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# Osteoclast Precursors: Regulation by Prostaglandins.

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# **Osteoclast Precursors: Regulation by Prostaglandins**

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B.A., California State University at Stanislaus, 2001

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**APPROVAL PAGE**

Master of Dental Science Thesis

**Osteoclast Precursors: Regulation by Prostaglandins**

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2009

## **SPECIAL RECOGNITION**

To my wife, Bonnie Michelle, for providing me with love and support,

And

To my family, Reza, Guity, and Kirk who have nourished and encouraged me throughout my educational journey.

Without their help, this work would not have been possible.

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## ABSTRACT

Bone resorption is dependent on the differentiation of osteoclast (OC) progenitor cells from the hematopoietic lineage into mature multinucleated OCs with the ability to resorb mineralized bone. Receptor activator of NF- $\kappa$ B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) are vital for osteoclastogenesis, while osteoprotegerin (OPG), the decoy receptor for RANKL, inhibits osteoclastogenesis. These factors are produced by osteoblasts (OBs), which are also the bone-forming cells. Prostaglandin (PG) E<sub>2</sub> is a potent stimulator of resorption. It stimulates osteoclastogenesis by acting on OBs to induce RANKL and inhibit OPG expression. Less is known about how PGE<sub>2</sub> acts on the hematopoietic lineage to directly regulate osteoclastogenesis. The goal of this project was to examine the effects of PGE<sub>2</sub> on OC formation from purified bone marrow macrophages (BMMs) in the presence of RANKL and M-CSF.

BMMs were isolated from murine long bones, expanded with M-CSF in petri dishes, replated in 96-well plates or 48-well plates, and treated with M-CSF and RANKL (M+R) or M+R plus PGE<sub>2</sub>. OCs, defined as cells with >3 nuclei staining for tartrate resistant acid phosphatase (TRAP), were counted in 3 wells per group on days 4-9 of culture. Counting was done either directly, under the microscope, or indirectly, with multiple digital images pieced together to create one seamless image of the entire well, which was then printed out for counting. Comparison of direct and indirect quantification of OC counts showed a 99.8% correlation. All experiments were quantified using the indirect method. In 48-well plates, M+R stimulated OC formation that peaked at d 7 or 8 in 3 independent

experiments, and PGE<sub>2</sub> inhibited OC formation induced by M+R at all time points and tended to delay the peak OC formation by 24 h. In 96-well plates, M+R alone had inconsistent results. In these 3 independent experiments the results showed: either stimulation, no effect leading to stimulation, or a biphasic effect with early inhibition and later stimulation. The OC formation peaked at d 4, 5, or d 7. Although effects of PGE<sub>2</sub> on formation of OCs from BMMs were variable in 96-well plates, PGE<sub>2</sub> consistently inhibited OC formation in 48-well plates. Our data suggest that direct effects of PGE<sub>2</sub> on OC precursors may oppose the indirect effects of PGE<sub>2</sub> to increase OCs via increasing RANKL in OBs.

## INTRODUCTION

**1. Bone Resorption** – Bone resorption is the process by which mineralized bone is broken down, and the calcium and phosphate in the mineral matrix is returned to the blood. Resorption is the first step in the bone remodeling cycle, which replaces “old” bone with “new” bone, and is coupled to bone formation by many locally produced factors, including some released from the bone matrix by the resorption process itself. The bone remodeling cycle is important for mineral homeostasis, for repair of damaged bone, and to allow bone to change its shape in response to mechanical loading. In particular, bone resorption is necessary for orthodontic tooth movement.

### Osteoclasts (OCs)

OCs are multinucleated cells (MNCs) that resorb mineralized bone. They develop from the monocyte-macrophage lineage of hematopoietic cells [1]. OCs express tartrate-resistant acid phosphatase (TRAP) activity and calcitonin receptors (CTRs). TRAP is an enzyme identified in both the ruffled border of the OC membrane and in the secretions in the resorptive space [2]. TRAP is highly expressed by OCs, macrophages, and neurons [3]. The exact function of TRAP is unknown, but many functions have been attributed to this protein, such as the generation of reactive oxygen species, iron transport, and regulation of cell growth and differentiation factor [4]. The CTR is a G protein-coupled receptor that binds calcitonin. CTR is more specific for OCs than TRAP [4]. Calcitonin is

involved in the maintenance of calcium homeostasis and can transiently inhibit OC activity [1].

### Osteoclastogenesis

Cell-to-cell contact between cells of the osteoblastic lineage and OC progenitors is necessary for OC differentiation [5]. Osteoblasts (OBs), which are derived from precursors in the mesenchymal cell lineage, support OC development by producing macrophage/monocyte-colony stimulating factor (M-CSF) and receptor activator of NF- $\kappa$ B ligand (RANKL). OC differentiation involves several major stages as illustrated in Figure 1. Hematopoietic stem cells (HSCs) give rise to colony forming unit-granulocyte/macrophages (CFU-GMs). M-CSF stimulates the proliferation of CFU-GMs into monocyte/macrophage cells that lack TRAP and CTR, two crucial OC markers. Mononuclear OC precursors differentiate into pre-fusion OCs (cells positive for both TRAP and CTR) in response to stimulation from M-CSF and RANKL. These factors bind their respective receptors, c-fms and RANK, expressed on OC precursors to stimulate OC formation. The pre-fusion OCs will further develop and differentiate by fusion to become MNCs (with continuous stimulation of M-CSF and RANKL).

Once MNCs become functional OCs, they present with a ruffled border and the ability to resorb mineralized bone matrix [6]. The ruffled border increases the surface area interface for bone resorption [7]. It also creates a 'Sealing Zone' around the resorption area, allowing the enzymes to have an isolated area with a low pH, thus providing the OC with the proper environment

needed for bone mineral dissolution [8]. RANKL continues to play an important role in activating OCs by stimulating formation of the ruffled membrane [1].

RANKL may also enhance OC survival [9].

### RANKL and Osteoprotegerin (OPG)

RANKL, also called osteoprotegerin ligand (OPGL) [10], is a member of the TNF superfamily. It is a cell surface molecule expressed not only by OBs but also by marrow stromal cells and activated T lymphocytes [5]. *In vitro*, RANKL and M-CSF have been shown to be sufficient for stimulating osteoclastogenesis [11]. Both RANKL knockout and RANK knockout mice have osteopetrosis secondary to complete absence of OCs in bone [12]. OB/stromal cells also produce osteoprotegerin (OPG), a soluble decoy receptor for RANKL. OPG inhibits RANKL function by competing with RANK for RANKL [13]. OPG knockout mice show decreased bone density [14] and defects in tooth eruption and osteoclastogenesis [14], while mice with over expression of OPG show increased bone density [15].

For some factors that stimulate resorption, such as parathyroid hormone, there are no known receptors on cells in the osteoclastic lineage, and their ability to stimulate resorption is reflected by their ability to stimulate RANKL and inhibit OPG (or to increase the RANKL:OPG ratio) in OBs. Other factors, such as prostaglandins, have receptors on OCs as well as on OBs and may, therefore, have effects on both lineages.

**2. Prostaglandins (PGs)** – PGs are 20-carbon polyunsaturated fatty acids produced by many cell types. Although OCs can produce PGs, the major source of PGs in bone is thought to be cells of the OB lineage. The production of PGs, especially PGE<sub>2</sub>, in OBs is stimulated by many resorption agonists (i.e., factors that increase the RANKL/OPG ratio), and PGE<sub>2</sub> is itself a potent stimulator of OC formation [15, 16]. Hence, the effects of other resorption agonists may be modulated by their induction of PGE<sub>2</sub>.

