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C-mpl Expression in Osteoclast Progenitors: A Novel Role for Thrombopoietin in Regulating Osteoclast Development

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Abstract

C-MPL EXPRESSION IN OSTEOCLAST PROGENITORS: A NOVEL ROLE FOR THROMBOPOIETIN IN REGULATING OSTEOCLAST DEVELOPMENT. Calvin L Barnes, Yougen Xi, Kimberly M Wilson, Mark C Horowitz, and Melissa A Kacena. Section of Bone Biology, Department of Orthopaedic Surgery and Rehabilitation, Yale University, School of Medicine, New Haven, CT.

A new paradigm has evolved in which multiple regulatory interactions between the skeletal and hematopoietic systems have been identified. Previous studies have demonstrated that megakaryocytes (MK) play a dual role in skeletal homeostasis by stimulating osteoblast proliferation and simultaneously inhibiting osteoclast (OC) development. Here we identify a novel regulatory pathway in which the main MK growth factor, thrombopoietin (TPO), directly regulates osteoclastogenesis. To study the role of TPO in OC development, spleen or bone marrow (BM) cells ($2x10^6$ cells/ml) or BM macrophages (BMM, $1x10^5$ cells/ml) from C57BL/6 mice , as a source of OC precursors, were cultured with M-CSF (30 ng/ml) and RANKL (50 ng/ml) to induce OC formation. TPO (0.1-1000 ng/ml) and/or primary MK (0-0.5%), derived from C57BL/6 fetal livers, were titrated into these cultures and OC were identified as tartrate resistant acid phosphatase positive ($TRAP^+$) giant cells with >3 nuclei. There was a significant, up to 15-fold reduction in OC formed when MK were added to all OC generating cultures, $p < 0.001$. Moreover, if OC generating cultures did not contain MK or MK progenitors, TPO treatment significantly enhanced OC formation up to six-fold, $p < 0.01$. This data demonstrates that MK are responsible for the inhibition of OC formation and that in cultures containing MK or MK progenitors such as BM or spleen cells, that TPO acts indirectly to inhibit OC formation by stimulating megakaryopoiesis, whereas in the absence of MK or MK progenitors TPO directly enhances OC formation. This conclusion is further supported by Real-Time PCR data which demonstrates that OC progenitors express c-mpl, the TPO receptor, albeit at low levels when compared to expression of c-mpl on MK. Finally, we have begun to dissect the c-mpl signaling pathway in OC progenitors. We have found that TPO induces tyrosine phosphorylation of several specific cellular proteins in the JAK/STAT pathway. Thus, TPO acts in a somewhat paradoxical manner by inhibiting OC formation through the stimulation of MK, while simultaneously playing a direct role in enhancing osteoclastogenesis.

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The Osteoclast

Bone is a dynamic tissue due to its intimate functional relationship to bone marrow (BM). BM contains osteogenic, hematopoietic, and immune cells. Other cell lineages are also present in BM and are supported by the stromal cell system. The close juxtaposition of these cells makes BM the focus for many of the regulatory interactions required for homeostatic development of bone. Changes in these regulatory interactions may result in altered bone formation or resorption, providing clues as to how skeletal homeostasis is maintained. Bone remodeling is a coupled process of bone resorption followed by bone formation. This is a constant, balanced process and the interruption of either bone resorption or formation leads to pathological conditions.

The mature, multi-nucleated osteoclast (OC) is the cell that resorbs bone. Early studies, using electron microscopy demonstrated the ultrastructure of the actively resorbing OC, and revealed distinguishing features. These consisted of large numbers of lysosomes, vacuoles, and a specialized portion of its plasma membrane that lies adjacent to bone actively being resorbed, now referred to as the “ruffled border” (1). This portion of the plasma membrane is intricately folded with finger-like extensions into the cytoplasm. Adjacent to the ruffled border is the sealing zone, which is directly apposed to the bone and is composed of F- actin and punctuate protrusions of cytoplasm known as podosomes that are devoid of organelles (2). The actin filaments and folds of the ruffled border provide the OC with a controlled surface area where hydrogen and proteolytic molecules can selectively dissolve bone. It is now known that when the OC is induced to resorb bone, acidic vesicles containing proton pumps are recruited to the ruffled border by the actin network (3-6). These proton pumps (H+ ATPases) secrete hydrogen ions

and are accompanied by the concomitant release of chloride ions from chloride channels (7). The net result of this process is creation of an acidic environment with hydrochloric acid which demineralizes bone. Cathepsin K, a lysosomal protease, has been shown to degrade the organic bone matrix following demineralization (8, 9).

OC are hematopoetic in origin. Some of the first evidence was discovered using parabiosis techniques in which osteopetrotic mice and rats had their circulatory system attached to their wild type, normal counterpart and osteoclastic activity was detected in the osteopetrotic animals (10-12). Many recent studies demonstrate that OC are of the monocyte/macrophage lineage, although the identity of the earliest OC precursor remains elusive. This ongoing research is outside the scope of this paper. For a complete review, consult Roodman (13). Currently there are 3 competing models of OC formation. The first model states that the hematopoetic stem cell gives rise to granulocyte-macrophage progenitor cell, CFU-GM, and that this cell produces OC. The second model proposes that the hematopoetic stem cell produces CFU-GM, which in turn produces monocytes and macrophages, of which some can be induced to form OC. The third model argues that the hematopoetic stem cell produces CFU-GM and CFU-O, a distinct progenitor cell which matures and fuses to form OC, and that CFU-GM does not directly produce OC (13).

However, in spite of the controversy concerning the earliest stem cell, it has been known for some time that in vitro maturation of macrophages into OC requires marrow stromal cells or osteoblasts (OB) (14). It is now known that OB and marrow stromal cells produce macrophage colony stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B ligand (RANKL) and that these two factors are necessary for

osteoclastogenesis (15-18). More recently, it was discovered that OB and stromal cells also produce the soluble mediator osteoprotegrin (OPG) which acts as a decoy receptor for RANKL, competing with RANK, thus inhibiting OC formation (18, 19). Fig. 1 is a model illustrating the actions of RANKL, M-CSF, and OPG in OC development.

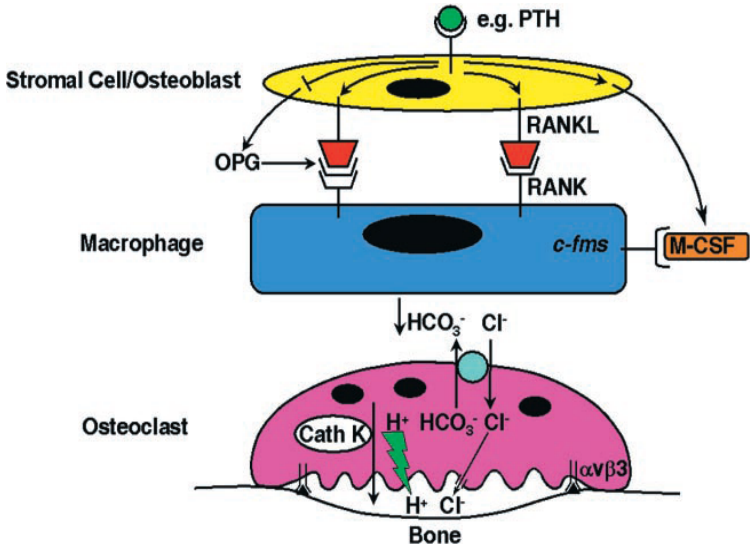

Figure 1. Model developed by Teitelbaum (42) depicting osteoclastogenesis and bone resorption. OB and stromal cells express RANKL and M-CSF which interact with their receptors, RANK and c-fms, respectively, on cells of the monocyte/macrophage lineage, initiating OC development. OPG acts as a decoy by binding RANKL and inhibiting OC formation. The mature OC attaches to the bone matrix and a ruffled membrane and sealing zone are created. This small area of bone matrix contained within the sealing zone is the microenvironment where bone resorption takes place. The acidic environment generated by the OC mobilizes the mineral phase of bone and allows for organic matrix degradation by cathepsin K.

