}$ C57BL/6 metatarsal explants treated with vehicle or 100 nM PTHrP as indicated and stained for CD31. Images acquired as in figure 2. (B) Angiogenic response to PTHrP is significantly less in MMP-9$^{-/-}$ than in WT metatarsal explants. Metatarsals from 7 WT and 6 MMP-9$^{-/-}$ litters of each genotype were treated with 100 nM PTHrP or vehicle. The mean increase in CD31^{+} area per litter for all litters ± SEM is reported. *: $p < 0.05$. for difference in treatment response between genotypes. PTHrP significantly stimulated angiogenesis in WT

but not in MMP-9$^{-/-}$ metatarsals, as determined by the ratio t-test comparing vehicle and PTHrP treated means for each litter.

3.4.3 MMP-9 is Required for OCL Stimulation of Angiogenesis *in vivo*

We stimulated OCL formation in both WT and MMP-9$^{-/-}$ C57BL/6 mice by supra-calvarial injection of RANKL to determine if OCLs increased angiogenesis by a mechanism requiring MMP-9. RANKL treatment induced dramatic changes in bone resorption and OCL formation in the outer table (Figure 3.3A). The resorbed area was replaced by non-calcified tissue resembling active periosteum and contained newly formed vessels not previously present in calcified tissue. Angiogenesis was induced by RANKL only in WT but not MMP-9$^{-/-}$ calvaria as measured by CD31^{+} vessel density or total CD31^{+} vessel area within the outer table of calvaria (Figure 3.3B). Angiogenesis in WT calvaria treated with RANKL was significantly different from RANKL treated MMP-9$^{-/-}$ calvaria when angiogenesis was quantified by vessel density, and nearly significant when angiogenesis was quantified by total vessel area. As with calvaria treated with PTHrP no significant differences in vessel size were detected (data not shown). MMP-9 was primarily expressed by OCLs, thus suggesting the MMP-9 important for OCL-stimulated angiogenesis is derived from OCLs themselves (Figure 3.3A). Surprisingly, the osteoclastogenic response to RANKL was blunted in MMP-9$^{-/-}$ calvaria (Figure 3.3A). This suggests that MMP-9 affects OCL-stimulated angiogenesis by decreasing the number of OCLs at the angiogenic site and will be analyzed in more detail in the next section.

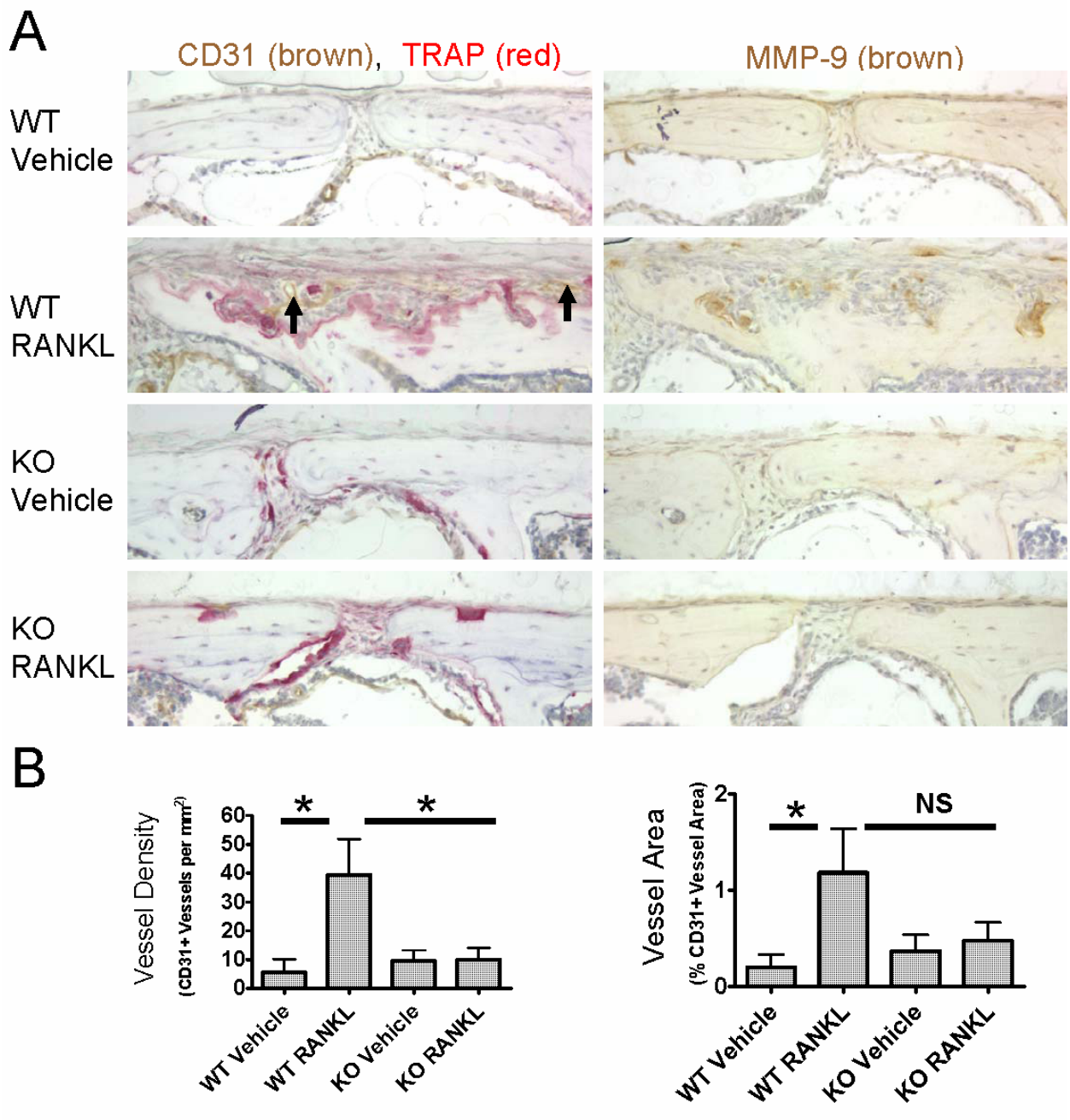


Figure 3.3. Osteoclasts stimulate angiogenesis in WT but not MMP-9$^{-/-}$ mice

(A) Supra-calvarial RANKL increases angiogenesis and MMP-9 expressing OCL formation to a greater extent in calvaria of wild type than MMP-9$^{-/-}$ mice. Serial sections of the outer table of calvaria stained for TRAP (red) and CD31 (brown) or MMP-9 (brown) and lightly counterstained with hematoxylin. Images are WT or MMP-9 KO injected supracalvarially with vehicle or m RANKL-GST as indicated. Arrows: CD31^{+} vessels in remodeling

bone. Outer surface is at the top of the image. (B) Quantification of RANKL induced vessel response by calculation of $CD31^+$ vessel density or area in the outer table of 10x objective images taken at the calvarial midline.

3.4.4 MMP-9 Stimulates Bone Angiogenesis Primarily through Autocrine Effects on OCL Migration

To further analyze the source of the MMP-9 responsible for the pro-angiogenic response of RANKL treatment in calvaria, I then determined the cell types expressing MMP-9 in the marrow spaces of mouse calvaria by singly or double labeling TRAP and MMP-9. MMP-9 expression was almost completely restricted to OCLs. Immunohistochemical analysis of mouse calvaria showed that MMP-9 was almost completely co-localized with the OCL marker TRAP (Figure 3.4). Only very rare MMP-9 positive, TRAP negative mononuclear marrow cells were detected.