### Regulation of PG Production

Conversion of arachidonic acid (AA), derived from the cellular lipid bilayer, by cyclooxygenase (COX), also called prostaglandin endoperoxide synthase, is the committed step in PG synthesis [14] (Figure 2). COX has two isoforms, COX-1 and COX-2, encoded by separate genes [14]. COX-1 is encoded by a 22 kb gene with 11 exons and COX-2 is encoded by a 8 kb gene with 10 exons [14]. Even though COX-1 and -2 are similar in their enzymatic mechanisms, their functional roles differ. COX-1 is constitutively expressed in nearly all tissues (including OBs). COX-1 is thought to produce the PGs responsible for “housekeeping” functions such as the maintenance of renal blood flow, platelet aggregation and gastric cytoprotection [17]. In contrast, COX-2 is inducible, and is responsible for acute PG responses [18]. COX-2 is induced in response to multiple factors, including cytokines and growth factors (IL-1, TNF- $\alpha$ , TGF- $\alpha$ , TGF- $\beta$ ), hormones (parathyroid hormone, 1,25(OH)<sub>2</sub>D<sub>3</sub>) and mechanical loading



of bone [19-21]. The induction of COX-2 is generally transient in most cells, with a return to baseline in 24-48 hours [14].

Non-steroidal anti-inflammatory drugs (NSAIDs) like acetylsalicylic acid (aspirin) and ibuprofen act to inhibit PG production by preventing binding of AA to the catalytic site in both COX-1 and COX-2. Newer NSAIDs, such as celecoxib (Celebrex), are selective for COX-2 [14].

### PGE<sub>2</sub> and OC Formation

PGE<sub>2</sub> can stimulate OC differentiation in various types of *in vitro* cultures and bone resorption in bone cultures [14]. Studies have shown that PGE<sub>2</sub> can indirectly increase osteoclastogenesis by increasing RANKL and decreasing OPG in OBs and can have direct stimulatory effects on the hematopoietic lineage to increase OC numbers as well [14]. On the other hand, other studies have reported that PGE<sub>2</sub> can inhibit the activity of mature OCs [22]. One study reported both inhibitory and stimulatory effects of PGE<sub>2</sub> on OC formation in hematopoietic lineage cells [8]. Hence, the effects of PGE<sub>2</sub> on OC formation and activity can be complex, perhaps in part because there are so many different model systems in which to study OC formation.

**3. Models for Studying Osteoclast Formation** – A number of different murine models to study osteoclastogenesis have developed over the years. All of them require the addition of some “resorption” agonist (some stimulator of RANKL) or the addition of RANKL itself. Some systems examine effects of indirect actions

of resorption agonists on OC formation, while others examine direct effects on the hematopoietic lineage.

### Bone Marrow Cultures

Bone marrow contains cells of both the hematopoietic and mesenchymal lineage. Hence, under appropriate conditions, whole bone marrow can be used to study either osteoblastic differentiation (called bone marrow stromal cell cultures) or osteoclastic differentiation (simply called bone marrow cultures). Although cells of the osteoblastic lineage make M-CSF, RANKL, and OPG, there is not enough RANKL made under basal culture conditions to stimulate OC formation. Hence, to differentiate OCs from bone marrow requires the addition of agonists that can increase RANKL production by cells of the mesenchymal lineage [23-25]. These cultures are generally used to assess the ability of various resorption agonists to stimulate the RANKL/OPG ratio and compare with numbers of OCs formed. Although these cultures do not require supplementation with either RANKL or M-CSF, addition of RANKL to these cultures can be examined to determine the maximal number of cells with the potential to develop into OCs.

In marrow cultures, PGE<sub>2</sub> generally stimulates OC differentiation by increasing RANKL and inhibiting OPG [24]. Since many resorption agonists also induce COX-2 in these cultures, some of the ability of these agonists to induce OC formation is dependent on their induction of PGs. This has been shown by

treating these cultures with NSAIDs to inhibit PG production or by using cells from COX-2 knockout mice [24].

### Spleen Cultures

Spleen cultures contain precursor OCs and no OB precursors. Therefore, these cultures can be used to examine direct effects of agonists on OC precursors. Since there are no osteoblastic cells present, the cultures require supplementation with M-CSF and soluble RANKL. If one wants to study the contribution of mesenchymal cells from one type of transgenic mouse and hematopoietic cells from another type, then co-cultures combining OBs from one type of transgenic mouse with spleen cells from another type can be used.

It has been shown that PGE<sub>2</sub> can increase the combined effects of RANKL and M-CSF to stimulate OC formation in some spleen cultures [25, 26]. For example, our lab found that there was a 50% reduction in OCs formed in the presence of RANKL and M-CSF when the spleen cells came from COX knockout mice [24]. This was explained as being due to increased expression of granulocyte monocyte-colony stimulating factor (GM-CSF), an inhibitor of OC formation, in COX-2 knockout spleen cultures [26]. However, that study measured OC formation only at one time point. In another study where OC formation was examined over multiple time points, we found that PGE<sub>2</sub>, in the presence of RANKL and M-CSF, had biphasic effects on OC formation [26]. PGE<sub>2</sub> decreased the number of OCs at 5-6 days of culture but increased the number of OCs at 8-9 days compared with cultures treated with RANKL and M-

CSF alone [24]. The increase in OC number at the later time point may have been due to the observation that PGE<sub>2</sub> decreased OC apoptosis at day 7. It was concluded that PGE<sub>2</sub> has an initial inhibitory effect on OC formation in spleen cell cultures and a later stimulatory effect mediated by the EP2 receptor [24]. Spleen cultures also contain T cells, and it was speculated that the T cells were responsible for the initial inhibitory effects of PGE<sub>2</sub> on osteoclastogenesis in this system. Hence, both stimulatory and inhibitory effects of PGE<sub>2</sub> on OC formation have been seen in spleen cultures.

#### Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs are a subset of white blood cells having a round nucleus, such as a lymphocyte or a monocyte, that can give rise to OCs in culture when treated with RANKL and M-CSF. The advantage of PBMCs above other cells is that blood is a readily accessible cellular material, and PBMCs can be isolated from whole blood relatively easily. Various studies have shown that PBMCs can display gene expression patterns characteristic for certain diseases, such as acute myeloid leukemia, atherosclerosis, and autoimmune diseases [27].

The effects of PGE<sub>2</sub> on human OC formation were examined in cultures of CD14<sup>+</sup> cells prepared from human PBMCs [28]. PGE<sub>2</sub> in the presence of RANKL and M-CSF inhibited OC formation in these cultures. The conditioned medium of CD14<sup>+</sup> cells pretreated with PGE<sub>2</sub> inhibited RANKL-induced OC formation not only in human CD14<sup>+</sup> cell cultures but also in mouse macrophage

cultures. The authors concluded that PGE<sub>2</sub> inhibits human OC formation in PBMC cultures through the production of an as yet unknown inhibitory factor.

#### RAW 264.7 Cultures

RAW264.7 cells are the only known clonal cell line that can give rise to OCs *in vitro*. The RAW 264.7 cell line is a functional murine macrophage cell line transformed by the Abelson Leukemia Virus. Hence, they may not reflect 'normal' OC development. These cells produce cytokines in response to lipopolysaccharide (LPS) and can make OCs in response to RANKL [29]. Since RAW 264.7 cells produce M-CSF, it is not necessary to add M-CSF to cultures.

Several studies have suggested that PGE<sub>2</sub> can enhance osteoclastogenesis in the RAW 264.7 cell line [29]. On the other hand, a study in our lab examining OC formation in RAW 264.7 cells treated with tumor necrosis factor (TNF- $\alpha$ ) found that inhibition of PGE<sub>2</sub> production in these cultures stimulated the formation of OC [30].

#### Bone Marrow Macrophage Cultures

Bone marrow macrophages (BMMs) are primary macrophages/monocytes isolated from bone marrow that can be differentiated into OCs [31-38]. BMMs are expanded in M-CSF and then treated with RANKL to stimulate OC formation. Compared to many other primary cells, BMMs are relatively homogenous, have a proliferative capacity, are transfectable, and have a lifespan longer than a week. In fact, BMMs can be grown up to three weeks without noticeable cell death or

altered morphology [39]. There is little known regarding the effects of PGE<sub>2</sub> on BMM cell cultures.

#### **4. OC Quantification**

Quantitative assessment of OC numbers are essential to allow statistical comparisons between treatment groups, and to facilitate reference databases for the study of metabolic bone diseases associated with increased bone resorption. Some methods have included scoring systems to evaluate TRAP staining intensity [40], and grid systems to evaluate TRAP distribution [41], however, TRAP levels are subjectively graded (e.g., 1 to 4) rather than actually quantifying cell numbers, stain density, or area percentages. Although many studies have provided valuable information regarding TRAP distribution and histomorphometry of resorbing cells, no study has offered a precise method for quantifying TRAP positive cells or OCs.