It is becoming increasingly evident that within the stroma of the marrow, there exist a complicated network of paracrine, cell-cell, and cell-matrix interactions that work

in concert to regulate the production and activity of OC. Most notably, TNF-α and IL-1 have a direct stimulatory effect on OB and marrow stromal cells to produce RANKL and M-CSF, thus promoting osteoclastogenesis (20). Moreover, TNF-α and IL-1 have been shown to stimulate the production of adhesion molecules leading to increased cell-cell interactions between bone stromal cells and hematopoietic cells (21). Also, IL-6 and IL-11 have been shown to stimulate osteoclastic activity (22, 23). Several other factors are known to inhibit osteoclastogenesis. In addition to OPG, these inhibitory molecules include, but are not limited to: interleukin (IL)-4, IL-10, IL-12, IL-13, IL-18, interferon gamma (IFN-γ), transforming growth factor beta (TGFβ), granulocyte-macrophage-colony stimulating factor (GM-CSF), OC inhibitory lectin (OCIL), calcitonin, amylin, and calcitonin gene-related peptide (24-38). Therefore, it is likely that additional undiscovered inhibitory cytokines exist.

The Megakaryocyte

Megakaryocytes (MK) arise from pluripotential, hematopoetic stem cells and pass through a series of identifiable stages of differentiation, culminating in terminally differentiated MK. The major function of MK is the formation of platelets. This occurs by differentiated MK becoming ploidy through a process termed endomitosis. Endomitosis consists of DNA replication and a mitotic event with sister chromatid separation, but no cytokinesis (39). This leads to the formation of the demarcation membrane system, an extensive system of membranes within the MK cytoplasm, which gives rise to proplatelets. Proplatelets are the presumed structure between MK and platelets that have been observed in vitro and in vivo (40). Proplatelets are long sinuous

processes that extend from the MK and either fragment to form platelets or, alternatively, attenuation points form pinching off newly formed functional platelets (41).

The molecular dissection of the MK differentiation pathway has been greatly facilitated by the identification of transcription factors required for the cells successful advance from stage to stage (Fig. 2). Loss of these specific factors precludes the cells from continued maturation, and results in the accumulation of cells at the latest stage of differentiation prior to the arrest. The selective loss of two different transcription factors, GATA-1 and NF-E2, which were originally thought to be required exclusively for erythroid lineage development, have now been shown to play a critical role in MK differentiation. Knock-down mice in the case of GATA-1, and knock-out mice in the case of NF-E2, exhibit a phenotype characterized by a marked megakaryocytosis and thrombocytopenia (43, 44). The thrombocytopenia is a result of the maturational arrest of MK differentiation in which GATA-1 halts differentiation at a stage between immature and mature MK, where as NF-E2 arrests development at a later stage just prior to terminal differentiation (Fig. 2).

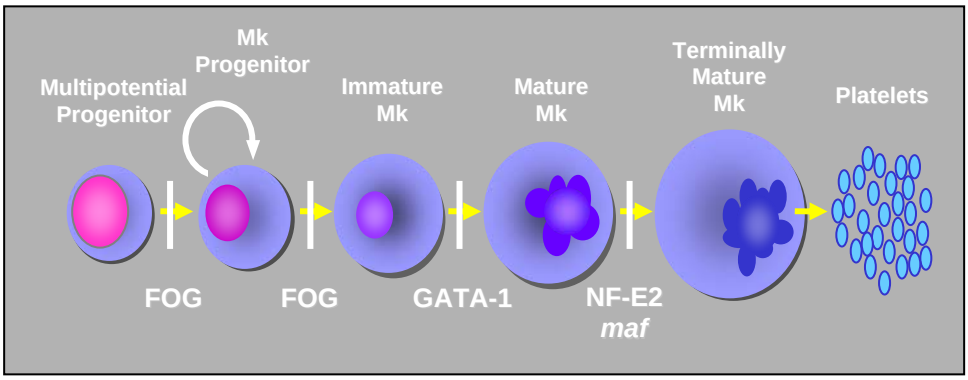

Figure 2. Schematic illustrating stages of MK differentiation and required transcription factors.

Thrombopoietin, C-mpl, and Megakaryocyte Signal Transduction

The cytokine, thrombopoietin (TPO) is the primary mediator of primitive megakaryocytic progenitor survival and proliferation, megakaryopoiesis, MK ploidy, and maturation (45-52). Its' receptor is the proto-oncogene c-mpl (53). The role of TPO and its receptor c-mpl in thrombopoiesis was elucidated in the early to mid 1990s. The pivotal experiments demonstrated that oligodeoxynucleotides antisense to c-mpl inhibited in vitro megakaryocytopoiesis (54). The role of c-mpl as the TPO receptor was further substantiated when it was shown that in murine models deficient in c-mpl, a thrombocytopenic phenotype existed (55). The ligand to c-mpl, TPO, was then purified and/or cloned by several research groups, clearly demonstrating its role as the primary regulator of thrombopoiesis (50, 51).

TPO is a 332 amino acid polypeptide containing two recognizable domains. The N-terminal region contains the receptor binding domain and is responsible for the biological activity of TPO (46). The N-terminal region has marked homology to erythropoietin (EPO) and its crystal structure has been identified as containing 4-α-helices arranged in an anti-parallel bundle fold (56, 57). This structural feature has been demonstrated in many cytokines including granulocyte colony-stimulating factor (G-CSF), GM-CSF, and growth hormone (GH) (58). The highly glycosylated, C-terminal region is thought to be responsible for protein secretion and stability (59). TPO was originally thought to be primarily produced by hepatocytes in the liver and convoluted tubular cells in the kidney. However, in the last decade it has become increasingly clear that TPO is also expressed in stromal cells within the BM (60-64). TPO has been

measured in blood in response to thrombocytopenic states caused by both antibody mediated and myelosuppressive techniques (65, 66).

C-mpl is a type I transmembrane protein belonging to the hematopoietic cytokine receptor family (67). Similar to the common beta chain (β_c) shared by IL-3, IL-5, and GM-CSF, the extracytoplasmic domain of c-mpl consists of duplicate cytokine receptor modules (CRM) (68). The cytoplasmic domain of c-mpl is highly conserved across species. There is 86% homology, at the amino acid level, between mouse and human mpl (69). The human c-mpl gene is composed of 12 exons (70). There are currently 4 identified splice variants of c-mpl. The predominant, full-length protein is referred to as the P-form. In humans, the P-form consists of a transmembrane domain and 122 cytoplasmic residues. The K-form diverges from the P-form after the 9^{th} cytoplasmic amino acid and terminates within intron 10, this protein does not transduce a proliferative signal and has no known biological activity; however, it is possible that mpl-K acts as a dominant negative receptor by heterodimerizing with mpl-P following ligand binding (71). The third splice variant, mpl-S, is a secreted form of mpl. Mpl-S results from alternative splicing of exon 8 directly to exon 11. This eliminates the transmembrane domain, the initial cytoplasmic residues, and the juxtamembrane WSXWS motif (70-72). The 4^{th} isoform, c-mpl-del, occurs from alternative splicing between exons 8 and 9 (lacks 72 bp), which results in the internalization of the receptor (73).

Two motifs in the cytoplasmic domain, box1 and box2, are conserved in the hematopoietic cytokine receptor family, and are 100% homologous between mouse and human mpl (69). Box1 appears to be required for signal transduction in all receptors examined, while box2 is not necessarily required for proliferative signals (74-78).

The signaling domain in c-mpl is 121 amino acids in mice and 122 amino acids in humans. It is immediately downstream of the hydrophobic transmembrane domain which is composed of 25 residues. While the c-mpl signaling domain is 91% homologous between mouse and human, it does not share significant homology to other proteins (79).

The signal transduction of TPO when it binds to c-mpl is very complex and is the subject of active research by several investigators. Evolution has seemingly fortified the TPO/c-mpl signal transduction mechanism with redundancy to ensure a proliferative signal will be transmitted (80). There is evidence that TPO signals proliferative events in target cells through a JAK/STAT pathway. It was demonstrated that when TPO binds to c-mpl, the receptor dimerizes, consequently tyrosine kinases, specifically JAK2 and TYK2, activate each other by transphosphorylation (81). It was later shown that JAK2, not TYK2 was required for mediated signaling (82). TPO is also known to induce activation of STAT5 and STAT3 following JAK2 activation (83). STAT proteins, once activated by tyrosine kinases, translocate to the nucleus and activate genes that signal cellular growth and division such as cyclin D and p21.