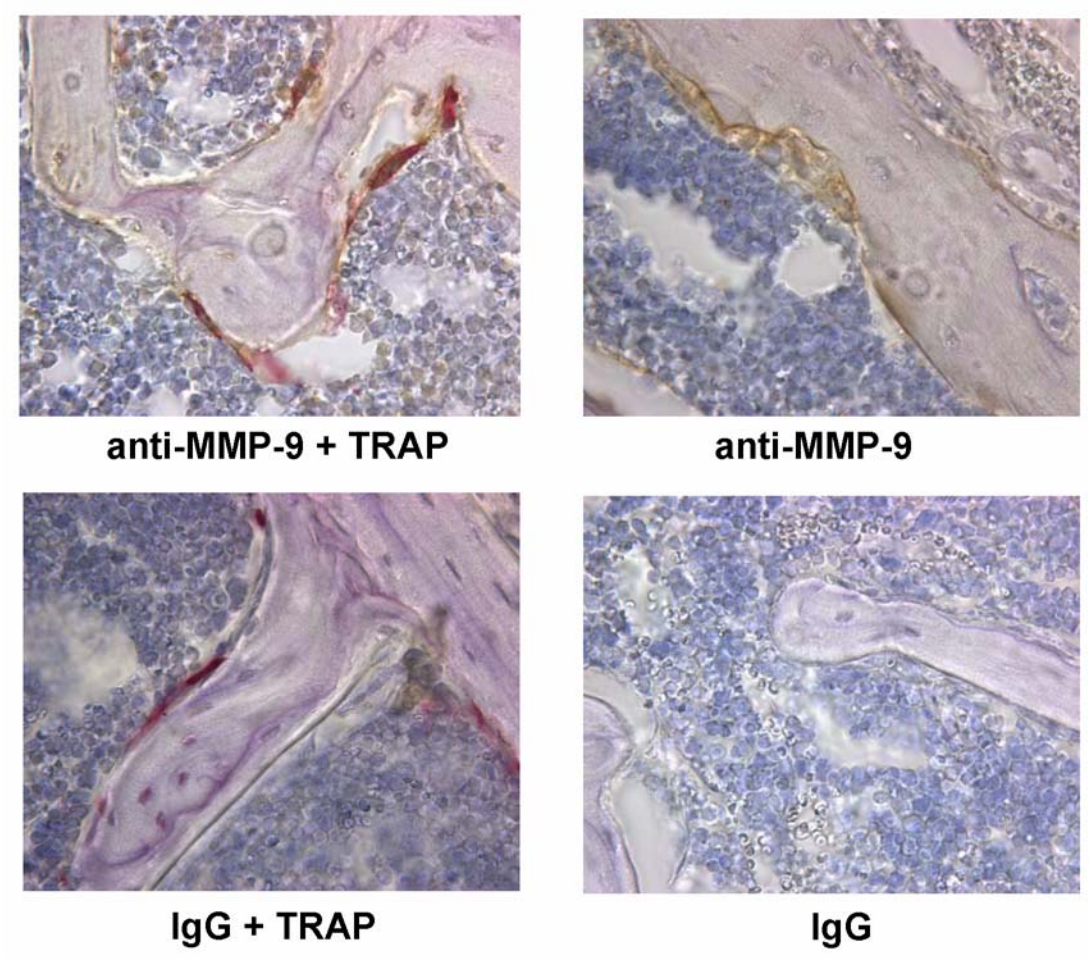


Figure 3.4. MMP-9 is predominantly expressed by osteoclasts in mouse calvaria.

Coronal sections of mouse calvaria stained for TRAP (red) or MMP-9 (brown) as indicated. Images acquired with a Nikon Eclipse E800 microscope with a Plan Apo objective (oil), NA of 1.4 and an Olympus America SN CG603057-H camera with Magnifire software. Original magnification x 60.

In the experiments comparing the angiogenic effects of OCL stimulation in explants or *in vivo* (Figure 3.2 and 3.3), I also analyzed the osteoclastic responses to determine if lack of MMP-9 affected OCL formation or recruitment. Osteoclast stimulation induced by PTHrP or RANKL was blunted in MMP$^{-/-}$ mice. Type I collagen C-terminal telopeptide (CTX), were measured in the conditioned media collected from days 1-3 and 4-6 of culture from all WT and MMP-9$^{-/-}$

PTHrP and vehicle treated explants. PTHrP significantly stimulated bone resorption in WT explants at both time points. However, PTHrP did not significantly stimulate resorption at 3 or 6 days in MMP-9$^{-/-}$ explants (Figure 3.5). Other investigators similarly reported lack of MMP-9 decreased OCL resorption in E17 metatarsal explants due to OCL migration or invasion defects.[161, 253]

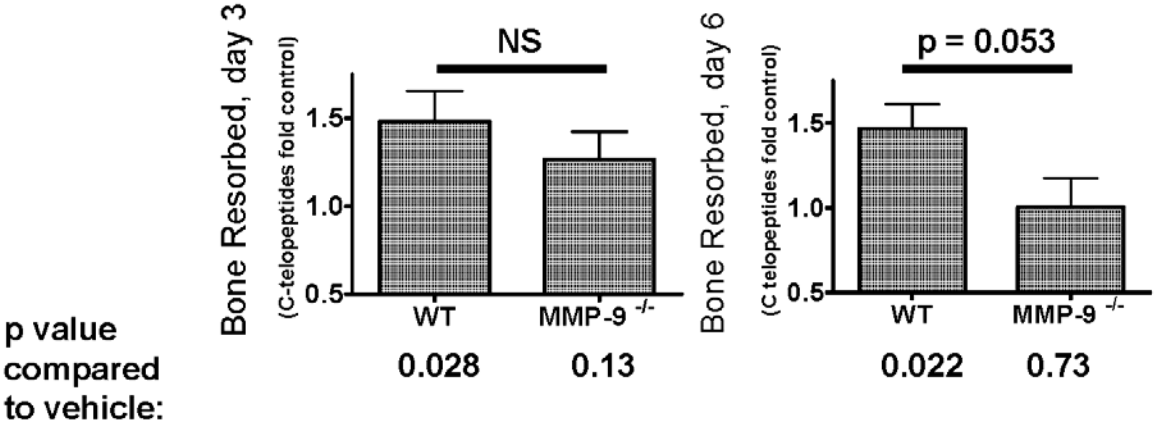


Figure 3.5. PTHrP increases bone resorption in WT but not in MMP-9$^{-/-}$ metatarsal explants.

CTX assayed from conditioned media collected from days 1-3 or 4-6 of culture of all 7 WT and 6 KO litters. *: $p < 0.05$. for treatment response compared to vehicle.

In parallel to our findings in metatarsal explants treated with PTHrP, OCL formation was stimulated by RANKL in WT but less so in MMP-9$^{-/-}$ animals in the calvarial outer table (Figure 3.6A). Resorption surface or TRAP^{+} area was significantly less in RANKL treated MMP-9$^{-/-}$ than RANKL treated WT calvaria. The OCL stimulation with RANKL among MMP-9$^{-/-}$ mice was dramatically blunted compared to WT animals and possibly absent. However, we were not able to find any reports in the literature that MMP-9 affects OCL differentiation directly. Therefore, to determine if the decrease in OCL numbers in RANKL treated MMP-9$^{-/-}$ mice

results from decreased OCL differentiation or precursor number, we assayed OCL differentiation *in vitro* and found that WT and MMP-9$^{-/-}$ mice formed similar numbers of OCLs (Figure 3.6B). To examine the relative angiogenic capacity of MMP-9$^{-/-}$ OCLs on a per cell basis, we calculated vessel number per OCL, which did not differ between genotypes (Figure 3.6C). Thus, MMP-9 is not of primary importance for OCLs to stimulate angiogenesis per se, but affects OCL numbers at the angiogenic site. Thus, MMP-9 most likely affects OCL-stimulated angiogenesis primarily by affecting their migration to the angiogenic site.