With the development of faster computers and better cameras, more sophisticated forms of image analysis have been introduced allowing images from the microscope to be captured and transmitted to a computer equipped with image analysis tools [41]. In our study, we will use digital imaging and computers, to standardize the size and location of the region of interest, quantify the positive OC staining area, document the reproducibility of the measurements, create a permanent record of the OC wells of interest, and demonstrate a direct correlation with the current "gold standard" of OC quantification performed directly under a microscope.

## **5. Bone Resorption, PGs and Orthodontic Tooth Movement**

Tooth movement is a coordinated array of events involving bone resorption and formation. The efficiency of bone resorption is the rate-limiting factor in tooth movement [41-44]. Bone is removed in front of the moving tooth by two mechanisms: frontal resorption at the periodontal ligament (PDL) interface and initial remodeling events (resorption cavities) in the cortical plate [45, 46]. Orthodontic force initiates a cascade of cellular proliferation and differentiation events in the PDL [47-51]. A variety of neurological, immune, and endocrine system responses, as well as local cytokines and intracellular messages, have been implicated in the osseous adaptive reaction as the root of the tooth is displaced [52]. These localized agents are likely mediators of the temporary discomfort noted during the initiation of orthodontic tooth movement. The role of local cytokines in the sustained bone modeling and remodeling events of tooth movement is largely unknown. However, PGs are thought to be important factors in the control of mechanically mediated bone adaptation [53].

Among other factors, the removal of osseous tissue during progressive tooth movement is directly related to the resorption rate and to OC recruitment. The OC resorption rate is largely controlled by metabolic factors [54]. There is currently no direct evidence to suggest that OCs are produced in the PDL. Pre-OCs can be derived from the bone marrow, enter the circulation and subsequently be delivered to the PDL and adjacent bone [14]. It has been shown that PDL cells also require cell-to-cell contact to stimulate osteoclastogenesis [55]. During the application of orthodontic forces, RANKL

has been seen in OBs, osteocytes, and fibroblasts [55]. Also, the RANKL/OPG ratio has been implicated in root resorption associated with heavy orthodontic forces applied in rats [56].

PGs are potent stimulators of bone resorption, and have been shown to be produced when teeth are mechanically loaded and to enhance tooth movement [14, 17]. For example Yamasaki et al [55] showed that orthodontic mechanical stress induced secretion of PGs in the periodontal tissues of rats stimulated OC bone resorption. When PGE<sub>1</sub> or PGE<sub>2</sub> were injected in the gingiva near the upper first molar in rats, OCs and alveolar bone resorption were observed [23, 57, 58]. On the other hand, the administration of indomethacin, an inhibitor of PG production, suppressed the appearance of OCs and bone resorption. Indomethacin also decreased the extent of resorption surfaces in response to orthodontic loading in miniature pigs [59]. Orthodontic forces have also been shown to increase cytokine production, such as TNF- $\alpha$  and IL-1, which can induce COX-2 expression and PG production in OBs and in cultured bone marrow cells [28]. Although these cytokines are themselves potent inducers of bone resorption, some of their effects on resorption may be mediated via their induction of COX-2 and PGs [26].



## RATIONALE

In order for tooth movement to occur, bone resorption has to take place on the compression side followed by bone deposition on the tension side. Thus, OCs play a crucial role in tooth movement. Although PGs have been shown to enhance orthodontic tooth movement, it is unclear if this enhancement involves not only PG stimulation of the RANKL/OPG ratio in OC supporting cells (PDL cells and/or OBs) but also stimulatory effects on cells of the osteoclast lineage. As discussed above, the effect of PGE<sub>2</sub> to increase RANKL/OPG is well documented, but PGE<sub>2</sub> has been shown to have both stimulatory and inhibitory effects on OC formation in different models used to study OC formation. One explanation for the differences seen in studies of the direct effects of PGE<sub>2</sub> on OC precursors may be the time points at which OC formation was quantified. In spleen cell cultures studied over an extensive time course, PGE<sub>2</sub> had biphasic effects, an initial inhibitory effect on OC formation with a later stimulatory effect [60]. Preliminary data from our lab, studying OC formation in cultured BMMs also suggest that PGE<sub>2</sub> may have inhibitory effects. One of the difficulties with studying BMMs is that very large numbers of OCs are formed, making counting under the microscope tedious, time-consuming, and operator dependent. The goals of this project were to develop a digital method of recording OC counts and to use this method to examine the effects of PGE<sub>2</sub> on OC formation from purified BMMs in the presence of RANKL and M-CSF over an extended time course.

## **HYPOTHESIS**

1. Digital imaging will strongly correlate with the conventional method of OC quantification.
2. PGE<sub>2</sub> will inhibit or delay OC formation in BMM cultures.

## **OBJECTIVES**

The overall objective of this research was to clarify the effects of PGE<sub>2</sub> on OC formation in BMM cultures. The specific objectives were:

1. To create a method that permitted convenient quantification of large numbers of OCs.
2. To examine the effects of exogenous PGE<sub>2</sub> on the differentiation of OC precursors into OCs in BMM cultures.
  - a) Examine the effects of a dose response of PGE<sub>2</sub> on BMM cells.
  - b) Examine the time course for effects of PGE<sub>2</sub> on BMM cells.

## MATERIALS AND METHODS

### 1. Materials

RANKL and M-CSF were obtained from R&D Biosystems (Minneapolis, MN). PGE<sub>2</sub> was obtained from Cayman Chemical Company (Ann Arbor, MI). Minimal Essential Medium Alpha (α-MEM), fetal calf serum (FCS), and trypsin/EDTA were purchased from Invitrogen (Carlsbad, CA). Leukocyte Acid Phosphatase Kit (TRAP stain) was purchased from Sigma (St. Louis, MO).

### 2. Bone Marrow Macrophage (BMM) Cultures

#### Isolation of BMMs

All animals used in this study were treated in accordance with protocols approved by the Animal Care and Use Committee of the University of Connecticut Heath Center. Long bones (femur and tibia) of 8 week old CD1 mice (males and females) were dissected free of adherent tissue and placed into sterile dishes on ice. Bone marrow was flushed with alpha MEM (without serum) under the hood using syringes with 25 gauge needle (1ml per bone). The bone marrow cells were then resuspended in alpha MEM + 10% FCS + 100 ng/ml M-CSF and plated at  $5 \times 10^6$  cells per/well in 100 mm petri dish (Fisher brand 08-757-12). Adherent cells, the bone marrow macrophages (BMMs), were lifted after 3 days with trypsin/EDTA, centrifuged and resuspended in alpha MEM +10% FCS + 30 ng/ml MCSF and 100 ng/ml RANKL and plated in either 48 or 96 well dishes for osteoclastogenesis experiments.

### Osteoclastogenesis

5,000 cells/well were plated in 96 well plates and 15,000 cells/well in 48 well plates. The media, which consisted of alpha MEM +10% FCS + 30 ng/ml M-CSF and 60 or 100 ng/ml RANKL, was changed every 2-3 days. Cells were cultured for a time course of 4-9 days, depending on the experiment. The cells were also subjected to different concentrations of PGE<sub>2</sub>, either 10<sup>-6</sup> M or 10<sup>-8</sup> M. Please see individual experiments under 'Results' section for exact time course and PGE<sub>2</sub> concentrations for each individual experiment.

### Expansion

Left over BMMs were expanded at 5X10<sup>6</sup> cells per/well in 100 mm petri dish in alpha MEM +10% FCS + 100 ng/ml M-CSF for future osteoclastogenesis experiments.

### 3. Tartrate Resistant Acid Phosphatase (TRAP) Staining

Cells were fixed at the end of culture with 2.5% glutaraldehyde. A Leukocyte Acid Phosphatase Kit was used to stain for TRAP following the manufacturer's instructions. The TRAP positive multinucleated cells (MNCs), with 3 or more nuclei, were either directly counted under the microscope at 20X total magnification or indirectly counted via digital imagery at 20X total magnification.