TPO has also been shown to signal proliferation in cells with a JAK-STAT deletion (84). This research group demonstrated that TPO mediated phosphorylation of Shc, vav, mitogen-activated protein kinase (MAPK) and Raf-1, which then lead to induction of c-fos and c-myc. Moreover, this group also demonstrated that wild-type and mutant c-mpl receptors activated phosphatidylinositol (PI) 3-kinase when stimulated with TPO and that TPO induced proliferation was suppressed in the presence of a PI 3-kinase inhibitor. It is now evident that the MAPK signaling cascade, Shc, Ras signaling, as well as PI 3-kinase, play some role in TPO signal transduction (85-90). More recently,

microarray profiles demonstrated increased kinase activity in Fyn and Lyn in human MKs after TPO stimulation, suggesting that Scr family kinases are also involved in TPO signal transduction (91). Protein Kinase C (PKC) may play a role as well, but its significance has not been tested (92, 93).

TPO, C-mpl and the Hematopoietic Stem Cell

There is a growing body of research demonstrating that TPO and c-mpl play some role in the stimulation, proliferation, and development of HSC. When c-mpl was first identified in 1993, it was shown to be expressed not only in MK and platelets, but in purified CD34+ cells from BM and blood monocytes by RT-PCR (54). However, more recently it was demonstrated by flow cytometry that 70% of murine HSC in BM are c-mpl positive and that genetic elimination of c-mpl reduces the potency of HSC to repopulate 7-fold (94). Dendritic cells, which develop from HSC, have also now been shown to express c- mpl (95). Additionally, TPO has been reported to have a synergistic effect with steel factor, an early HSC growth factor, and IL-3 to promote formation of multilineage HSC colonies (96). Soluble c-mpl has also been shown to act in synergy with steel factor to produce formation of multilineage colonies (97). Also, TPO has been shown to act synergistically with granulocyte colony stimulating factor (G-CSF) to promote neutrophil recovery in myelosuppressed mice (98).

Perhaps the most convincing evidence is from the study of c-mpl deficient mice. It has been shown that mice with a c-mpl knockout have lower numbers of not only MK progenitors and MK, but erythroid, granulocyte-macrophage, and multilineage precursor cells as well (99). C-mpl mice now serve as a model system to study the human disease

congenital amegakaryocytic thrombocytopenia (CAMT). CAMT is characterized by an essential thrombocytopenia which develops into a pancytopenia / aplastic anemia later in life. The disease has been shown to be caused by mutations in c-mpl, which further suggests a role of TPO and its receptor in HSC survival and development (100).

This body of evidence demonstrates that c-mpl is not conserved strictly to MK and is expressed on other cells at various stages of maturity in the hematopoietic lineage. This research also suggests that TPO can act synergistically with other cytokines to promote growth of granulocytes, macrophages, and erythroid progenitor cells. Therefore, given that OC are also of the hematopoietic lineage, it is plausible that c-mpl may be expressed on OC precursors and that TPO may play a direct role in osteoclastogenesis.

The Megakaryocyte and Bone

Evidence for a role of MK in bone formation comes from data indicating that MK express mRNA for, and secrete the bone matrix protein osteocalcin (39). The expression of osteocalcin by BM MK and peripheral blood platelets is significant in that these are the only known extra-osseous cells that secrete osteocalcin (39). In addition to osteocalcin, MK or platelets also secrete other matrix proteins including osteonectin, bone sialoprotein, and osteopontin (40, 41, 43, 44). More recently, bone morphogenetic proteins -2, -4, and -6 have been immunolocalized in MK (101). These data suggest that MK could, under the appropriate circumstances, contribute to bone formation by the secretion of these proteins. On the other hand, MK have also been reported to stimulate the differentiation of OB as defined by enhanced expression of procollagen (102).

A limited amount of evidence also suggests a possible role for MK in OPG mediated bone remodeling. In two investigations, immunohistochemical analysis showed that MK stained positive for an OPG specific antibody (102, 103). Bord et al (102) also showed that OPG expression increased in estradiol treated MK cultures as culture duration increased, MK maturation also increased. In another study, MK expression of OPG was documented using RT-PCR and immunolocalization techniques (104). Based on immunostaining, these authors suggested that larger, more mature MK contained more OPG. However, this latter study did not find demonstrable levels of OPG in human MK conditioned medium (CM). While not determined experimentally, it was theorized that the OPG, if secreted, binds significant amounts of the MK expressed RANKL (102, 105). On the other hand, OPG was detected in CM from MK isolated from C57BL/6 and GATA-1 mice (450±62 and 242±17 pg/ml, respectively) (106). Since MK from GATA-1 deficient mice are arrested at an immature stage of differentiation, this data lend further support to the hypothesis that more mature MK contain higher quantities of OPG (107). Moreover, the fact that MK appear to be a source of OPG implicates a potential role for MK in regulating OPG levels and RANKL expression and implies that MK could be involved in bone resorption and therefore bone remodeling.

Additional evidence for a role of MK in bone remodeling comes from studies of mice deficient in GATA-1 and NF-E2, the previously described transcription factors required for MK differentiation. Mice deficient in these transcription factors have a phenotype characterized by increased numbers of immature MK, a concomitant drastic reduction of platelets, and a striking increased bone mass (108-110). A marked 2-3-fold increase in trabecular bone volume and bone formation indices were observed in these

mice. A 20-150-fold increase in trabecular bone volume was measured for the entire femoral medullary canal. In vivo OB number and bone formation parameters were significantly elevated. When wild-type or mutant OB were cultured with MK from GATA-1 or NF-E2 deficient mice, OB proliferation increased 3-6-fold by a mechanism that required cell-to-cell contact. These observations demonstrated a novel interaction between MK and OB which resulted in an increased bone mass, a here-to-fore, unrecognized anabolic pathway in bone (110).

Cytokines Involved in Megakaryopoiesis and Osteoclastogenesis

Many of the pleiotropic cytokines that stimulate megakarypoiesis also play some role in osteoclastogenesis. As Fig. 3 depicts, these cytokines include but probably are not limited to IL-11, stem cell factor (SCF), leukemia inhibitory factor (LIF), IL-6, and G-CSF (111). IL-11, in addition to its role in many stages of MK differentiation, is known to stimulate macrophages (112, 113). Further IL-11 has been shown to upregulate RANKL expression in cells of the OB lineage, which in turn can enhance OC formation (114). Similarly, SCF, the ligand of c-kit, acts in the early stages of megakaryopoiesis and is also known to enhance osteoclastogenesis (115, 116).

LIF has been documented as being involved with the differentiation of multipotential progenitors into MK progenitors as well as the differentiation of MK progenitors into immature MK (111). LIF has been implicated in modulating OC differentiation by two separate mechanisms. First, LIF appears to upregulate RANKL expression in cells of the OB lineage while simultaneously decreasing OPG expression

(117). Additionally, LIF may upregulate c-fms expression in osteoclast precursors (118). Both of these effects enhance osteoclastogenesis.

IL-6 promotes megkaryopoiesis, megakaryocyte differentiation, and raises platelet levels in vivo (119-121). Like IL-11, IL-6 has been implicated in OC differentiation through the upregulation of RANKL by cells of the OB lineage. In addition to increasing RANKL expression, OPG expression is reduced with IL-6 stimulation, leading to enhanced osteoclastogenesis (122). G-CSF is important for the normal differentiation of multipotential progenitors into MK progenitors (111). G-CSF is also important in OC development. G-CSF facilitates the development of OC progenitors from HSC and overexpression of G-CSF in vivo induces osteopenia as a result of increased numbers of OC (123, 124).

Lastly, TPO, the main MK growth factor is critical for all stages of megakaryopoiesis (47, 113, 125). It is unknown whether TPO has a direct effect on osteoclastogenesis; however, given the wealth of data demonstrating an overlap of cytokines involved in both megakaryopoiesis and osteoclastogenesis, it is plausible that TPO could potentially directly affect osteoclastogenesis. The purpose of this current research endeavor is to determine whether TPO directly affects osteoclastogenesis.

Thrombopoietin and Bone

There are only a few studies examining the role of TPO in skeletal homeostasis. A myelofibrotic syndrome with osteosclerosis can be induced in mice by either repeated injections with TPO or by infecting mice with a virus vector carrying the TPO gene (126-

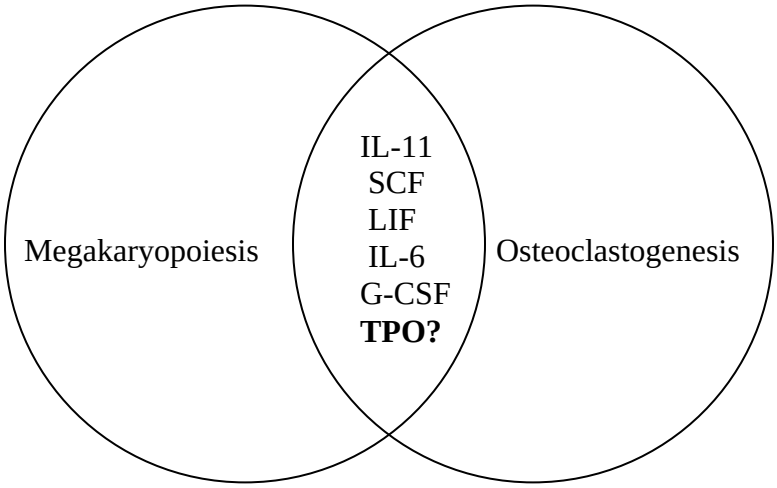

Figure 3. Pleiotropic cytokines involved in megakaryopoiesis and osteoclastogenesis.