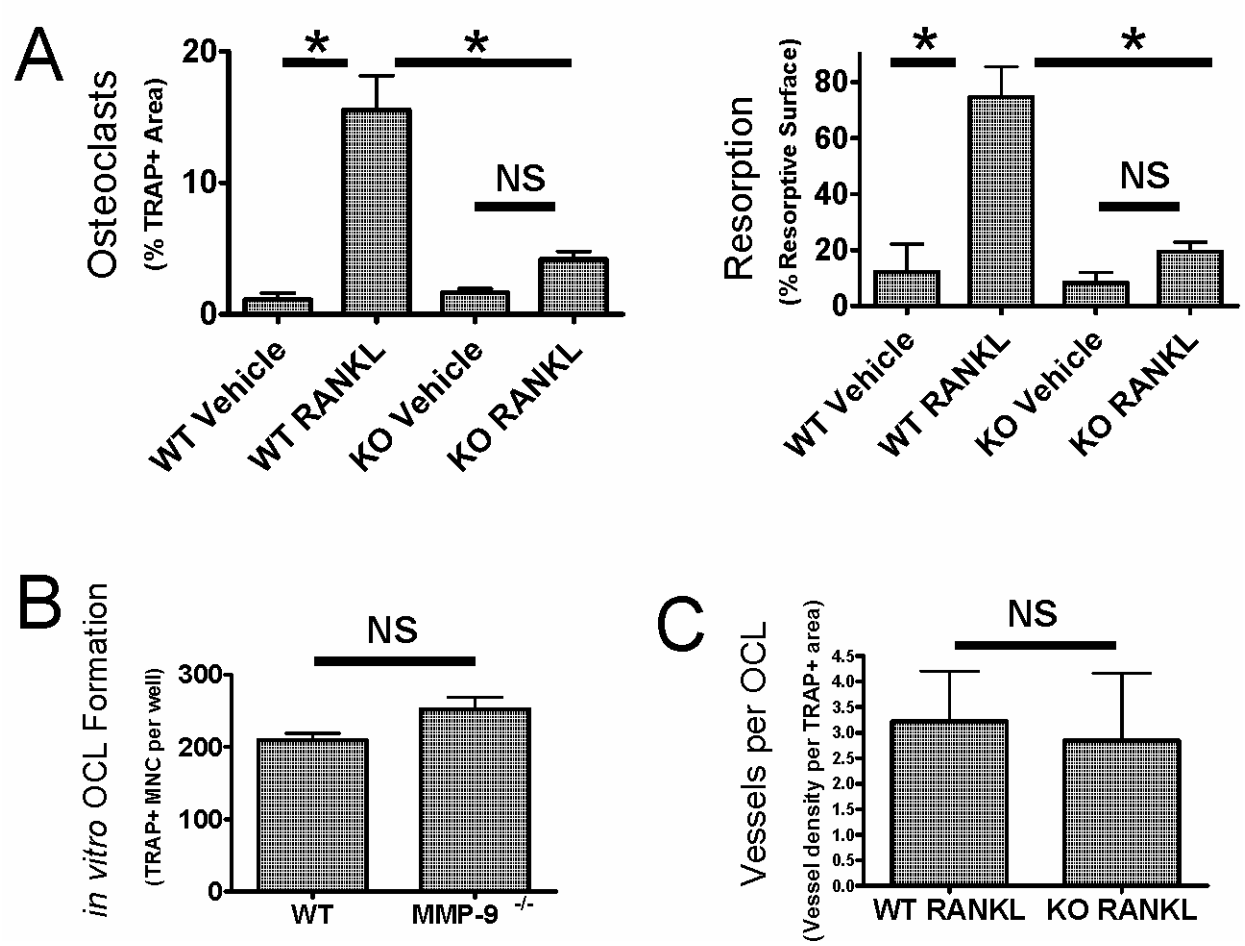


Figure 3.6. Lack of MMP-9 decreases RANKL-stimulated osteoclastogenesis *in vivo* but not *in vitro*.

(A) Total percent $TRAP^+$ area of the calvarial outer table or resorptive surface of the outer table sub-periosteal surface. (B) *In vitro* Osteoclast formation from bone marrow from WT and MMP-$9^{-/-}$ C57BL/6 mice was quantified by counting $TRAP^+$ multi-nucleated cells. (C) Vessels per OCL was calculated by dividing vessel density by $TRAP^+$ area for each RANKL treated animal. Mean ± SEM. *: $p < 0.05$.

Because MMP-9 is known to stimulate angiogenesis by VEGF release from matrix in other tissues, we determined if VEGF signaling was increased with OCL stimulation *in vivo*. Increased VEGF signaling induced by proteases such as MMP-9 can be analyzed using monoclonal antibodies which recognize VEGF only in complex with its receptors. Therefore we determined the number of VEGF / receptor complex positive vessels using the 11b5 monoclonal antibody, which recognizes VEGF in complex with VEGF-R1 or R2.[278] Labeling with this antibody, we found that PTHrP non-significantly increased 11b5+ vessel density (Figure 3.7B). The labeling of $11b5^+$ vessels was less clearly defined than $CD31^+$ vessels, suggesting that other cell types in addition to endothelial cells, such as pericytes, are also labeled (Figure 3.7A). A minority of the vessels were $11b5^+$, as illustrated by the observation that the $11b5^+$ vessel density was only about 20% of the $CD31^+$ vessel density (Figure 3.7A and comparing Figures 3.7B and 2.4C). In some highly angiogenic states linked to MMP-9, greater than 90% of vessels are VEGF / receptor complex positive.[13] Furthermore, we were unable to detect $11b5^+$ vessels induced in angiogenic areas of the calvaria outer table from animals treated with RANKL (same areas as illustrated in Figure 3.3). Therefore, these data suggest that increased VEGF signaling due to release from matrix may contribute to OCL induced angiogenesis, but other mechanisms are likely to be involved.

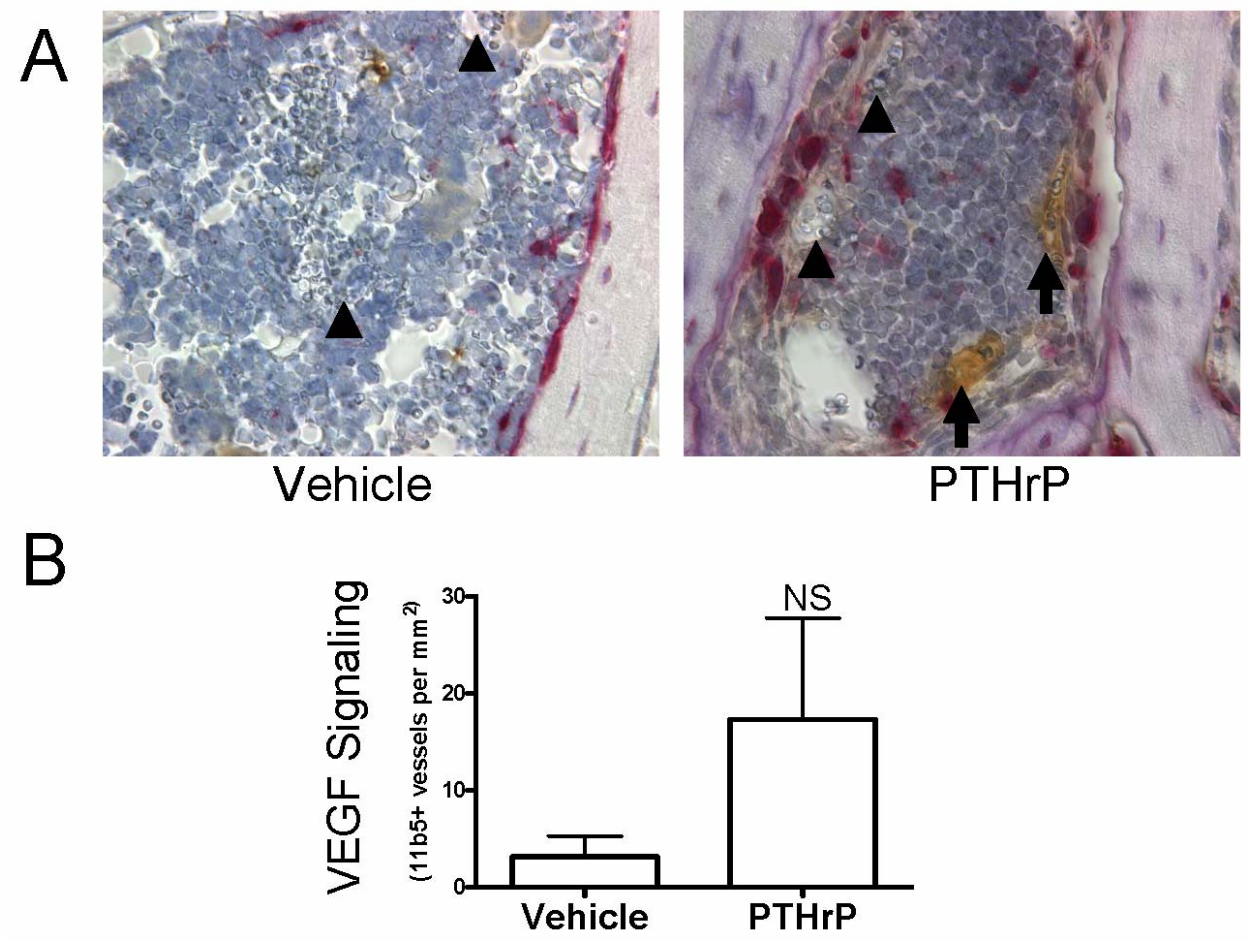


Figure 3.7. PTHrP may increase VEGF signaling *in vivo*.

VEGF / receptor complex positive vessels in PTHrP treated calvaria. (A) Para-sagittal marrow spaces in vehicle or PTHrP treated calvaria. Sections stained for VEGF / receptor complexes with the 11b5 monoclonal antibody (brown), TRAP (red) and counterstained with hematoxylin. Arrows: 11b5+ vessels. Arrowheads: 11b5 negative vessels. Original magnification x 60 (B) Number of $11b5^{+}$ vessels per mm^2 of para-sagittal marrow area. Mean ± SEM.

3.4.5 MMP-9, MCP-1, IL-8 or Osteopontin Are Not Required for Angiogenic Activity of OCL Conditioned Media

To further verify the mechanism used by OCL to stimulate angiogenesis, we examined the angiogenic activity of candidate angiogenic factors secreted by OCL using the HUVEC / fibroblast *in vitro* angiogenesis assay. These experiments suggested that none of these factors are important on their own for angiogenic action of OCLs directly upon endothelial cells.

We examined possible direct angiogenic activity of OCL-derived MMP-9 with a neutralizing antibody to MMP-9 *in vitro*, using an antibody concentration 3 fold higher than previously reported to neutralize MMP-9 activity.[277] Neutralization of MMP-9 activity did not affect angiogenesis in the basal state. When stimulated by OCL conditioned media MMP-9 inhibition surprisingly had a slight stimulatory effect on angiogenesis (Figure 3.8). Thus, OCL-derived MMP-9 is unlikely to stimulate endothelial cells directly.