#### 4. OC Quantification

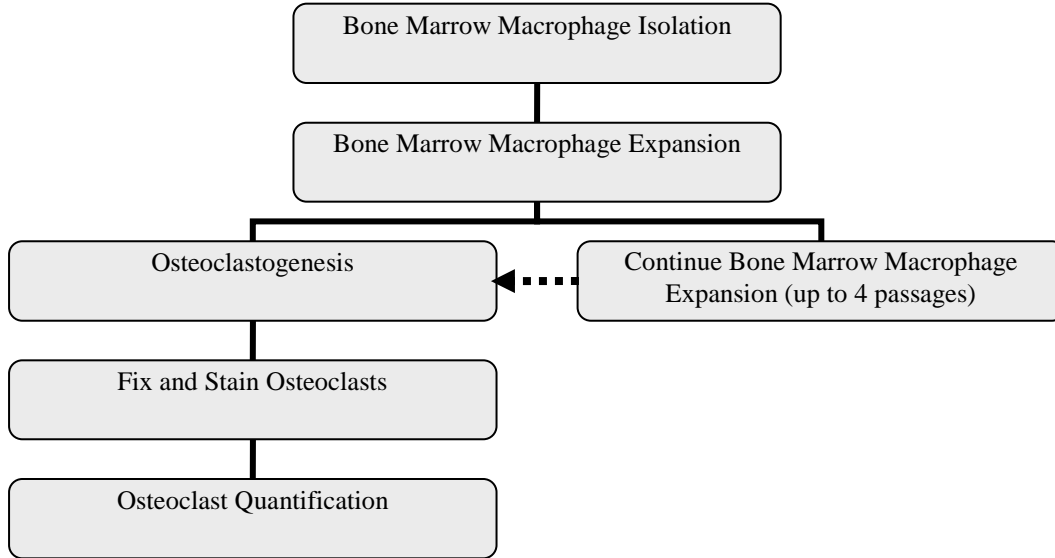
##### Direct OC Quantification

The TRAP stained plates were placed under a high power light microscope (Olympus IX70, Melville, New York) and viewed at 20X total magnification. Due to the high power magnification needed to view the OCs nuclei, it was not possible to view the entire well under one field of view. In order to view the entire well, the built in eyepiece grid was used to ensure proper tracking of the well during quantification. Each TRAP positive cell with 3 or more nuclei was counted using a handheld click counter. An OC tally of each well from each plate was recorded and graphed.

##### Indirect OC Quantification

The TRAP positive plates were placed under a high power light microscope (Nikon Eclipse TE2000-U, Tokyo, Japan) and viewed at 20X total magnification. The microscope was connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, MI) and the images were captured using Windows Spot Advanced software version 4.1 (Diagnostic Instruments, Sterling Heights, MI). Due to the high power magnification needed to view the OCs nuclei, it was not possible to view the entire well under one field of view. Multiple images were merged into one complete and seamless image using Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA), printed using a photo quality color printer, Hewlett-Packard Color Laser Jet 4700dn (Hewlett-Packard, Palo Alto, CA), and the OCs were quantified.

## 5. Design Overview



## 6. Statistical Analysis

All values are depicted as the mean  $\pm$  standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism® for Microsoft Windows®, version 4.0 (GraphPad Software, La Jolla, CA). To compare multiple treatment groups, differences were examined by two-way analysis of variance (ANOVA) followed by the Bonferroni test.

## RESULTS

### 1. Comparison of Direct and Indirect OC Quantification

Our first goal of this project was to create a method that will permit a reliable and convenient quantification of large numbers of OCs. The wells of 48 well plates were divided into sextants, and six different images of the same well were captured and later pieced back together to create one seamless image (Figure 3). The wells of 96 well plates were divided in half, and two different images of the same well were captured and later pieced back together to create one seamless image (Figure 4). The images were then printed and the OCs counted.

Only 'normal' and 'healthy' appearing multinucleated TRAP positive cells were counted. 'Ghost' cells, cells with degraded membranes, or cells with less than 3 nuclei were not quantified (Figure 5).

Following the protocol listed above, a correlation between direct (directly under the light microscope) OC quantification and indirect (using the digital photography method) OC quantification was compared (Figure 6, Table 1) and graphed (Figure 7). The results of the correlation experiments showed a 99.81% correlation between the direct and indirect quantification of OC counts (Figure 7). Since the indirect quantification method has many advantages over the direct method, all the remainder of our experiments were quantified using the indirect method.

## 2. Effects of PGE<sub>2</sub> on OC Formation

The second aim was to examine the direct effects of exogenous PGE<sub>2</sub> on the differentiation of OCs in BMM cultures. For these experiments, we added PGE<sub>2</sub> to BMM cells from CD1 mice, in the presence of M-CSF and RANKL, and observed OC formation. We did a total of six experiments. Since we found that the size of the wells in the dishes used affected the results, we have divided the experimental results according to the size of the wells.

### Effects of PGE<sub>2</sub> using 48 well plates

Experiment 1 was done in 48 well dishes (Table 2, Fig. 8). Cultures were treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml), with and without PGE<sub>2</sub> (10<sup>-6</sup> M). OCs were counted on days 5, 6, 7, and 8. OC counts peaked at day 7 for both control (RANKL and M-CSF) and treatment (PGE<sub>2</sub>) groups. PGE<sub>2</sub> decreased the peak OC count by 84%.

Experiment 2 was also done in 48 well dishes (Table 3, Fig. 9). Cultures were treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml), with and without PGE<sub>2</sub> (10<sup>-6</sup> M or 10<sup>-8</sup> M). OCs were counted on days 6, 7, 8 and 9. OC counts for the control group peaked at day 7. OC number in the PGE<sub>2</sub> 10<sup>-8</sup> M group peaked at day 8. OC number in the PGE<sub>2</sub> 10<sup>-6</sup> M group showed a slow steady increase without a true peak. For both treatments, PGE<sub>2</sub> had an inhibitory effect on OC formation, with PGE<sub>2</sub> 10<sup>-6</sup> M being more inhibitory than PGE<sub>2</sub> 10<sup>-8</sup> M. PGE<sub>2</sub> 10<sup>-8</sup> M decreased the peak OC count by 36% and PGE<sub>2</sub> 10<sup>-6</sup> M decreased the peak OC count by 93%.



Experiment 3 was also done in 48 well dishes (Table 4, Fig. 10). Cultures were treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml), with and without PGE<sub>2</sub> (10<sup>-6</sup> M or 10<sup>-8</sup> M). OCs were counted on days 5, 6, 7, 8 and 9. OC counts for the control group and the PGE<sub>2</sub> 10<sup>-8</sup> M group both peaked on day 7, while OC numbers for the PGE<sub>2</sub> x 10<sup>-6</sup> M group peaked at day 8. PGE<sub>2</sub> had an inhibitory effect on OC formation, with PGE<sub>2</sub> X 10<sup>-6</sup> M being more inhibitory than PGE<sub>2</sub> 10<sup>-8</sup> M. Peak OC count was decreased by 14% in the PGE<sub>2</sub> 10<sup>-8</sup> M group and by 41% in the PGE<sub>2</sub> 10<sup>-6</sup> M group.

#### Effects of PGE<sub>2</sub> using 96 well plates

Experiment 4 was done in 96 well dishes (Table 5, Fig. 11). Cultures were treated with M-CSF (30 ng/ml) and RANKL (60 ng/ml), with and without PGE<sub>2</sub> (10<sup>-6</sup> M or 10<sup>-8</sup> M). This was the only experiment where 100 ng/ml of RANKL was not used. OCs were counted on days 6, 7, 8 and 9. OC counts for all 3 groups peaked at day 7. Both PGE<sub>2</sub> groups had a stimulatory effect on OC formation, with PGE<sub>2</sub> 10<sup>-8</sup> M being more stimulatory than the PGE<sub>2</sub> 10<sup>-6</sup> M group. PGE<sub>2</sub> 10<sup>-8</sup> M increased the peak OC count by 89% and PGE<sub>2</sub> 10<sup>-6</sup> M increased the peak OC count by 36.7%.