128). The fibrotic disease was prominent in mice over expressing the factor at 9 months of age. TGFβ-1 and platelet derived growth factor (PDGF), which are secreted by MK, were increased 2-fold and 5-fold, respectively, in the platelet-poor plasma of the TPO overexpressing mice as compared to controls (128). These data suggest that the MK secrete increased amounts of TGFβ-1 and PDGF, which are responsible for the myelofibrosis and osteosclerosis. This theory is also supported by recent work by Vanucchi et al (129). These studies have shown that GATA-1 low mice and TPO overexpressing mice both have a myelofibrotic phenotype and increased TGFβ-1 levels in the extracellular fluid, therefore implicating the MK as the source of TGFβ-1 increase that leads to myelofibrosis (129). Moreover, it has been shown that in mice with TGFβ-1 deficient BM, TPO overexpression does not induce myelofibrosis (130).

Conversely, another study suggests that upregulation of OPG causes an osteosclerotic phenotype in TPO overexpressing mice by inhibiting osteoclastogenesis (131). These authors theorized that the OPG was derived from the stromal cells. In light of the data showing that OPG is secreted by MK and the reports of OPG being expressed or localized in MK, it is possible that MK could also be directly contributing to the OPG levels described in the TPO overexpressing mice (102-104). Alternatively, like MK, other non-mesenchymal/stromal cells could also be contributing to the increased OPG levels.

A recent study by Kakumitsu et al (132) also supports a role of OPG and TGFβ-1 in the myelofibrotic, osteosclerotic phenotype in TPO overexpressing mice. Using transgenic mice that continuously secrete TPO, this group demonstrated that the mice develop anemia, myelofibrosis, and osteosclerosis. These mice had elevated numbers of MK, with an accompanying significant increase in the plasma levels of TGFβ-1 and OPG. Interestingly, these mice also demonstrated an increased number of peripheral neutrophils in the blood, granulocytic immature cells, myeloid precursor cells, and erythroid precursor cells in the BM despite the myelofibrosis and subsequent anemia, further implicating a role for TPO in HSC survival and development.

Another investigation suggested that the mechanism of the increased bone phenotype in TPO overexpressing mice was due to the inhibition of osteoclastogenesis (133). In this study the effect of TPO on OC formation in vitro was examined. The data showed that TPO reduced OC number in a dose-dependent manner. However like the other previously mentioned studies, Wakikawa et al (133) reported that MK number and maturation increased in these experiments.

The confounding factor in all of these studies is the presence of MK. Because TPO stimulates MK proliferation, adding TPO to BM co-culture systems that contain MK and/or MK progenitors, will by definition increase the number of MK in the culture compared to cultures containing no TPO. Therefore, it is possible that the increased number of MK is responsible for the inhibition of OC formation seen by Wakikawa et al (133). Indeed, recent studies have shown that MK cultured in the absence of TPO, inhibit OC formation in a dose-dependent manner (106). In addition, MK CM (TPO-free) also inhibited OC formation in a dose-dependent manner (106). Therefore it appears that TPO may play an indirect role in bone resorption by inhibiting OC formation by increasing MK.

The role of TPO in bone homeostasis remains unclear due to the presence of MK in these prior studies. The purpose of our research is to better characterize the relationship between TPO and OC and to determine if a direct relationship exists independently of MK.

Hypothesis and Aims

From our review and analysis of the literature, there were three aspects which highlight the purpose of this research. First, it was evident that c-mpl was not only expressed in MK, but in other cells derived from HSC such as dendritic cells. Furthermore, it was demonstrated that TPO acts synergistically with other cytokines to stimulate growth of erythroid, granulocyte, and macrophage progenitors and therefore was not solely restricted to cells of the MK lineage. Second, it was becoming increasingly evident that several cytokines stimulate both megakaryopoiesis and osteoclastogenesis such as IL-11 and G-CSF. Third and last, there was substantial evidence supporting an indirect role for TPO in inhibition of osteoclastogenesis by promoting megakaryopoiesis; however, a more precise relationship between TPO and OC without the confounding effects of MK has yet to be studied or reported in the literature. To this end, we hypothesized that **OC precursors express c-mpl, the TPO receptor, and that TPO directly affects OC formation in vitro**. The following specific aims test this hypothesis:

Specific Aim I. To verify the ability of MK to decrease OC formation in vitro. OC progenitors were cultured in the presence of M-CSF and RANKL to promote osteoclastogenesis. Once OC were formed, cells were fixed, stained for tartrate resistant acid phosphatase (TRAP), and $TRAP^{+}$ multinucleated (>3) cells were counted. This control population was then compared to a population of OC progenitors that had been exposed to MK or MK CM to determine if a significant decrease in OC number had occurred.

Specific Aim II. **To test the effects of TPO to on OC formation in vitro in the absence of MK and/or MK precursors.** OC progenitors that did not contain MK or MK precursors (Pax5-/- spleen cell line [SCL] and BMM) were cultured in the presence of M-CSF and RANKL with or without TPO. Once OC were formed, cells were fixed, stained for TRAP, and $TRAP^+$ multinucleated (>3) cells were counted.

Specific Aim III. To characterize the expression of c-mpl in OC progenitors. C-mpl expression in OC progenitors was examined using Real-Time PCR (RT-PCR). RNA was isolated from MK and OC progenitor cells. The RNA was converted to cDNA, and subsequently purified prior to PCR amplification. MK served as a positive control for expression of c-mpl. C57BL/6 BMM, RAW264.7, and Pax5-/-SCL were the OC progenitor cells that were utilized.

Specific Aim IV. To begin dissecting the signal transduction pathway that occurs when OC are stimulated with TPO. In Aim IV, we tested the hypothesis that TPO induces receptor dimerization, tyrosine phosphorylation, and a series of signaling events that results in the enhancement of OC development. Primary BMM derived from 6-8 week-old C57BL/6 mice or the Pax5-/- SCL OC progenitors were used. Cells were serum starved overnight and then stimulated with 100 ng/ml of recombinant murine TPO for 0, 1, 3, 5, 10, or 30 minutes. As a positive control, primary MK derived from the fetal livers of E13.5-15.5 C57BL/6 mice or mpl-transfected, TPO dependent Ba/F3 cells were serum starved and then stimulated with TPO for 0 or 10 minutes. Lysates were collected and immunoblotted with antiphosphotyrosine antibodies (αP-Tyr).

Materials and Methods

1. <u>Preparation of BM Cells:</u>** Tibiae and femurs were dissected from 6-10 week old C57BL/6 mice, the epiphyses removed, and the marrow flushed with 2-3 mls of ice cold α-MEM with 10% FCS (vol/vol) using a 27 gauge needle and syringe. A single cell suspension was prepared and the cells were washed 2x prior to use.

2. <u>Preparation of Whole Spleen Cells:</u> Spleens from adult C57BL/6 mice were dissected out and placed in a sterile culture dish containing PBS with 2% FCS (vol/vol). The fibrous capsule was then broken, releasing spleen cells which were made into single cell suspensions. Fibrous tissue was removed and cells were washed 2x.

3. <u>Preparation of Fetal Liver cells:</u> Although fetal livers and adult spleens are good sources of MK, substantially more MK (at least 5x, data not shown) are obtained from fetal liver preparations (MA. Kacena). Fetuses were dissected from pregnant mice at E13.5-15.5. The livers were removed and single cell suspensions made by forcing cells thru sequentially smaller gauge needles (18G, 20G, 23G). Cells were washed 2x with DMEM + 10% FCS and then seeded (5 fetal livers/100 mm dish) in 100 ml culture dishes, in DMEM + 10% FCS + 1% murine TPO (128). After 3-5 days, when the cells become confluent, MK were obtained by separating them from the lymphocytes and other cells using a one-step albumin gradient to obtain a 95% pure MK population (134). The bottom layer was 3% albumin in PBS (Bovine Albumin, protease free, fatty acid poor, Serologicals Proteins Inc.), the middle layer was 1.5% albumin in PBS, and the top

layer contained the cells to be separated. All of the cells sedimented through the layers at 1g for approximately 40 minutes at room temperature. The MK fraction was collected from the bottom of the vessel. The non-MK (NM) derived fraction was composed of the remaining cells.