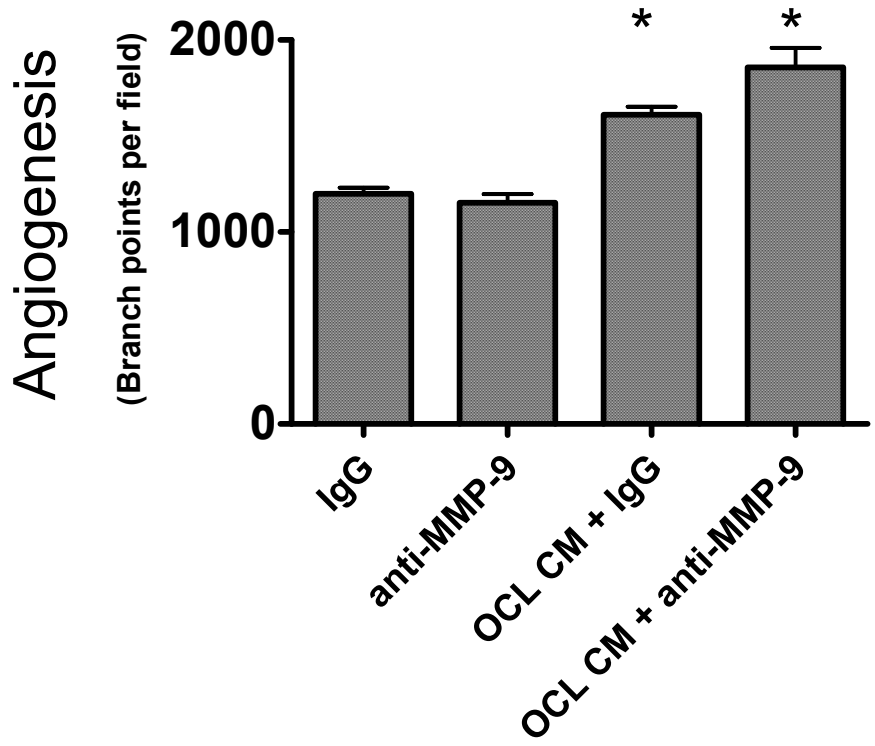


Figure 3.8. MMP-9 inhibition does not decrease the angiogenic activity of osteoclast conditioned media *in vitro*.

Concentrated OCL conditioned media or control media was combined with angiogenesis assay media and pre-incubated with 3 μg/ml neutralizing anti-MMP-9 or control IgG for 1 hour at 37°C, then used to stimulate the HUVEC / fibroblast angiogenesis assay. The assay was stained for CD31 and angiogenic tube formation was quantified with Metamorph software.

Because Tanaka et al reported that osteopontin is important for the angiogenic activity of OCLs in the HUVEC / fibroblast *in vitro* angiogenesis assay, we tested the angiogenic activity of recombinant osteopontin in the same assay.[5] However, we were not able to replicate their results that 1 μg/ml rh osteopontin stimulated angiogenesis (Figure 3.9). In our system, the working concentration of osteopontin derived from OCL conditioned media is also approximately 1 μg/ml as measured by ELISA (data not shown).

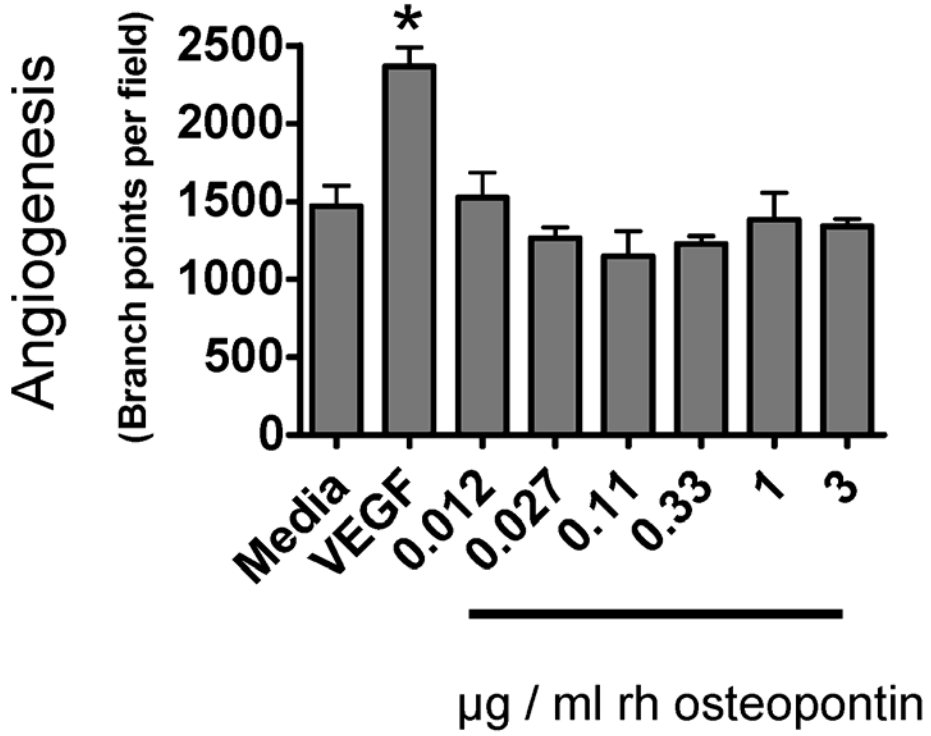


Figure 3.9. Recombinant osteopontin does not stimulate angiogenesis *in vitro*.

2 µg / ml rh VEGF or indicated concentrations of rh osteopontine were added to the HUVEC / fibroblast angiogenesis assay. Mean ± SEM *: $p < 0.05$ from media control.

In the human OCL angiogenesis arrays, IL-8 and MCP-1 were the most highly expressed non-proteinase angiogenic factors. Therefore, I also investigated if they were important for the angiogenic activity of OCL conditioned media. I first performed ELISA experiments to determine the concentrations of MCP-1 and IL-8 secreted by OCLs. IL-8 was low ng / ml and MCP-1 was at high pg / ml at the working concentrations in OCL CM used for *in vitro* angiogenesis assays (data not shown).

To determine if IL-8 was required for the pro-angiogenic activity of human OCL conditioned media, I first performed an IL-8 dose response curve in the presence or absence of IL-8 neutralizing antibody. I chose a concentration range that was obtainable using human OCL

conditioned media. I did not observe any stimulatory effect at rh IL-8 concentrations up to 9 ng/ml (Figure 3.10A). Curiously, 1 ng/ml IL-8 slightly inhibited angiogenesis. This suggests that human OCLs probably do not secrete enough IL-8 under these conditions to explain their stimulation of the assay. In a separate experiment, no stimulation was seen with 100 ng / ml IL-8 (data not shown).

Similarly, I also neutralized IL-8 in OCL conditioned media added to the human *in vitro* angiogenesis assay (Figure 3.10B). As was suggested by the IL-8 dose response curve, IL-8 neutralization had no effect on the capacity of OCL conditioned media to stimulate angiogenesis. However, IL-8 neutralization did reduce the stimulation of the assay by recombinant VEGF. This observation may be due to inhibition of endothelial cell autocrine IL-8.[239] Thus, IL-8 does not appear to be an important factor in OCL stimulation of angiogenesis.

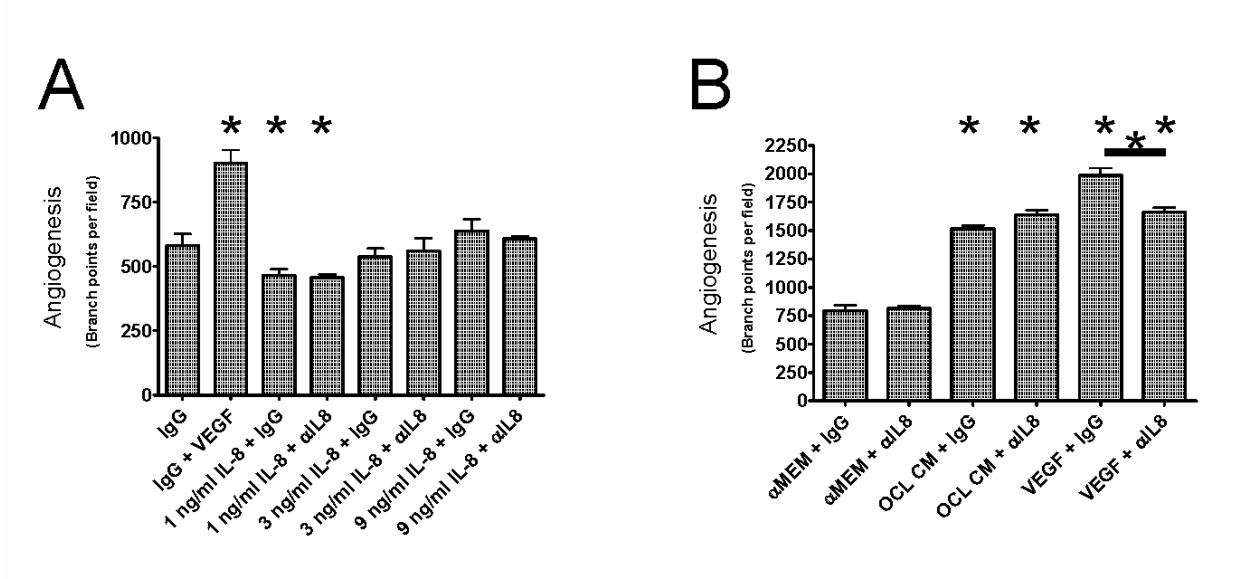


Figure 3.10. IL-8 is not responsible for the angiogenic activity of osteoclast conditioned media.