Experiment 5 was also done in 96 well dishes (Table 6, Fig. 12). Cultures were treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml), with and without PGE<sub>2</sub> (10<sup>-6</sup> M). OCs were counted on days 4, 5, 6, and 7. There was no appreciable difference in OC counts at days 4 and 5, for the control group or the PGE<sub>2</sub>10<sup>-6</sup> M group. At later time points, however, PGE<sub>2</sub> had a stimulatory effect

(days 6, and 7.) Thus, our conclusion from this experiment was that PGE<sub>2</sub> 10<sup>-6</sup> M had a no effect early on leading to a later stimulation of OCs.

Experiment 6 was also done in 96 well dishes (Table 7, Fig. 13). Cultures were treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml), with and without PGE<sub>2</sub> (10<sup>-6</sup> M). OCs were counted on days 4, 5, 6, and 7. OC counts peaked at day 5 for both the control group and the PGE<sub>2</sub> 10<sup>-6</sup> M group. In this experiment, PGE<sub>2</sub> 10<sup>-6</sup> M had an initial inhibitory effect, and a later stimulatory effect. Thus, our conclusion from this experiment was that PGE<sub>2</sub> 10<sup>-6</sup> M had a biphasic effect.

Please see Table 8 for the overall experimental results for all 6 experiments.

## DISCUSSION

We developed an “indirect” method for quantifying OC numbers. Multiple high power fields were photographed and merged together to give prints of whole wells of cells. This method of counting OCs showed a 99.8% correlation with the direct counting of OC under a microscope. It also gave us a hard copy of the data that could be filed for later review by other investigators.

The experiments to study effects of PGE<sub>2</sub> on OC formation were unexpectedly complicated by finding that results varied with size of the well used to culture OCs. Three independent experiments using 48 well dishes showed that PGE<sub>2</sub> had an inhibitory effect on osteoclastogenesis. The higher dose of PGE<sub>2</sub> (10<sup>-6</sup> M) was more inhibitory than the lower dose of PGE<sub>2</sub> (10<sup>-8</sup> M). In these experiments, the peak OC number consistently occurred on day 7-8 in both control (RANKL + M-CSF) cultures and in PGE<sub>2</sub>-treated cultures.

In contrast, the 96-well experiments had inconsistent results. In these 3 independent experiments the results showed: either stimulation, no effect leading to stimulation, or a biphasic effect with early inhibition and later stimulation. The OC formation peaked at d 4, 5, or d 7. Although effects of PGE<sub>2</sub> on the formation of OCs from BMMs were variable in 96-well plates, PGE<sub>2</sub> consistently inhibited OC formation in 48-well plates. In addition, the higher dose of PGE<sub>2</sub> (10<sup>-6</sup> M) was less stimulatory than the lower dose of PGE<sub>2</sub> (10<sup>-8</sup> M).

We do not think that this variability is the result of plating density. The 48 and 96 well plates have surface growth areas of 0.95 cm<sup>2</sup> and 0.32 cm<sup>2</sup> respectively. The 48 well plates were plated with 15,000 cells per well, while the

96 well plates were plated with 5,000 cells per well. Hence, our plating densities were similar at 15,600-15,800 cells per  $\text{cm}^2$ . However, there was a difference in the ratio of circumference to growth area in the dishes. The 48 well plate has a 3.45 cm circumference or 3.6 cm per  $\text{cm}^2$  of growth area, while the 96 well plate has a 2 cm circumference or 6.2 cm per  $\text{cm}^2$  of growth area. There may be greater “edge effects” in the smaller well, such that more cells are sensing the well wall instead of other cells. Interaction with the well wall might cause cells to react differently than if they were interacting with other cells

Others have also observed variability in these types of models to study OC formation. One group of researchers had a similar situation to ours, so they performed 46 similar experiments for a time course of 26 days [60]. Unexpectedly, they found that OC numbers changed in a manner much more complex than current knowledge could predict [60]. They observed synchronized waves of OC formation and death and OC oscillations [60]. In other words, when they cultured cells for a longer time period, they observed a second wave of OC formation after the first wave disappeared (i.e., all initial OCs had died). They also observed that the second wave had a greater amplitude than the first [60]. In several experiments, they even observed a third wave of OC formation, larger than the previous two waves. They examined different plating densities but did not see an affect on the rate of OC formation. However, they found that experiments performed with low concentration of RANKL (10 ng/ml) did not exhibit this oscillating OC formation pattern, and the experiment groups with a high concentration of RANKL (100 ng/ml) developed OCs with oscillatory

behavior more frequently [60]. They concluded that RANKL concentration, not plating density, significantly affects the probability of the experiment to exhibit oscillations in osteoclast number [60].

Five of our 6 experiments were done using a 'high' concentration of RANKL (100ng/ml). Experiment 4 was also done using relatively 'high' concentration of RANKL (60ng/ml). Perhaps our experiments were exhibiting the 'oscillations' described above but we did not follow them long enough to see these oscillations. It is possible that the effects of PGE<sub>2</sub> were simply to delay, relative to the control cultures, the wave of OC formation, causing the PGE<sub>2</sub> treated wave to be offset from the control treated wave (Figure 14). If this were the case, whether PGE<sub>2</sub> caused stimulation, inhibition, or a biphasic event would simply depend on where the measurement was made relative to the positions of the control and PGE<sub>2</sub> treated waves. As can be seen from the hypothetical situation illustrated in Figure 14, the conclusion taken from the counts at days 4, 5, and 6 would be that PGE<sub>2</sub> has an inhibitory effect, while at day 7 there was no significant difference, and at days 8, 9, and 10 PGE<sub>2</sub> was stimulatory. Whether or not this delay in OC formation will be important *in vivo* is not known. It is unrealistic to have such micro-controlled environments for days to weeks at a time, when *in vivo*, these environments may last for only minutes to hours. .

Clearly these data suggest that we should examine the effects of PGE<sub>2</sub> on OC formation using lower doses of RANKL, where oscillations are not expected to occur. Further examination is needed to determine whether the size of the culture well produces a different outcome when plated at the same density. The

effects of looking at cell growth rate and the age of the mice from which the BMMs were isolated may also be important factors in osteoclastogenesis that require further examination. We would also like to develop mice that have COX-2 deleted specifically from OC precursors. We could then look at the resorption response *in vivo* to agents that stimulate resorption.

## **SIGNIFICANCE**

Overall, our study provides new information about the quantification of OCs and the process of osteoclastogenesis by taking into account the dose response and time course associated with BMM cultures using PGE<sub>2</sub>. Our study also highlights the difficulties in creating such models in a biologically accurate manner. This study gives us a better understanding of the role of osteoclastogenesis and bone remodeling and its mechanism of action via PGs. It gives orthodontists greater insight into the mechanisms involved in stimulation and inhibition of tooth movement. This in turn could improve the quality and efficiency of treatment.

## TABLES

Direct Microscope Count								
Solution	Day 6a	Day 6b	Day 7a	Day 7b	Day 8a	Day 8b	Day 9a	Day 9b
M	0	0	0	0	0	0	0	0
M + R	665	543	754	829	531	648	338	237
M + PGE <sub>2</sub> <sup>-6</sup> M	0	0	0	0	0	0	0	0
M + R + PGE <sub>2</sub> <sup>-6</sup> M	12	21	50	47	44	57	51	62
M + PGE <sub>2</sub> <sup>-8</sup> M	0	0	0	0	0	0	0	0
M + R + PGE <sub>2</sub> <sup>-8</sup> M	213	203	361	318	565	520	236	238
Indirect Digital Photo Count								
Solution	Day 6a	Day 6b	Day 7a	Day 7b	Day 8a	Day 8b	Day 9a	Day 9b
M	0	0	0	0	0	0	0	0
M + R	769	638	866	942	609	754	385	278
M + PGE <sub>2</sub> <sup>-6</sup> M	0	0	0	0	0	0	0	0
M + R + PGE <sub>2</sub> <sup>-6</sup> M	11	19	48	44	46	54	53	68
M + PGE <sub>2</sub> <sup>-8</sup> M	0	0	0	0	0	0	0	0
M + R + PGE <sub>2</sub> <sup>-8</sup> M	247	184	419	368	593	565	266	266