4. <u>Preparation of MK CM:</u> MK were isolated as described above, washed 2x, and seeded into 6-well plates at a concentration of $1x10^6$ cells/ml. They were cultured in α-MEM supplemented with 10% FCS. After 72 hrs, the CM was centrifuged, sterile filtered, aliquoted, and stored at -20ºC.

5*. <u>Preparation of Bone Marrow Macrophages (BMM):</u> BM cells were prepared as above. $5x10^7$ cells/ml were seeded into a 100 mm tissue culture dishes, in α-MEM supplemented with 10% FCS and 20 ng/ml of M-CSF (R&D Systems). After 3 days in culture, cells were lifted with trypsin, and were adjusted to a concentration of 100,000 cells/ml for OC generation as described below.

6*. <u>Preparation of Pax5-/- SCL:</u> Pax5-/- spleen cells ($2.5x10^6$ cells/ml) were prepared as above, and were cultured in 100 mm tissue culture plates in α-MEM with 10% FCS for 10 days. The nonadherent cells were washed away and the adherent cells were released from the dish by treatment with EDTA/trypsin (0.032%). Pax5-/- SCL is a purified monocyte/macrophage OC progenitor cell line developed by our laboratory, which does not contain MK, lymphocytes, or other accessory cells. The Pax5-/-SCL was chosen because OC develop from these cells in ~3 days, when cultured with M-CSF and

RANKL to induce OC formation. The Pax5-/- SCL was transferred and fed weekly (135).

7*. <u>In Vitro OC-like Cell Formation Models:</u> OC-like cells were generated by two previously described methods (136, 137). First, co-cultures containing $2x10^6$ spleen cells or BM cells/ml and 20,000 primary calvarial OB/ml were grown in α-MEM supplemented with 10% FCS and 10^{-8} M $1,25(OH)_2D_3$. The media was changed every other day for 6-8 days. Second, $2x10^6$ spleen cells or BM cells/ml or 100,000 Pax5-/- SCL or BMM were cultured in α-MEM supplemented with 10% FCS and 30 ng/ml of M-CSF (R&D Systems) and 50 ng/ml RANKL (R&D Systems). Media was changed every third day for 6-12 days (until OC formed). In each of the above models, once OC formed, the cells were fixed with 2.5% glutaraldehyde in phosphate buffered saline for 30 minutes at room temperature, stained for TRAP using a kit from Sigma, and $TRAP^+$, multinucleated (>3) OC cells were counted. TPO was titrated (0-1000ng/ml) into cultures.

8*. <u>RNA Extraction and RT-PCR:</u> Cells were washed 2x with PBS. RNA was isolated from the cells using a NucleoSpin II RNA Purification kit (BD Biosciences) incorporating an on-column DNase treatment to remove contaminating genomic DNA. For RT-PCR, cDNA was prepared from 5μg of total RNA using Sprint PowerScript Reverse Transcriptase (BD Biosciences) and oligo$(dT)_{12-18}$ primers. The cDNA was purified using an Amicon YM30 filter device (Millipore). Quantitative RT-PCR was

performed on a Cepheid Smart Cycler using Platinum Taq polymerase (Invitrogen) and Sybr Green I (Molecular Probes) incorporation.

The quantitative comparison between samples was calculated using comparative C_T. The data was normalized by subtracting the difference of the threshold cycles (C_T) between the gene of interest (c-mpl) and the C_T of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This value is defined as the ΔC_T. To compare the relative expression of c-mpl in two types of cells (e.g. primary C57BL/6 MK vs. primary C57BL/6 BMM) the $\Delta\Delta C_T$ must be calculated. $\Delta\Delta C_T$ for this example is the absolute value of the difference between the ΔC_T for the MK and the BMM or $|\Delta C_T$ MK - ΔC_T BMM|. The relative difference (fold increase or decrease) in expression is calculated as $2^{\Delta\Delta CT}$. The following were the sequences used for the c-mpl:

forward primer: 5' TCACCTTGGTGACTGCTCTG

reverse primer: 5' GGACTTAGGGCTGCAGTGTC

The following were the sequences used for GAPDH:

forward primer: 5' CGTGGGGCTGCCCAGAACAT

reverse primer: 5' TCTCCAGGCGGCACGTCAGA

9*. <u>Cell Stimulation and Lysis:</u> Seventy-five percent confluent cultures of C57BL/6 BMM and Pax5-/- SCL, C57BL/6 MK, or Ba/F3 cells were placed in reduced serum media (0.5%) for 16-18 hours. The cells were stimulated with human recombinant TPO (100 ng/ml) for 0, 1, 3, 5, 10, and 30 minutes. At the end of the incubation period, cells were immediately rinsed with two changes of ice-cold PBS containing inhibitors (100 μM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 200 μM sodium

orthovanadate). After aspirating the PBS/inhibitors, the cells were placed (adherent cells were scraped) in the appropriate volume of lysis buffer containing 20 mM Tris, pH7.2, 150 mM NaCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, and 5.0 mM EDTA. The following protease and phosphatase inhibitors were also added to the lysis buffer: 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 50 mM sodium fluoride. Lysates were centrifuged for 15 minutes a 12,000xg at 4°C and supernatants were collected. Protein concentration was determined using a Micro BCA protein assay kit with BSA as the standard. Each sample used for Western blotting was adjusted to contain equivalent amounts of protein.

10. <u>Western Blotting:</u>** Cell lysates (200-500 μg) were pre-cleaned with Protein A-Sepharose for 1 hour at 4°C. Protein A-Sepharose was removed and cell supernatants were incubated in the appropriate antibody (e.g. αP-Tyr) for 2-16 hours at 4°C. The antigen-antibody complexes were recovered during a 30-60 minute incubation using rabbit anti-mouse immunoglobulin and protein A-Sepharose or protein A-Sepharose alone. The immune complexes were washed 3-5 times with lysis buffer and the immunoprecipitated proteins were eluted into SDS-PAGE sample buffer (3% SDS, 60mM Tris, pH 6.9, 2 mM EDTA, 4% glycerol) by heating the samples to 100°C for 5 minutes.

11*. <u>Statistical Analysis:</u> Statistical significance from controls was determined using student's t –test. For all figures, the data are presented as an average with the error bar representing the standard deviation.

* I conducted these experiments and generated data from them.

** I was trained in these procedures, but did not perform them regularly or generate data from them.

Results

MK Inhibit OC Development In Vitro

Spleen cells ($2x10^6$ cells/ml) from C57BL/6 mice, as a source of OC precursors, were cultured with M-CSF (30 ng/ml) and RANKL (50 ng/ml) to induce OC formation. It should be noted that similar data was obtained regardless of whether OC were generated using spleen or BM cells as the source of OC precursors. Therefore, to avoid redundancy we show only the data using spleen cells. C57BL/6 or GATA-1 deficient MK were titrated into the culture wells and OC were identified as those cells that were $TRAP^+$ with >3 nuclei. Spleen cells ($2x10^6$ cells/ml) from C57BL/6 mice as a source of OC precursors, demonstrated up to a 15-fold reduction in OC number when 5,000 or 10,000 MK were added to the spleen cell cultures, $p < 0.001$ (Fig. 4). This data also shows that the MK from GATA-1 deficient mice are also able to inhibit OC formation in vitro, but that the inhibition was significantly less pronounced, $p < 0.04$, than that seen with equivalent numbers of C57BL/6 MK.

To rule out the possibility that the MK-induced OC inhibition was non-specific, spleen cells treated with M-CSF and RANKL were cultured with MK, the non-MK (NM) derived fraction (see preparation of fetal liver cells in Materials and Methods), or C57BL/6 thymocytes as an unrelated cell type. A dose-dependent inhibition of OC formation was again observed (Fig. 5). In contrast, at all concentrations tested, neither thymocytes nor the non-MK cell fraction inhibited OC development. These data suggest that the inhibition of OC formation was MK specific. Next, we confirmed that MK were also able to inhibit OC formation in the co-culture model system. In these experiments

C57BL/6 spleen cells ($2x10^6$ cells/ml) were cultured with freshly prepared C57BL/6 calvarial OB (20,000 cells/ml). Cultures were treated with 10^{-8} M $1,25(OH)_2D_3$ to enhance OC differentiation. C57BL/6 MK inhibited OC formation in a cell-number dependent manner (data not shown).