(A) rh $VEGF_{165}$ (2 ng/mL) or rh IL-8 was added to the HUVEC / fibroblast *in vitro* angiogenesis assay after a 30 minute pre-incubation at the indicated concentrations with mouse IgG or mouse anti-human IL-8 at 0.5 μg/mL. Number of branch points were quantified. (B) rh $VEGF_{165}$ (2 ng/mL) or CM from purified human OCLs was added to the human endothelial cell / fibroblast *in vitro* angiogenesis assay after a 30 minute pre-incubation at the indicated concentrations with mouse IgG or mouse anti-human IL-8 at 1 μg/mL. Mean ± SEM. * $p < 0.05$ by ANOVA.

We also tested whether MCP-1 might be an important OCL derived angiogenic factor because it was also highly expressed on the human OCL quantitative PCR array. However, doses of MCP-1, including doses higher than could be obtained from OCL CM, failed to stimulate the human *in vitro* angiogenesis assay (Figure 3.11)

Thus, it seems unlikely that IL-8, MCP-1 or osteopontin at least by themselves are the key angiogenic factor in human OCL conditioned media.

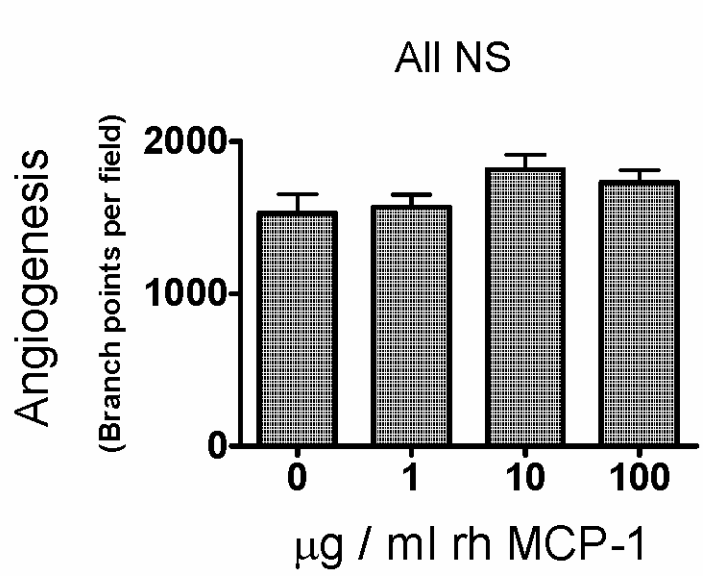


Figure 3.11. Recombinant MCP-1 does not stimulate angiogenesis *in vitro*.

rh MCP-1 added to human fibroblast / HUVEC co-culture angiogenesis assay at doses indicated. Mean ± SEM

3.5 CONCLUSIONS

In studies to examine the mechanism(s) responsible for OCL stimulation of angiogenesis we determined that MMP-9 plays an important role in OCL stimulation of angiogenesis, as well as bone remodeling, with both the angiogenic and bone resorptive effects of PTHrP being absent in MMP-$9^{-/-}$ metatarsal explants. Similarly, the pro-angiogenic and bone resorptive effects of RANKL were reduced in MMP-$9^{-/-}$ calvaria *in vivo*. The reduced angiogenesis seen in MMP-$9^{-/-}$ explants or mice treated with PTHrP or RANKL most probably reflects the decreased OCL numbers and activity in MMP-$9^{-/-}$ mice at the site where angiogenesis occurs, compared to WT controls. The number of vessels per OCL was not different between genotypes, suggesting that OCLs lacking MMP-9 do not have an intrinsic angiogenic deficit once they have formed and

migrated to the proper location. Similarly, PTHrP stimulated both angiogenesis and resorption in wild type bone explants, but did not stimulate either angiogenesis or resorption in explants lacking MMP-9.

The observed reductions in stimulated OCL formation or function in MMP-9 mice$^{-/-}$ are most likely due to reduced OCL migration rather than direct effects of MMP-9 on OCL differentiation or matrix solubilization. Explants from E17 MMP-9$^{-/-}$ mouse metatarsals but not more mature bones show a lower level of basal resorption but not OCL number, compared to wild type due to delayed OCL invasion of the primary ossification center (growth plate) rather than a requirement for MMP-9 in biochemical matrix solubilization.[161] Likewise, MMP-9 is required for migration of OCL or OCL precursor cell lines *in vitro*.[253-255] MMP-9$^{-/-}$ and WT mice did not display different levels of OCL formation *in vitro*, showing that the decreased resorption or OCL numbers in explants or *in vivo* was not due to defective differentiation or decreased precursor numbers. Because of our observations that MMP-9 is important for both bone resorption and angiogenesis under conditions of increased osteoclastogenesis, it may be possible clinically to inhibit both bone destruction and angiogenesis with a MMP-9 inhibitor. These findings are summarized in Firure 3.12.

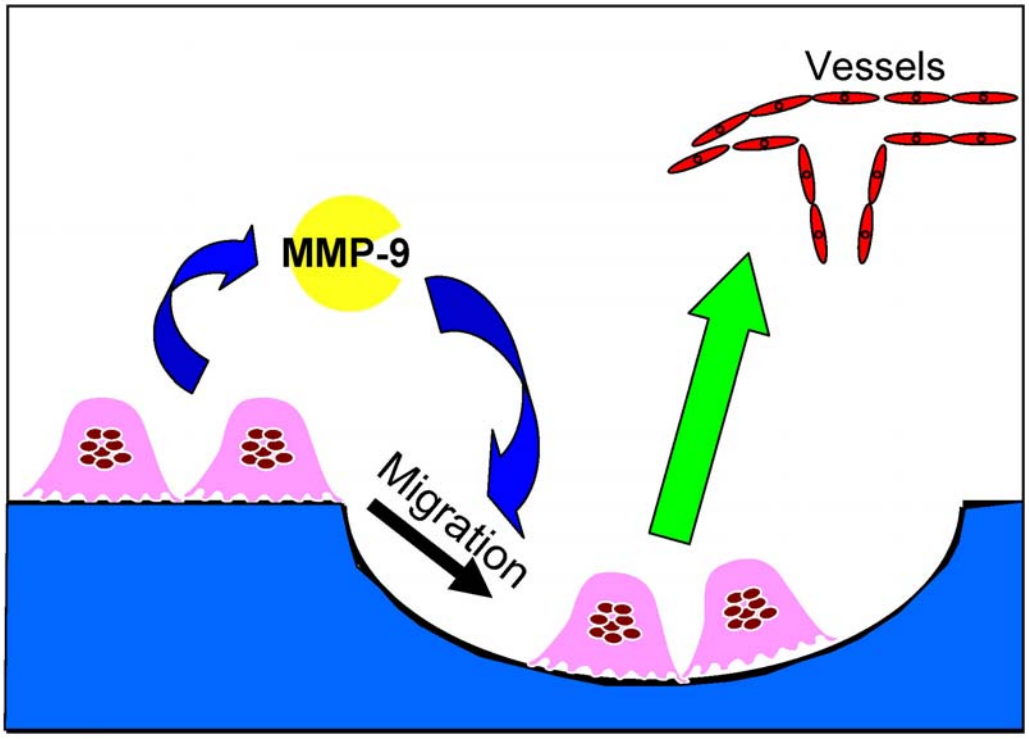


Figure 3.12. MMP-9 is important for osteoclast stimulated angiogenesis due to its effects on osteoclast migration.