**Table 1:** Osteoclast (OC) counts: comparison of direct and indirect quantification methods.

Indirect Digital Photo Count for Experiment #1								
Solution	Day 5a	Day 5b	Day 6a	Day 6b	Day 7a	Day 7b	Day 8a	Day 8b
M	0	0	0	0	0	0	0	0
M + R	207	227	462	536	621	697	396	403
M + PGE <sub>2</sub> <sup>-6</sup> M	0	0	0	0	0	0	0	0
M + R + PGE <sub>2</sub> <sup>-6</sup> M	35	57	45	68	107	104	64	59

**Table 2:** OC quantification for Experiment 1.



Indirect Digital Photo Count for Experiment #2								
Solution	Day 6a	Day 6b	Day 7a	Day 7b	Day 8a	Day 8b	Day 9a	Day 9b
M	0	0	0	0	0	0	0	0
M + R	769	638	866	942	609	754	385	278
M + PGE <sub>2</sub> <sup>-6</sup> M	0	0	0	0	0	0	0	0
M + R + PGE <sub>2</sub> <sup>-6</sup> M	11	19	48	44	46	54	53	68
M + PGE <sub>2</sub> <sup>-8</sup> M	0	0	0	0	0	0	0	0
M + R + PGE <sub>2</sub> <sup>-8</sup> M	247	184	419	368	593	565	266	266

**Table 3:** OC quantification for Experiment 2.

Indirect Digital Photo Count for Experiment #3						
Solution	Day 5a	Day 5b	Day 5c	Day 6a	Day 6b	Day 6c
M+R	50	32	44	146	126	147
M+R+PGE <sub>2</sub> <sup>-6</sup> M	5	3	3	15	30	29
M+R+PGE <sub>2</sub> <sup>-8</sup> M	27	22	23	96	103	83
Solution	Day 7a	Day 7b	Day 7c	Day 8a	Day 8b	Day 8c
M+R	272	303	316	249	287	223
M+R+PGE <sub>2</sub> <sup>-6</sup> M	77	75	74	152	183	191
M+R+PGE <sub>2</sub> <sup>-8</sup> M	263	247	256	224	241	222
Solution	Day 9a	Day 9b	Day 9c			
M+R	101	106	91			
M+R+PGE <sub>2</sub> <sup>-6</sup> M	34	63	49			
M+R+PGE <sub>2</sub> <sup>-8</sup> M	86	91	103			

**Table 4:** OC quantification for Experiment 3.

Indirect Digital Photo Count for Experiment #4								
Solution	Day 6a	Day 6b	Day 6c	Day 6d	Day 7a	Day 7b	Day 7c	Day 7d
M	0	0	0	0	0	0	0	0
M+R	61	54	35	35	233	159	191	187
M+PGE <sub>2</sub> <sup>-6</sup> M	0	0	0	0	0	0	0	0
M+R+PGE <sub>2</sub> <sup>-6</sup> M	88	61	72	68	239	274	263	277
M+PGE <sub>2</sub> <sup>-8</sup> M	0	0	0	0	0	0	0	0
M+R+PGE <sub>2</sub> <sup>-8</sup> M	76	97	83	126	442	323	339	351

Indirect Digital Photo Count for Experiment #4								
Solution	Day 8a	Day 8b	Day 8c	Day 8d	Day 9a	Day 9b	Day 9c	Day 9d
M	0	0	0	0	0	0	0	0
M+R	47	26	27	26	39	17	19	35
M+PGE <sub>2</sub> <sup>-6</sup> M	0	0	0	0	0	0	0	0
M+R+PGE <sub>2</sub> <sup>-6</sup> M	176	165	232	210	111	97	112	88
M+PGE <sub>2</sub> <sup>-8</sup> M	0	0	0	0	0	0	0	0
M+R+PGE <sub>2</sub> <sup>-8</sup> M	62	57	35	35	33	39	37	32

**Table 5:** OC quantification for Experiment 4.

Indirect Digital Photo Count for Experiment #5						
Solution	Day 4a	Day 4b	Day 4c	Day 5a	Day 5b	Day 5c
M + R	117	137	148	78	136	133
M + R + PGE <sub>2</sub> <sup>-6</sup> M	162	102	105	137	128	117

Indirect Digital Photo Count for Experiment #5						
Solution	Day 6a	Day 6b	Day 6c	Day 7a	Day 7b	Day 7c
M + R	13	21	19	21	22	19
M + R + PGE <sub>2</sub> <sup>-6</sup> M	45	106	120	41	45	57

**Table 6:** OC quantification for Experiment 5.

Indirect Digital Photo Count for Experiment #6						
Solution	Day 4a	Day 4b	Day 4c	Day 5a	Day 5b	Day 5c
M + R	83	59	73	101	90	71
M + R + PGE <sub>2</sub> <sup>-6</sup> M	16	24	16	66	33	54

Indirect Digital Photo Count for Experiment #6						
Solution	Day 6a	Day 6b	Day 6c	Day 7a	Day 7b	Day 7c
M + R	17	17	4	12	8	6
M + R + PGE <sub>2</sub> <sup>-6</sup> M	32	59	48	17	35	31

**Table 7:** OC quantification for Experiment 6.

Overall Experimental Results		
<u>Experiment #</u>	<u>Well Size</u>	<u>PGE<sub>2</sub> Effect on OC Formation</u>
1	48	Inhibition
2	48	Inhibition
3	48	Inhibition
4	96	Stimulation
5	96	No Effect → S
6	96	Biphasic (I → S)

**Table 8:** Overall Experimental Results.

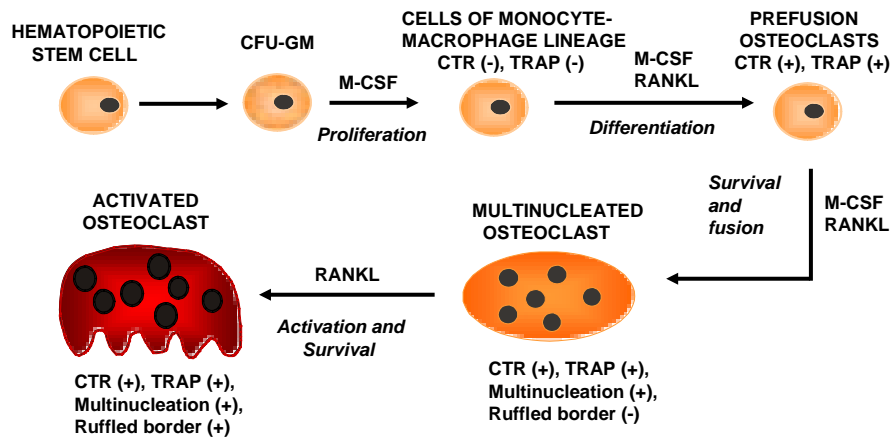
Inhibition (I) – The Total OC counts of the experiment group (group with PGE<sub>2</sub>) was less than the control group (group without PGE<sub>2</sub>).

Stimulation (S) – The total OC counts of the experiment group (group with PGE<sub>2</sub>) was more than the control group (group without PGE<sub>2</sub>).

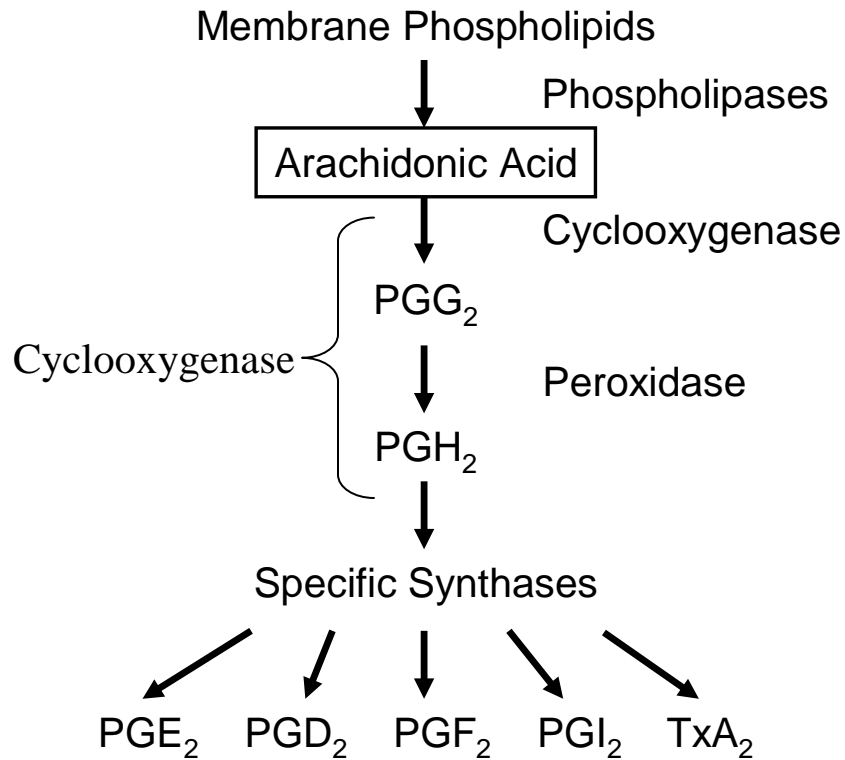
No Effect → S – The experiment group and control groups showed no appreciable difference between OC counts at early time points. At later time points, the experiment group showed stimulation.