Furthermore, inhibition of OC formation was also observed in the Pax5-/- SCL model system. When just 2500 C57BL/6 MK (5%) were added to the SCL cultures, there was a statistically significant, greater than 8-fold reduction in OC number recorded when compared to control, $p < 0.001$. However, adding higher numbers of MK did not further reduce OC number (Fig. 6).

Next, we generated MK CM and tested the ability of this CM to inhibit OC formation. Here we cultured $1x10^6$ C57BL/6 MK/ml, $1x10^6$ GATA-1 deficient MK/ml or $5x10^5$ MEG-01 (a megakaryoblastic cell line) cells/ml for 3 days (pretested and shown to have highest activity at day 3, data not shown) before collection. As Fig. 7 depicts, C57BL/6 MK, GATA-1 deficient MK, and MEG-01 CM significantly inhibited OC formation in a dose-dependent manner, $p < 0.001$. For C57BL/6 MK CM the ED_{50} was approximately equal to 2% (vol:vol). These data indicate that a soluble factor(s) secreted by either primary MK or a MK cell line was capable of inhibiting OC differentiation.

The data presented above demonstrate that MK cultured in the absence of TPO, inhibit OC formation in a dose-dependent manner. Further, TPO-free MK CM also inhibited OC formation in a dose-dependent manner. This data, performed in our laboratory, serves as the preliminary results from which the following TPO experiments were designed.

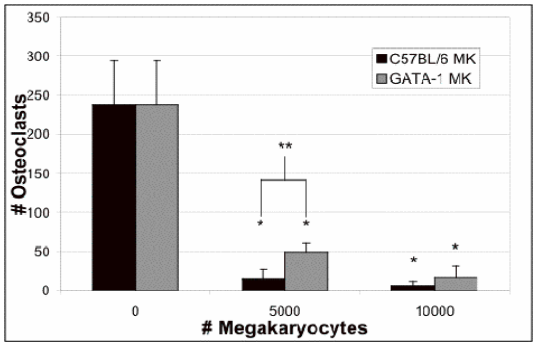

Figure 4. Inhibition of OC formation by MK after 9 days in culture. Both C57BL/6 and GATA-1 deficient MK markedly inhibited OC formation in vitro (up to 15-fold reduction, * = $p < 0.001$), although inhibition was reduced in cultures containing GATA-1 deficient MK (** = $p < 0.04$). Triplicate cultures were tested within individual experiments and experiments were repeated 3 times.

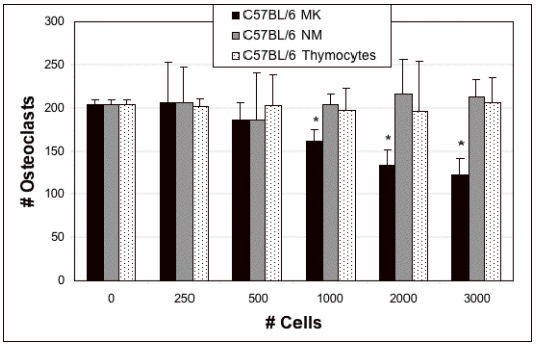

Figure 5. Dose-dependent inhibition of OC by C57BL/6 MK after 9 days in culture. MK, non-MK (NM), or thymocytes were titrated into spleen cell cultures and were stimulated to induce OC formation. MK inhibited OC in a dose-dependent manner (* = $p < 0.008$). NM and thymocytes did not inhibit OC development at any of the concentrations tested. Triplicate cultures were tested within individual experiments and experiments were repeated twice.

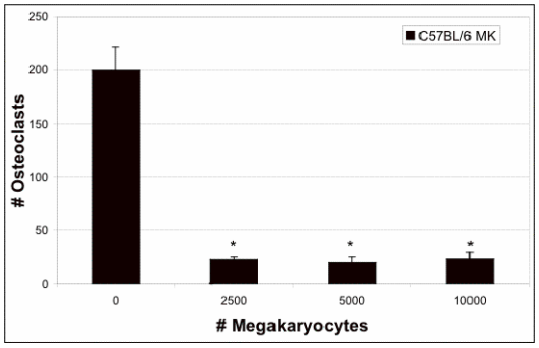

Figure 6. A greater than 8-fold reduction in OC formation by C57BL/6 MK (BSA-gradient separated on day 4) was seen when the Pax5-/-SCL was used as a source of OC precursors (* = $p < 0.001$). Triplicate cultures were tested within individual experiments and experiments were repeated 3 times

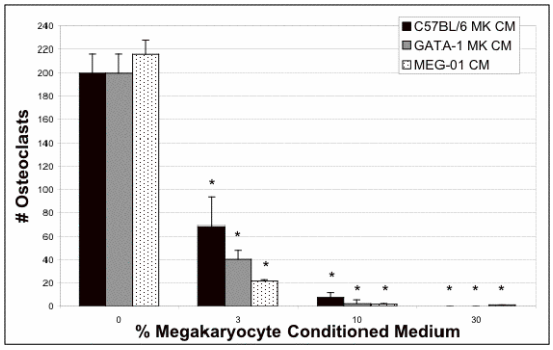

Figure 7. CM concentration-dependent inhibition of OC development (4 days in culture) in Pax5-/-SCL culture model by C57BL/6 MK CM, GATA-1 deficient MK CM, and MEG-01 CM (* = $p < 0.001$). Triplicate cultures were tested within individual experiments and experiments were repeated 3 times

TPO Enhances OC Formation in Vitro in MK-Free Cultures

OC generated from Pax5-/- SCL cultured in the absence of MK demonstrated an increase in cell number by 50-70% when stimulated with TPO, $p < 0.04$ (Fig. 7). Recombinant human TPO was titrated into the OC-like cell formation models at day 0. As shown in Fig. 8, with just 1 ng/ml of human recombinant TPO, OC number was 70% higher than in cultures without TPO stimulation. Similarly, OC generated from C57BL/6 BMM in the absence of MK were increased in a dose-dependent manner when cultured with TPO. A six-fold increase in OC number was observed when these cells were cultured with 100 ng/ml of TPO, $p < 0.01$ (Fig. 9).

This data clearly demonstrates that TPO itself enhances OC formation in vitro. However, if OC generating systems are used which contain MK and/or MK progenitors, the effects of the MK inhibiting OC formation appears to outweigh the effects of TPO enhancing OC formation, resulting in a net inhibition of OC formation.

C-mpl Expression in OC Progenitors

To determine whether OC progenitors express c-mpl, RT- PCR analysis was completed. As described in the Materials and Methods section, the comparative C_T method was used to compare c-mpl expression in different types of cells. GAPDH mRNA served as an internal control for the c-mpl mRNA. For each cell type used, mRNA was extracted separately from 3 different cultures of cells, and the RT-PCR was done in duplicate (the RAW264.7 OC progenitor cell line mRNA is based on only one mRNA culture). All data are reported as the normalized minimum threshold cycle (ΔC_T)

or the PCR amplification cycle at which the reporter dye fluorescence crossed the selected threshold or baseline.