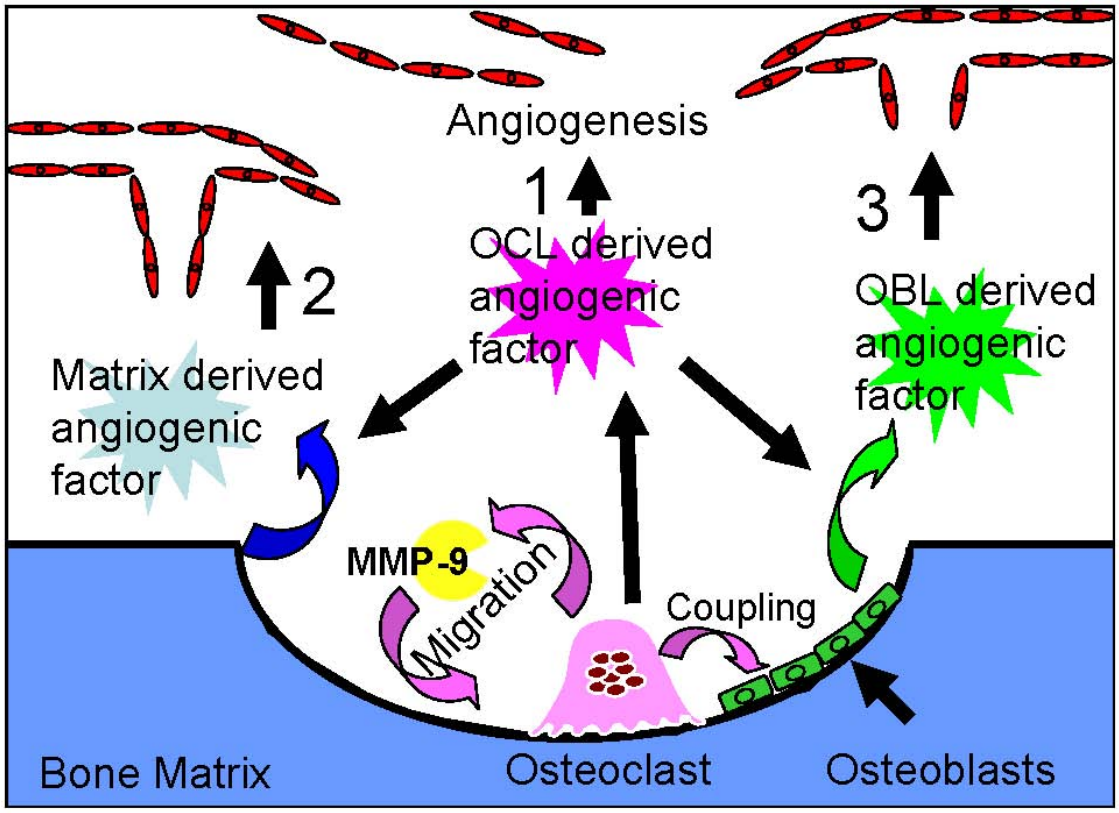
MMP-9 is predominantly expressed by OCLs in bone and is expressed at high levels, suggesting that the MMP-9 required for OCL stimulation of angiogenesis is secreted by OCLs themselves. I speculated in the introductory chapter that OCLs may stimulate angiogenesis through MMP-9 or other proteinase mediated released of VEGF from extracellular matrix. This molecular action of MMP-9 occurs in bone and is important for OCL recruitment into the primary ossification center.[161] Further, MMP-9$^{-/-}$ mice have delayed vessel invasion into the primary ossification center.[14] We investigated the possibility that OCLs stimulate angiogenesis by VEGF release by examining the level of VEGF in complex with its receptors *in vivo* with an antibody that specifically recognizes these complexes. Our data that a minority of vessels in PTHrP-treated mice, and no vessels in RANKL-treated mice were positive for VEGF /

receptor complexes suggests that OCLs stimulate angiogenesis primarily by other processes. The molecule(s) that act on vessels directly as a result of OCL stimulation *in vivo* remain unclear.

I investigated how OCLs may stimulate angiogenesis by secretion of an angiogenic factor, that acts directly on endothelial cells by analyzing the angiogenic factors important for angiogenic activity of OCL conditioned media. I did not find that any of the OCL-derived angiogenic factors were important on their own for direct stimulation of endothelial cells. Tanaka *et al* have reported OCLs stimulate angiogenesis in cell culture, and attributed the activity of their conditioned media to osteopontin.[5] They showed that rh osteopontin stimulated *in vitro* angiogenesis, which was neutralized by an antibody to αvβ3 integrin, and that a neutralizing antibody to osteopontin neutralized the angiogenic activity of OCL conditioned media. However, in contrast to these results, I was unable to detect any pro-angiogenic effects of rh-osteopontin at doses up to 3 fold the reported dose. The basis for these differences is unknown. These investigators did not determine if OCL-secreted osteopontin contributed to angiogenesis *in vivo*. Likewise I found that MMP-9 inhibition did not decrease the angiogenic activity of OCL conditioned media. Therefore, OCL-derived MMP-9 is unlikely to stimulate angiogenesis by direct action on endothelial cells. Similarly, recombinant IL-8 did not stimulate *in vitro* angiogenesis at doses attainable by OCL conditioned media, and an IL-8 neutralizing antibody did not inhibit the angiogenic activity of OCL conditioned media. Likewise, recombinant MCP-1 did not stimulate *in vitro* angiogenesis at doses attainable by OCL conditioned media. Therefore, IL-8 and MCP-1 are also unlikely to be responsible for the angiogenic activity of OCL conditioned media.

Because I found that MMP-9 is required for OCL stimulated angiogenesis predominantly due to its effects on OCL migration, it is likely that there are additional factors produced as a result of increased osteoclastogenesis that are important for vessel stimulation. Three possible sources of these factors are diagrammed in Figure 3.13. Most simply, OCLs may stimulate angiogenesis through direct secretion of an angiogenic factor (mechanism #1). However, more complex mechanisms may also occur. OCLs may stimulate angiogenesis by release of one of the many angiogenic factors stored in extracellular matrix (mechanism #2) Alternatively, OCLs may require a linked osteoblastic response to stimulate angiogenesis or may stimulate expression of an angiogenic factor in osteoblasts or other cells (mechanism #3). Since osteoblast-derived VEGF is clearly important for angiogenesis, osteoblasts and OCLs may cooperate to stimulate angiogenesis through OCL stimulation of osteoblastic production of VEGF.

My findings that MMP-9 affects both angiogenesis and OCLs suggests osteoclastogenesis and angiogenesis are linked in some situations. Linkage between OCLs and vessels occurs most likely in situations where OCL invasion is required such as fetal long bones before invasion of the growth plate, long bone fracture healing or our observed remodeling of calvaria induced by RANKL. OCLs most likely invade from the periosteum in these situations. Such linkage between osteoblasts and angiogenesis occurs during development of long bones.[15] Linkage between OCLs and vessel formation could result from endothelial cell stimulation of OCL formation. Endothelial cells can increase OCL formation by several mechanisms including increased RANKL expression on their surface, thereby stimulating OCL formation when co-cultured with OCL precursors.[140] Endothelial cells may also regulate the recruitment of OCL precursors to remodeling sites from the vascular compartment.[141]



1. Direct secretion of an angiogenic factor
2. Release of an angiogenic factor from matrix.
3. Induction of an angiogenic factor in osteoblasts or other cells.

Figure 3.13. Possible sources of direct acting angiogenic factors.

In summary I have shown that OCLs contribute to angiogenesis *in vitro* and *in vivo* by a mechanism requiring MMP-9. MMP-9 is important for OCL resorption and formation, likely due to its previously reported effects on OCL invasion or migration rather than differentiation or matrix solubilization. OCL-stimulated angiogenesis is deserving of further study to more precisely define the molecular mechanism(s) involved and to identify the physiological and pathological settings in which OCL-stimulated angiogenesis plays a role.

4.0 GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

4.1 GENERAL CONCLUSIONS

4.1.1 The Osteoclast Is an Angiogenic Cell in Bone

I have shown in this dissertation that osteoclasts are important for bone angiogenesis in a bone organ culture system and an *in vivo* model. Prior to these studies, a possible role for the osteoclast in angiogenesis was unclear, with no published reports that osteoclasts were involved in angiogenesis *in vivo*. There were reports that osteoclast conditioned media stimulated angiogenesis *in vitro* published during the course of my studies.[5, 150]. However, two studies specifically reported that osteoclasts were not important for angiogenesis *in vivo*.[4, 149] Integrating our results with those in the literature suggests that osteoclasts are important for angiogenesis at some, but not all, anatomic sites.

The studies reporting that osteoclasts were not important for angiogenesis *in vivo* used bisphosphonates and / or osteopetrotic (lacking osteoclasts) mice to abolish osteoclast activity, and analyzed developmental angiogenesis in mouse tail vertebrae or in the epiphysis of the tibia.[4, 149] I did not observe any increase in angiogenesis with osteoclast stimulation with PTHrP in femorae, but did find a different pattern of vessels in the metaphysis adjacent to the growth plate. I have not analyzed vertebrae for an angiogenic response to PTHrP treatment. In

my *in vivo* studies using RANKL, I did not analyze sites other than the calvaria because the RANKL was injected locally. In the discussion section of the paper reporting the phenotype of MMP-9$^{-/-}$ mice, the authors reported that the phenotype of osteopetrotic mice (lacking osteoclastes c-fos$^{-/-}$ and op/op) mirrored the phenotype of MMP-9$^{-/-}$ mice; lengthened growth plate and delayed vessel invasion on the metaphyseal side of the growth plate. Of note, these investigators did not observe any angiogenic defects in epiphyses of MMP-9$^{-/-}$ or osteopetrotic mice. Thus it is likely that the relationship of osteoclasts to angiogenesis is different in epiphyses and metaphyses. Therefore, it must be determined at each bone site of interest if osteoclasts do or do not contribute to angiogenesis.