Biphasic (I → S) – When the experiment group showed inhibition at early time points and stimulation at later time points.

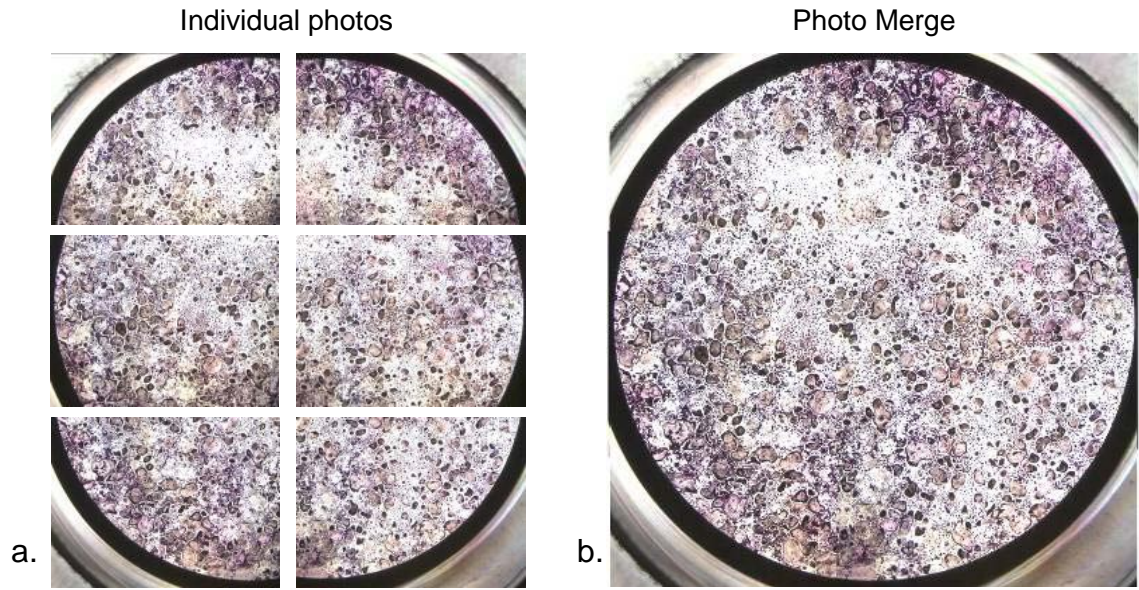
## FIGURES



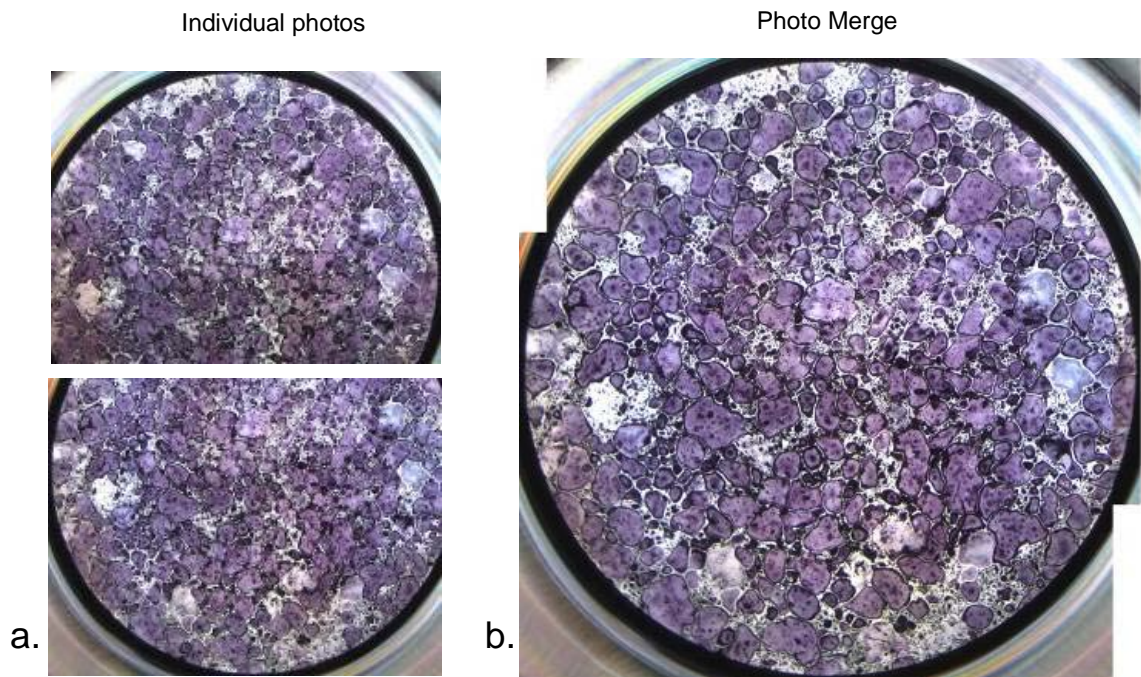
**Figure 1:** Osteoclast (OC) differentiation pathway.



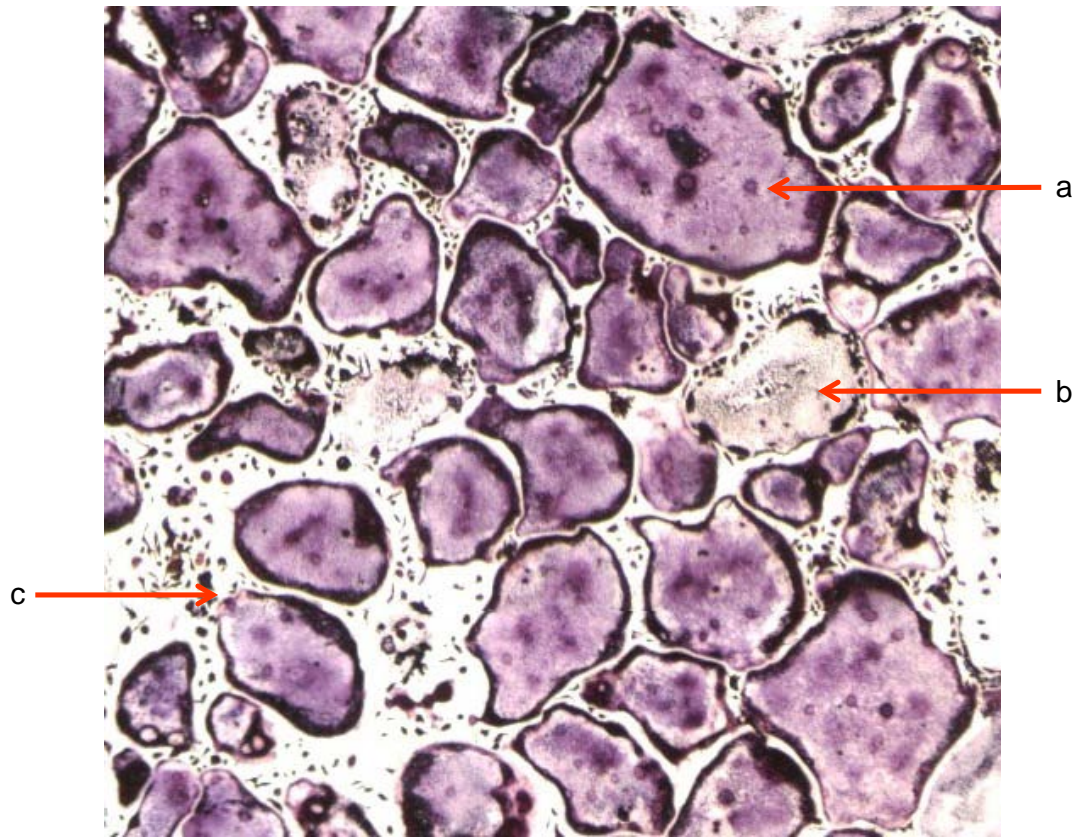
**Figure 2:** Arachidonic acid (AA) metabolic pathway leading to the production of prostaglandins (PG).