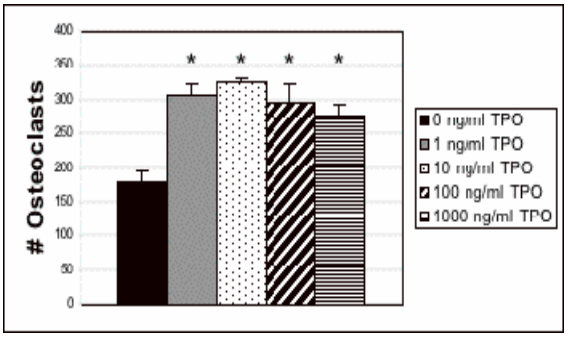

Figure 8. Stimulation of Pax5-/-SCL OC precursors in the absence of MK cells with TPO (1-1000 ng/ml) enhances OC formation by 50-75% (* = $p < 0.04$). Duplicate cultures were tested within individual experiments and experiments were repeated 3 times

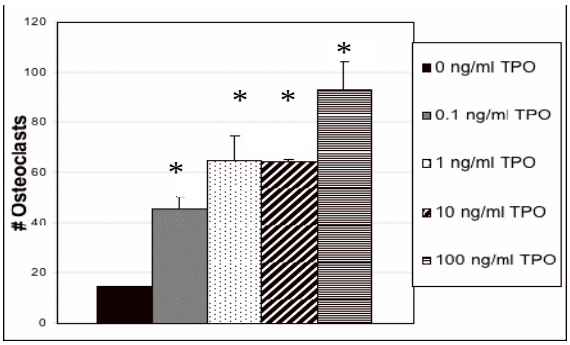

Figure 9. Stimulation of C57BL/6 BMM in the absence of MK with TPO (0.1-100 ng/ml) enhances OC formation by 6-fold (100ng /ml TPO, * = $p < 0.01$). Duplicate cultures were tested within individual experiments and experiments were repeated 3 times

As shown in Fig. 10, the ΔC_T for c-mpl expression in primary C57BL/6 MK was 25.0±1.1. For OC progenitors, the ΔC_T for c-mpl expression was 29.5±1.7 for primary C57BL/6 BMM, 33.08±0.04 for RAW264.7 cells, and 33.2±2.2 for Pax5-/- SCL. Although MK express significantly more c-mpl than do OC progenitors ($2^{|\Delta CT\ (MK)-\ \Delta CT\ (BMM)|} = 2^{|25.0-29.5|} \approx 22.6$, a 22.6-fold increase), this data demonstrates that OC progenitors express c-mpl (<35 C_T is considered legitimate c-mpl expression). This conclusion is further supported by our negative control, B9 cells (B cell). In B9 cell specimens, the minimum threshold level was not exceeded with 40 cycles. This demonstrates the specificity of c-mpl expression. Therefore, this expression data, in combination with the functional data that shows that TPO enhances OC formation in vitro, implies that TPO is

interacting with its receptor, c-mpl, on OC progenitors, and that this interaction begins a cascade of signaling events which eventually results in enhanced OC formation.

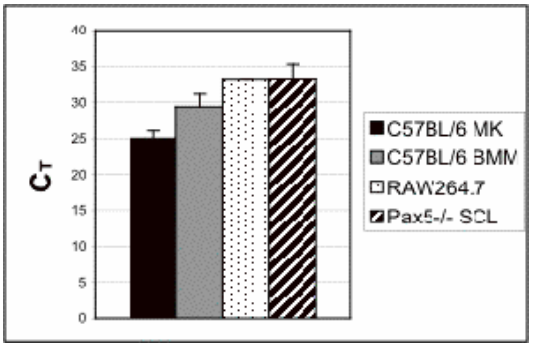

Figure 10. Expression by RT-PCR of c-mpl in C57BL/6 MK, C57BL/6 BMM, RAW264.7, and Pax5-/-SCL. All OC progenitor cells tested express c-mpl, albeit at lower levels than primary MK. The data represent the average ΔC_T from duplicate testing of three sets of mRNA (only one set of RAW264.7 mRNA was analyzed).

TPO Induces Tyrosine Phosphorylation of Specific Cellular Proteins in OC Progenitors

In MK and platelets, signal transduction by TPO has been shown to involve tyrosine phosphorylation of several specific cellular proteins such as: JAK2, TYK2, STAT3, STAT5, mpl, Shc, vav and SHIP. As shown in Fig. 11, TPO treatment of C57BL/6 BMM induced tyrosine phosphorylation of proteins with the following apparent mol wt: ~105-115, ~95, ~52, and ~46 kD. The majority of the proteins appeared to be rapidly tyrosine phosphorylated within 1 minute after TPO stimulation. Phosphorylation of the proteins reached maximal levels within 3-5 minutes after stimulation and significantly diminished by 30 minutes. While immunoprecipitation of the specific

cellular proteins has yet to be completed, these molecular weights are consistent with signaling molecules known to by tyrosine phosphorylated when MK are stimulated with TPO. Whether these same signaling molecules are tyrosine phosphorylated when OC progenitors are stimulated with TPO is the subject of future research; however, this data demonstrates a signaling cascade in OC progenitors when stimulated with TPO providing more evidence for a direct interaction between TPO and OC.

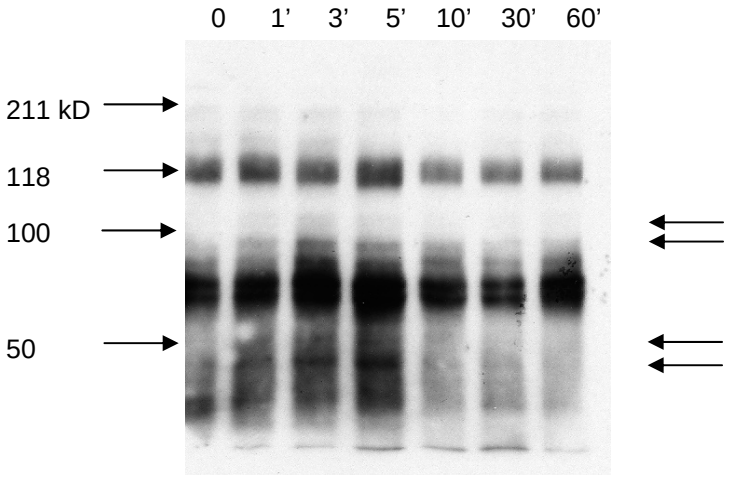

Figure 11. TPO induces tyrosine phosphorylation in C57BL/6 BMM. BMM were treated with vehicle alone (0') or TPO (100 ng/ml) for the various time periods indicated. Equal amounts of protein solubilized in lysis buffer (25 μg/time point) were separated on 7.5% SDS-PAGE, transferred to nitrocellulose and probed with antiphosphotyrosine antibodies. Bound antibody was detected in the ECL reaction. The arrows on the right-hand side indicate proteins that become tyrosine phosphorylated in response to TPO.

Discussion

In the last decade, a new paradigm has emerged in which MK have been shown to play an important role in skeletal homeostasis. In brief, data demonstrate that MK may act as an anabolic stimulator of bone formation by expressing/secreting bone related proteins, or by directly enhancing OB proliferation and differentiation (39, 40, 41, 43, 44, 101, 102, 110, 138). Simultaneously, MK express and/or secrete several factors that are known to be involved in osteoclastogenesis (37, 102, 103, 104, 105, 139-143). And, recent studies demonstrate that MK inhibit OC formation in vitro (106). The net result is that increases in MK can lead to increases in bone volume (110, 144).

One in vivo example of increases in MK leading to an increase in bone is TPO overexpressing mice. Mice overexpressing TPO, the main MK growth factor, have an approximate 4-fold increase in MK number and develop myelofibrosis with osteosclerosis (126-128). These mice have significantly elevated levels of TGFβ-1, PDGF, and OPG, all of which are known to be secreted by MK (106, 128, 131, 132, 143). It was hypothesized that these elevated levels were responsible for the myelofibrosis and osteosclerosis seen in TPO overexpressing mice.

To further dissect this hypothesis, Chagraoui and colleagues (130, 131) performed several elegant experiments. First they showed that when irradiated wild-type recipient mice were adoptively transferred with TGFβ-1-/- HSC that were infected with a retrovirus encoding murine TPO protein, TPO overexpression did not induce the myelofibrosis or osteosclerosis. However, when wild-type TPO-overexpressing HSC were adoptively transferred, myelofibrosis and osteosclerosis was seen within 16-weeks (130). Next, they performed mix and match adoptive transfer experiments with OPG.

They demonstrated that all mice had elevated TGFβ-1 levels and that all mice developed myelofibrosis, suggesting that TGFβ-1 and not OPG is critical for myelofibrosis to occur. Next, they demonstrated that when wild-type recipient mice were adoptively transferred with either wild-type or OPG-/- HSC that were infected with a retrovirus encoding murine TPO protein, that serum OPG levels were elevated and osteosclerosis developed. Interestingly, in OPG-/- recipient mice, an osteoporotic bone phenotype was observed (131). This data suggests that OPG secreted by the BM stromal cells and OB in the wild-type recipient mice is responsible for the osteosclerosis seen in TPO overexpression.

In vitro studies by Wakikawa et al (133) also suggested that the osteosclerosis seen in TPO overexpressing was due to inhibition of osteoclastogenesis. Their experimental design utilized a co-culture model with BM cells and stromal cells which inevitably contained MK and MK precursors. Their results demonstrated TPO mediated inhibition of osteoclastogenesis in a dose-dependent manner. However, Wakikawa et al (133) speculated the inhibitory effect of TPO on osteoclastogenesis in their co-culture system to be due to increased thrombopoiesis and production of MK cytokines, namely TGFβ-1 and PDGF. They found that antibodies to TGFβ-1 and PDGF, when introduced into their model system, antagonized TPO induced inhibition of osteoclastogenesis.