Of note, in both the organ culture system and calvaria stimulated with RANKL, osteoclast-stimulated angiogenesis was accompanied by a large amount of tissue modeling or remodeling. During the time course of the metatarsal cultures, osteoclasts invade the primary ossification center. With supra-calvarial RANKL treatment, the angiogenesis occurred in areas where calcified tissue was replaced by non-calcified tissues resembling active periosteum. This process is reminiscent of endochondral ossification, where osteoclasts and vessels invade from the periosteum. Thus, physiological processes accompanied by changes in tissue composition are likely candidates for an important role of osteoclasts in angiogenesis.

If osteoclasts do primarily stimulate angiogenesis in situations of tissue modeling or remodeling, they are likely to stimulate angiogenesis by complex mechanisms involving several cell types. As discussed in Chapter 3, osteoclasts and osteoblasts may co-operate to stimulate angiogenesis, and angiogenesis and osteoclastogenesis may be linked in some situations due to endothelial cell stimulation of osteoclastogenesis.

4.1.2 MMP-9 Is Required for Osteoclast-Stimulated Angiogenesis

Because MMP-9 is primarily expressed by osteoclasts in bone and is important for angiogenesis in fracture healing and endochondral ossification, I determined if MMP-9 was important for osteoclast-stimulated angiogenesis.[14, 164] My results were similar in both experimental systems. MMP-9 blunted both the pro-angiogenic effects and osteoclastogenic effect of osteoclast stimulatory agents in metatarsal explants treated with PTHrP and calvaria treated with RANKL. The importance of osteoclastic MMP-9 for angiogenesis resulted primarily from its capacity to stimulate osteoclast migration, a capacity which was reported prior to my studies.[12, 253] The number of vessels per osteoclast did not differ between genotypes. This suggests that lack of MMP-9 did not reduce angiogenesis in our studies because of an angiogenic defect intrinsic to the osteoclast, but because of a reduced number of osteoclasts at the angiogenic site. Likewise, osteoclast differentiation *in vitro* was not inhibited by lack of MMP-9. Thus, MMP-9 likely decreased the number of osteoclasts at the angiogenic site due to decreased migration rather than precursor number or differentiation. In MMM-$9^{-/-}$ calvaria, there was a small increase in osteoclastogenesis but no increase in angiogenesis. Therefore, future studies need to determine if an intrinsic, albeit minor, angiogenic defect of MMP-$9^{-/-}$ osteoclasts is present. However, I cannot make this conclusion from the current data.

4.2 FUTURE DIRECTIONS

The studies presented in this dissertation are extremely novel and should fundamentally change the way that angiogenesis in bone is understood. Thus, they open up many new avenues

for research. Firstly, studies should be performed to extend the findings presented here. In particular, studies are required to definitively show if the MMP-9 required for osteoclast-stimulated angiogenesis is derived from osteoclasts themselves. Also, a more definitive examination of the anatomic sites where osteoclasts are important for angiogenesis is required. Secondly, it is very important that the mechanisms used by osteoclasts to stimulate angiogenesis are worked out in more depth. I have shown that MMP-9 is required for osteoclast-stimulated angiogenesis, but the molecule(s) which directly acts on endothelial cells has yet to be determined. Lastly, because angiogenesis in bone is important to many disease processes, further studies based on this work may uncover novel therapeutic approaches, increase our understanding of pathophysiology or explain the effects of anti-osteoclast therapeutics. Each of the sub-headings below could comprise a publication-sized unit. Many of these studies could be performed using the experimental techniques established in this dissertation.

4.2.1 Extension of Findings

An important series of studies suggested by our observations that osteoclasts stimulate angiogenesis by a MMP-9 dependent mechanism would be to specifically knock out MMP-9 in the OCL and perform similar studies as were performed in Chapter 3. These types of experiments would permit the determination if the lack of osteoclastic MMP-9 blunts the pro-angiogenic effects of PTHrP on metatarsal explants or RANKL on calavaria. We and others have shown that MMP-9 is primarily expressed by OCLs in bone. Therefore, it is likely that the MMP-9 required for OCL-stimulated angiogenesis is secreted by OCLs themselves. An OCL specific MMP-9 knockout would resolve this question. Our laboratory created a mouse line expressing Cre-recombinase controlled by the TRAP promoter, which is expressed in OCL and

committed OCL precursors. However, to our knowledge, no one has created a floxed MMP-9 line, likely due to the viability of MMP-$9^{-/-}$ animals. Therefore, to create an OCL-specific MMP-$9^{-/-}$ mouse, the gene must be floxed and bred to the existing TRAP-Cre mouse line. An OCL specific MMP-$9^{-/-}$ mouse may also benefit the study of additional anatomic sites where OCLs may be involved in angiogenesis. In this dissertation, I examined OCL-stimulated angiogenesis in detail in calvaria, and to a limited extent in long bones, but not in vertebrae.

Before the creation of an OCL-specific MMP-9 knockout, useful studies can be performed to further explore how OCLs play a role in angiogenesis. We intended to further confirm that PTHrP stimulated angiogenesis *in vivo* because of its OCL stimulatory effects, by blocking osteoclastogenesis with osteoprotegerin (OPG) or RANK-Fc in PTHrP and vehicle treated animals. This will allow us to determine if PTHrP is able to stimulate angiogenesis *in vivo* in the absence of OCLs. However, we were unable to acquire sufficient quantities of osteoprotegerin or RANK-Fc that was active in our *in vivo* system. Commercially available OPG-Fc from R&D systems had minimal activity. The only formulations of OPG-Fc or RANK-Fc reported to be active *in vivo* were developed by Amgen.[279] Unfortunately, Amgen declined to provide us with these reagents. Similarly, PTHrP treatment of MMP-$9^{-/-}$ and wild type mice would show if MMP-9 is required for angiogenesis stimulated by PTHrP. These studies are ongoing.

In preliminary studies not presented here, an OCL-specific (TRAP promoter driven) transgenic mouse for the matrix proteinase ADAM8 had increased calvarial vessel density, but these studies are not yet confirmed. These studies would support an angiogenic role for OCLs and suggest a role for ADAM-8 in OCL-stimulated angiogenesis.

To perform the studies described in this section, it will be necessary to secure a reliable source of RANKL that is active *in vivo*. The mouse-RANKL-GST used *in vivo* in these studies was generously provided by Dr. F. Patrick Ross. Dr. Ross may not be able to provide any additional RANKL. We did not observe any OCL stimulation with a dose of 2 μg per mouse per day in a volume of 200 μl of rh RANKL from R&D Systems. A dose of 2 μg (R&D Systems) or 6 μg (Peprotech) of mouse RANKL per day for 3 days injected over the femur was used to locally stimulate osteoclastogenesis at that site.[280] Therefore it is likely that some commercial formulations of RANKL will be active in our system. Alternatively, a purification protocol for m RANKL-GST will have to be adopted.

4.2.2 Further Mechanistic Studies

Further dissection of the mechanisms responsible for osteoclast-stimulated angiogenesis is needed. Is OCL secretion of osteopontin or a yet unidentified angiogenic factor that directly acts on vessels important for OCL-Stimulated angiogenesis *in vivo*? Tanaka et al reported that osteopontin is required for the angiogenic activity of OCL conditioned media [5], but I was not able to replicate some of their findings. Osteopontin may not be important for OCL-stimulated angiogenesis *in vivo* because it is a very abundant protein (2% of non collagenous protein) and is primarily produced by osteoblasts rather than OCLs.[221] A possible role for osteopontin in OCL-stimulated angiogenesis *in vivo* or in organ culture could be tested by stimulating OCLs in mice or in metatarsals in wild type and osteopontin$^{-/-}$ mice and determining if lack of osteopontin blunted the pro-angiogenic effects.

Alternatively, are there intermediary cell types that respond to OCL and in turn produce angiogenic factors, and which cells are these? Osteoblastic production of VEGF is a reasonable

candidate for possible OCL-stimulated angiogenesis due to an intermediary cell type. Osteoblast VEGF is well-known to be involved in angiogenesis in bone.[15] OCLs may stimulate expression of an angiogenic factor such as VEGF in osteoblasts, or may increase angiogenesis induced by osteoblasts due to coupled bone formation. I investigated a possible requirement for VEGF in OCL-stimulated angiogenesis by determining if metatarsal angiogenesis stimulated by PTHrP was blocked by a VEGF neutralizing antibody. However, the VEGF neutralizing antibody greatly diminished the basal level of angiogenesis in the assay, making the results uninterpretable (data not shown). However, a similar approach may be used in organ culture or *in vivo* with an existing line of mice specifically lacking HIF-1α in osteoblasts and thus having greatly reduced osteoblastic VEGF expression. If osteoblastic VEGF is important for OCL-stimulated angiogenesis, angiogenesis stimulated by PTHrP or RANKL should be blunted in studies employing these mice.

Are angiogenic factors released from matrix important for the angiogenic potential of OCLs? Our studies suggest that OCL release of matrix bound VEGF is not primarily responsible for their ability to stimulate angiogenesis. However, other factors, notable TGF-β, may be released by OCLs to stimulate angiogenesis. TGF-β may stimulate angiogenesis directly or by induction of VEGF in osteoblasts.[216] TGF-β is likely to come from calcified bone matrix.[10, 270] Therefore, the $src^{-/-}$ mouse, in which OCLs form but do not resorb bone may be used to study a possible role for calcified matrix resorption and TGF-β.[53] These studies will have to be interpreted in light of possible effects of lack or src upon endothelial cells.