Thus, it remains unclear if TPO has a direct role in osteoclastogenesis, independent of MK. To elucidate whether TPO has a direct effect on osteoclastogenesis, we did the following. First, we examined the relationship between MK and MK CM and osteoclastogenesis. It is evident from our data that MK inhibit OC formation in vitro. Spleen cells from C57BL/6 mice as a source of OC precursors demonstrated up to a 15-fold reduction in OC number when 5,000 or 10,000 MK were added to the cell cultures

(Fig. 4). Furthermore, inhibition of OC formation was also observed in the Pax5-/- SCL model system (Fig. 6). Additionally, MK CM was shown to inhibit OC formation in a dose-dependent manner, suggesting that a soluble factor released by MK mediates OC formation inhibition (Fig. 7).

Next, we studied the relationship between TPO and OC, independent of MK. Our results clearly indicate that TPO exerts a direct effect on OC formation in vitro. Experiments in which OC were generated in the absence of MK (Pax5-/- SCL) demonstrated an increase in cell number by 50-70% when stimulated with TPO (Fig. 8). Also, up to a six-fold increase in OC number was observed after culturing C57BL/6 BMM cells (no MK) with TPO (Fig. 9). Interestingly, the response of C57BL/6 BMM cells to TPO was greater than that of the Pax5-/- SCL cells. This data suggests that primary cells such as C57BL/6 BMM are more responsive to TPO stimulation than cells lines such as Pax5-/- SCL. Moreover, since primary cells more closely model physiological response, these results further substantiate a direct role of TPO in OC formation. The argument can be made that primary cells inevitably contain some degree of contaminating cells and that the results could be spurious. However, all non-adherent cell were removed from cultures, so contaminating cells were adherent. Furthermore, as cells were induced to become OC, and since only OC were assessed in this assay by TRAP staining, only BMM or other OC precursors were responding to TPO stimulation.

It is evident from this data that the presence of MK or MK precursors would confound any attempt to elucidate a possible direct effect between TPO and osteoclastogenesis. Wakikawa et al (133) observed that TPO inhibited in vitro osteoclastogenesis in a dose-dependent manner. However, their co-culture model

contained MK and MK precursors. We confirmed the dose-dependent osteoclastogenesis that Wakikawa et al (133) observed by introducing increasing numbers of MK into OC progenitor cell cultures (Fig. 5). This suggests that Wakikawa et al (133) observed an indirect inhibitory effect of TPO on osteoclastogenesis due to increased megakaryopoiesis.

Since the MK mediated inhibition of OC formation dominates the direct effect of promoting osteoclastogenesis by TPO, the implications of this direct relationship between TPO and OC progenitors remains unclear. As detailed in the literature review, at least 70% of HSC are c-mpl positive, and mature, hematopoietic non MK cells such as dendritic cells express the TPO receptor (94, 95). Moreover, there is a growing body of research that suggests synergistic relationships between TPO and other growth cytokines such as G-CSF and IL-3 are essential to stem cell survival and proliferation (96, 98). Carver-Moore et al (99) have shown that mice with a c-mpl knockout have lower numbers of not only MK progenitors and MKs, but erythroid, granulocyte-macrophage, and multilineage precursor cells as well. It is well established that OC are hematopoietic in origin. Moreover, our data demonstrate that OC progenitors express c-mpl, albeit at low levels when compared to MK (Fig. 10). Specifically, we found that the level of expression of c-mpl on OC progenitors is approximately 22.6-fold less than that found in MK. This suggests that MK most likely bind the majority of TPO, and hence cause a net inhibition of osteoclastogenesis when stimulated with TPO, an indirect effect. However, our data suggests that there is also a concomitant, direct, TPO-mediated signal to OC progenitors that promotes osteoclastogenesis. Given the previously described research pointing to a crucial role for c-mpl in stem cell survival for multi-lineages of

hematopoietic cells, the direct stimulation of OC formation by TPO may be necessary for the survival and proliferation of this specific HSC population that produces OC.

Our preliminary data suggests not only that c-mpl is expressed in OC progenitors, but more importantly, it is functional. We observed rapid onset of tyrosine phosphorylation by Western blotting with 1 min with maximal activity at 3-5 min, tapering off by 30 minutes (Fig. 11). Drachman et al (145) demonstrated that TPO binding to c-mpl induces tyrosine phosphorylation evident within 1 minute, generally peaking between 5-10 minutes, and decreasing by 60 minutes. In their experiments, BaF3 cells expressing the c-mpl receptor had a dramatic increase in tyrosine phosphorylation in response to TPO. The most prominent phosphotyrosine proteins migrated at 52 kD, 95 kD, with several bands between 115 and 125 kD, and 140 kD; less intense bands were seen at 45, 65-75, and 110 kD. Our results of tyrosine phosphorylation occurring at ~105-115, ~95, ~52, and ~46 kD show a great deal of overlap. Drachman et al (145) identified through immunoprecipitation, phosphorylated Shc at 52 kD and c-mpl dimer at 95 kD doublet as well as JAK2 at 125 kD. Although immunoprecipitation assays are needed to identify the proteins being phosphorylated in our study, we did observe tyrosine phosphorylation at 52 kD and 95 kD suggesting that most likely, TPO/c-mpl signaling is well conserved among cell types. But it is plausible that other proteins could migrate at these weights. More importantly, this data clearly demonstrates that the c-mpl receptor on OC precursors is functional and that TPO stimulation initiates a time-dependent tyrosine phosphorylation cascade in OC precursors, a cell signaling event. This further supports a direct role for TPO in osteoclastogenesis.

We have demonstrated that TPO as well as MK both have direct and opposing effects on OC formation. We propose the following model (Fig. 12): TPO is secreted by cells of the OB lineage. Secreted TPO can bind to either cells of the MK lineage or cells of the OC lineage. When TPO binds to cells of the MK lineage, MK proliferation and differentiation occurs. Increases in MK number result in increases in OB number by a direct cell-to-cell mechanism. Increases in MK number also results in an increase in the secretion of soluble mediator(s) that inhibit OC formation. Simultaneously, TPO can bind c-mpl on OC progenitors, which results in an increase in OC formation and/or survival of HSC precursors of OC.

In closing, our research has demonstrated a novel role for TPO as a stimulatory cytokine promoting osteoclastogenesis. Moreover, we have also shown that not only is c-mpl expressed on OC progenitor cells, but is also a functional receptor. It has become evident through the literature that MK play a role in skeletal homeostasis. However, until now, the role of TPO has never been studied independently of MK. Our research is the first to examine the relationship between TPO and OC independently of MK. We have shown that TPO plays a direct role in skeletal homeostasis by enhancing OC development. Future research will be aimed at dissecting the TPO signaling pathways in OC, as well as functional bone resoprtion by OC stimulated with TPO. Such research will provide even more insight and knowledge about the relationship between TPO and OC, in the hopes of continuing to reveal the mechanisms underlying skeletal homeostasis and the roles of TPO and MK in bone remodeling.

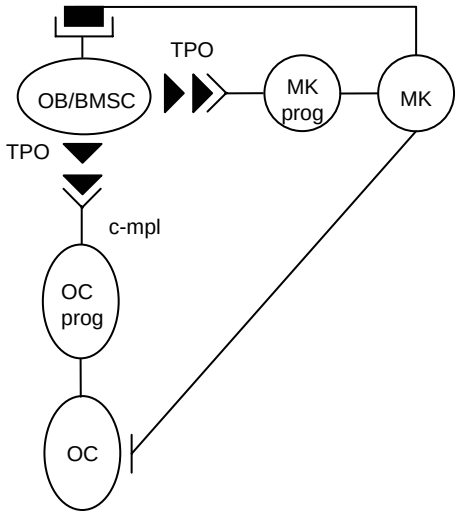

Figure 12. Model of how TPO both directly and indirectly regulates skeletal homeostasis. TPO that is secreted by OB/bone marrow stromal cells (BMSC) can bind to either MK or OC progenitors. C-mpl signaling in MK progenitors results in enhanced MK proliferation. MK then interact with OB/BMSC via direct cell-to-cell contact which in turn upregulates OB/BMSC proliferation (positive feedback loop). At the same time, MK secrete a factor that inhibits OC formation. On the other hand, TPO can bind c-mpl on OC progenitors, which results in increased OC formation.

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