4.2.3 Role of Osteoclast-Stimulated Angiogenesis in Myeloma and Tumor Metastases

Much of the current research into bone metastases has been influenced by the "vicious cycle" hypothesis. In this model, tumor cells increase OCL formation by secreting factors such as IL-6. The resultant increased numbers of OCLs increase tumor growth due to release of factors such as TGF-β from matrix.[109] The increased numbers of OCLs induced by the tumor could increase angiogenesis. However, increased angiogenesis could also result from the increased tumor burden. Therefore, it is very challenging to directly study the contribution of OCL-stimulated angiogenesis to bone metastases. The discovery of the importance of MMP-9 for OCL-simulated angiogenesis may allow the more direct study OCL-stimulated angiogenesis in bone metastases. Because MMP-9 is predominantly expressed by OCLs, the use of a host mouse lacking MMP-9 may allow the study of OCL-stimulated angiogenesis. However, two studies suggest that this line of research may not yield dramatic results. Host derived MMP-9 has been reported to be important for angiogenesis (but not tumor burden) in a prostate xenograft model of bone metastasis.[281] Similarly, a recent report found that host derived MMP-7, but not MMP-9 was important to increase tumor burden in a model of breast cancer bone metastases, but the authors did not study angiogenesis.[257] Therefore, we will need to carefully consider the results of these studies before investing resources in the study of MMP-9 in bone metastases. If we decide to undertake these studies, we have techniques and models readily available. OCLs may not need to migrate to stimulate angiogenesis in tumors, so MMP-9 may be less important for OCL-stimulated angiogenesis in this setting.

If a OCL-specific MMP-9$^{-/-}$ mouse line is not created, we could study the effects of lack of host MMP-9 in two bone metastasis models: B16 melanoma ingrafted in genotype matched C57BL/6 or C57BL/6 MMP-9 $^{-/-}$mice, and 5TGM multiple myeloma ingrafted in in RAG-1$^{-/-}$,

MMP-9 $^{-/-}$ or RAG-1$^{-/-}$, MMP-9$^{+/+}$ mice. Important parameters include angiogenesis, tumor burden and bone resorption. We will be able to rapidly begin studies in the B16 model because the cells are of the same genetic background as the MMP-9$^{-/-}$ mice, which we have available, and concurrently establish a colony of RAG-1$^{-/-}$, MMP-9$^{-/-}$ mice. Myeloma metastasis will be established by two methods, intratibial, and supracalvarial injection, both of which have been used successfully in our laboratory. We chose to examine the calvarial site in addition to the more commonly examined long bones, because of our data showing the importance of OCLs and MMP-9 to angiogenesis in calvaria. In addition to its clinical relevance, myeloma is an interesting model to use to examine the contribution of OCL MMP-9 to angiogenesis because of the osteoblast suppression caused by myeloma cells. In our previous studies, it has been difficult to determine if osteoblasts were also required for OCLs to stimulate angiogenesis.

4.2.4 Role of OCL-Stimulated Angiogenesis in Fracture Repair

Osteoclasts are important for fracture healing as shown by delayed fracture healing or non-unions in osteopetrotic (ia/ia) rats or RANK$^{-/-}$ mice.[166, 282] Furthermore, consistent with our findings, non-stabilized tibial fractures have less vessel density in RANK$^{-/-}$ than WT mice.[166] Likewise, MMP-9$^{-/-}$ mice have increased numbers of non-unions and less vessel invasion of non-stabilized tibial fractures. However, the source of MMP-9 at this site is especially uncertain due to MMP-9 expression by inflammatory cells.[164] Thus, the literature suggests that osteoclast-derived MMP-9 is important for angiogenesis in fracture healing, and fracture healing in general. The distinction between stabilized and non-stabilized fractures is important because non-stabilized fractures heal by way of a cartiliage intermediate, which is

invaded by vessels and OCLs in a similar fashion as the primary ossification center during development, and in the fetal metatarsal assay employed in this dissertation.

If an OCL-specific MMP-$9^{-/-}$ mouse is created, it will be used in a tibial non-stabilized fracture model to determine if OCL-derived MMP-9 is important for angiogenesis and long bone fracture healing. However, angiogenesis in membranous (flat) rather than long bone fractures is poorly understood. Our results that RANKL stimulated angiogenesis is blunted in MMP-$9^{-/-}$ mice also suggests that OCL-derived MMP-9 is important for angiogenesis and fracture healing of membranous bone. To test this hypothesis, we will examine angiogenesis and healing of the calvarial defect model in global or OCL specific MMP-$9^{-/-}$ mice.[283] If OPG-Fc or RANK-Fc is available, we will also inhibit osteoclastogenesis, and determine if OCLs themselves are important for angiogenesis and fracture healing in membranous bone.

4.2.5 Role of OCL –Stimulated Angiogenesis in Bisphosphonate Treated Mice

An understanding of OCL-stimulated angiogenesis is of great importance to understanding the mechanism of action of bisphosphonates. Bisphosphonates are anti-resorptive agents first used for osteoporosis. They have shown great efficacy in bone metastases, and inhibit tumor growth and angiogenesis in bone.[190] Micro-molar concentrations of bisphosphonates inhibit endothelial cells *in vitro*.[191] However, it is unclear if this mechanism of action of bishphonates is realistic *in vivo* because OCLs are exposed to much higher levels of the drugs than endothelial cells.[189] Therefore, my findings that OCLs are important for bone angiogenesis, may help to explain why bisphosphonates are angiogenic *in vivo*. Moreover, bisphosphonates have also been found to inhibit tumor angiogenesis stimulated by macrophage

derived MMP-9 in other tissues.[13, 284] Therefore, bisphosphonates may inhibit OCL-stimulated angiogenesis by inhibiting their migration as well as their survival or resorption.

The *in vivo* models and techniques established in this dissertation could be used to determine if the anti-angiogenic effects of bisphosphonates are due to their effects on OCLs. In vitro experiments with bisphosphonates are difficult to interpret, as the cell types affected by the drugs may be very different due to the pharmacokinetics and deposition of the drugs in bone matrix. I found that zoledronate inhibits metatarsal angiogenesis and osteoclastogenesis in parallel at micro-molar concentrations. However, it was difficult to determine if this was due to toxicity or specific effects on OCLs and endothelial cells (data not shown). To address this question, if bisphosphonates block the angiogenic effects of PTHrP or RANKL *in vivo*, this would suggest that their anti-angiogenic effects are due at least in part to OCL inhibition. Interpretation of this study would require that bisphosphonates had no effect on vessels among the animals treated with the vehicle for PTHrP or RANKL. Furthermore, to determine if bisphosphonates may inhibit OCL-stimulated angiogenesis by inhibiting OCL expression of MMP-9, their effects on OCL MMP-9 expression could be determined *in vitro*. They should also be able to inhibit OCL-induced angiogenesis *in vivo* at the same doses required to inhibit MMP-9 expression.

Bisphosphonates are associated with osteonecrosis of the jaw (ONJ), especially in cancer patients. However, the mechanism by which they may cause ONJ is unknown. A recent report from a phase III trial of the RANKL monoclonal antibody denosumab found a comparable number of cases of ONJ in denosumab and zoledronate treated groups (Amgen website, August 3, 2009). This suggests that OCL inhibition in general, rather than specific properties of bisphosphonates, may cause ONJ. Bisphosphonate suppression of bone remodeling is one of the

most popular hypotheses for ONJ pathogenesis.[196] Decreased angiogenesis due to bisphonates may also play role in ONJ. Recently, ONJ was reported patients treated with the anti-VEGF antibody bevacizumab.[197] Furthermore, a small retrospective study found more cases of ONJ in cancer patients treated with bisphosphonates and bevacizumab than bisphosphonates alone.[198] ONJ is also linked to dental extraction or other surgery. Because of their ability to inhibit remodeling of the callus of fixed fractures in long bones, bisphosphonates may also contribute to ONJ due to affects on healing of dental trauma, such as extraction.[167, 168]

One could use the rat root socket healing after extraction model to study the possible importance of angiogenesis stimulated by OCLs and MMP-9 to pathogenesis of ONJ. This model is beginning to be used as an approximation of ONJ pathogenesis. Inhibition of OCLs or MMP-9 may inhibit both healing and angiogenesis in this model. A specific MMP-9 neutralizing antibody has been used *in vivo*, However, this may prove cost prohibitive.[285] Small molecules that are less specific for MMP-9 are available for *in vivo* use.[286] If sufficient quantities of *in vivo* active OPG-Fc or RANK-Fc are available, the role of osteoclasts in root socket angiogenesis and healing could also be studied.

In summary, osteoclasts stimulate angiogenesis and MMP-9 is important for this process. Future studies are needed to confirm and extend our findings in normal and pathological conditions associated with increased osteoclast number and activity.

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Another bibliography entry.

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