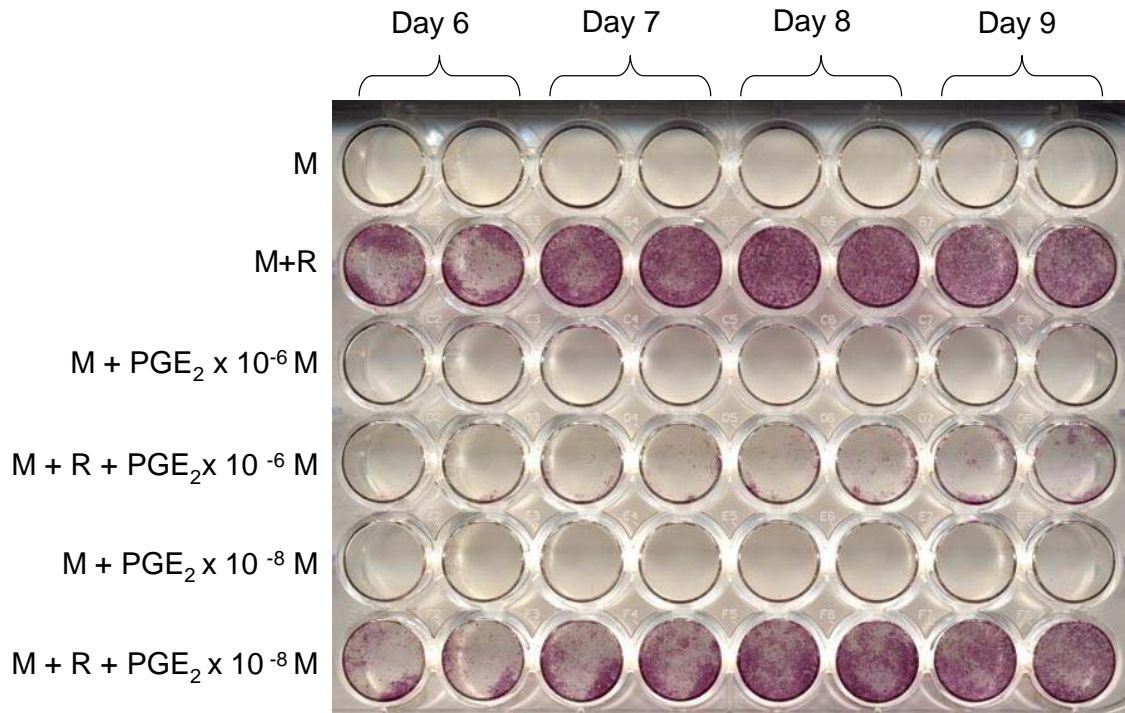
**Figure 3:** 48 well photo merge. a) The wells of 48 well plates were divided into sextants, and six different images of the same well were captured. b) Reconstruction of 48 well dish creating one seamless image.



**Figure 4:** 96 well photo merge. a) The wells of 96 well plates were divided in half and two different images of the same well were captured. b) Reconstruction of 96 well dish creating one seamless image.

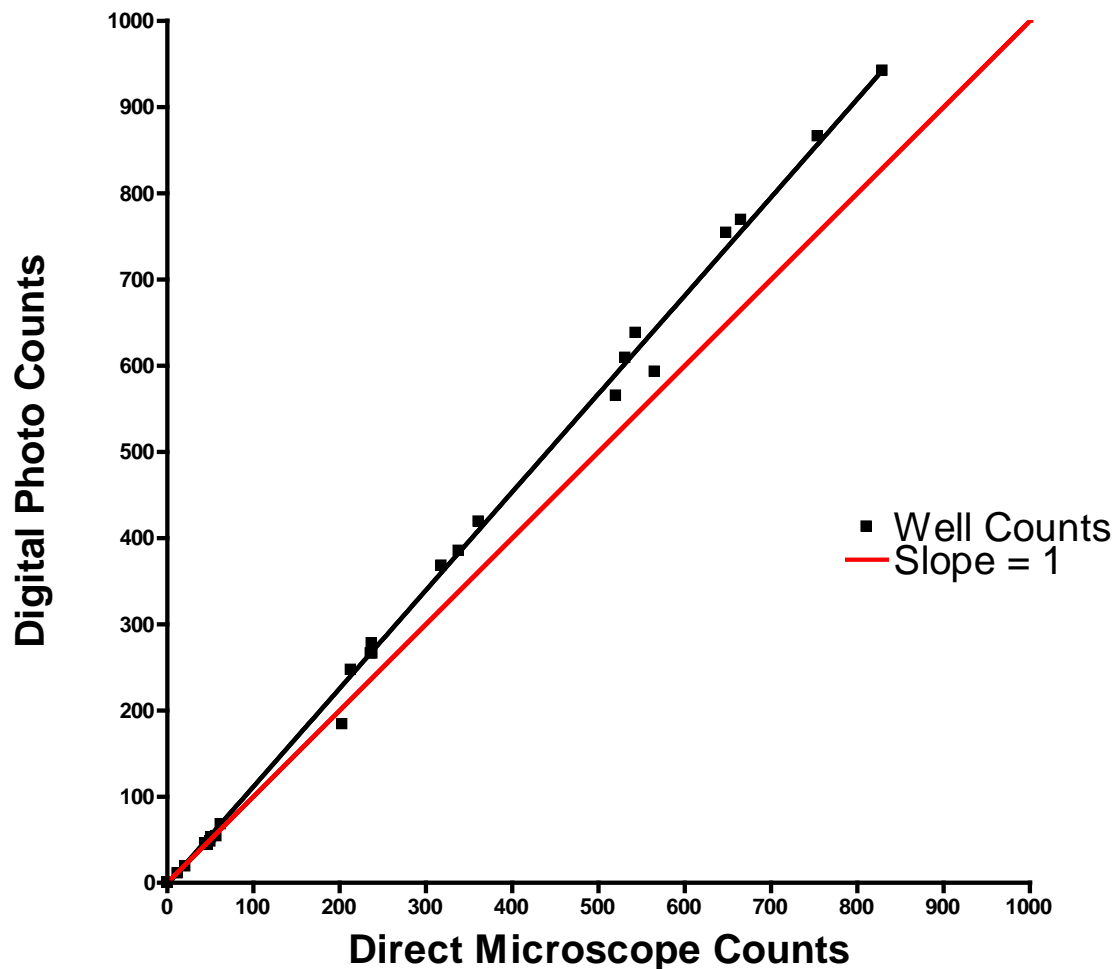


**Figure 5:** Expanded view of an experimental well. a) “Normal” healthy looking OC with multiple nuclei. b) “Ghost” OC showing complete degradation of the cell membrane. c) TRAP positive OC showing partial cell membrane degradation and cell fragments. Note: only “Normal” appearing OCs were quantified. ‘Ghost’ cells, cells with degraded membranes, or cells with less than 3 nuclei were not quantified. Cells b and c would not be quantified.



**Figure 6:** Tartrate resistant acid phosphatase (TRAP) stained bone marrow monocyte/macrophage (BMM) cells from CD-1 mice. BMM Cells from CD1 mice were cultured with macrophage-colony stimulating factor (M-CSF or M), receptor activator of NF- $\kappa$ B ligand (RANKL or R), and different PGE<sub>2</sub> concentrations over a 4 day time course. Cultures were TRAP stained and TRAP positive multinucleated cells (MNC) were directly counted under a light microscope and indirectly counted using the digital photography method. The results were charted and graphed.

## Correlation Microscope vs. Digital Photo Count

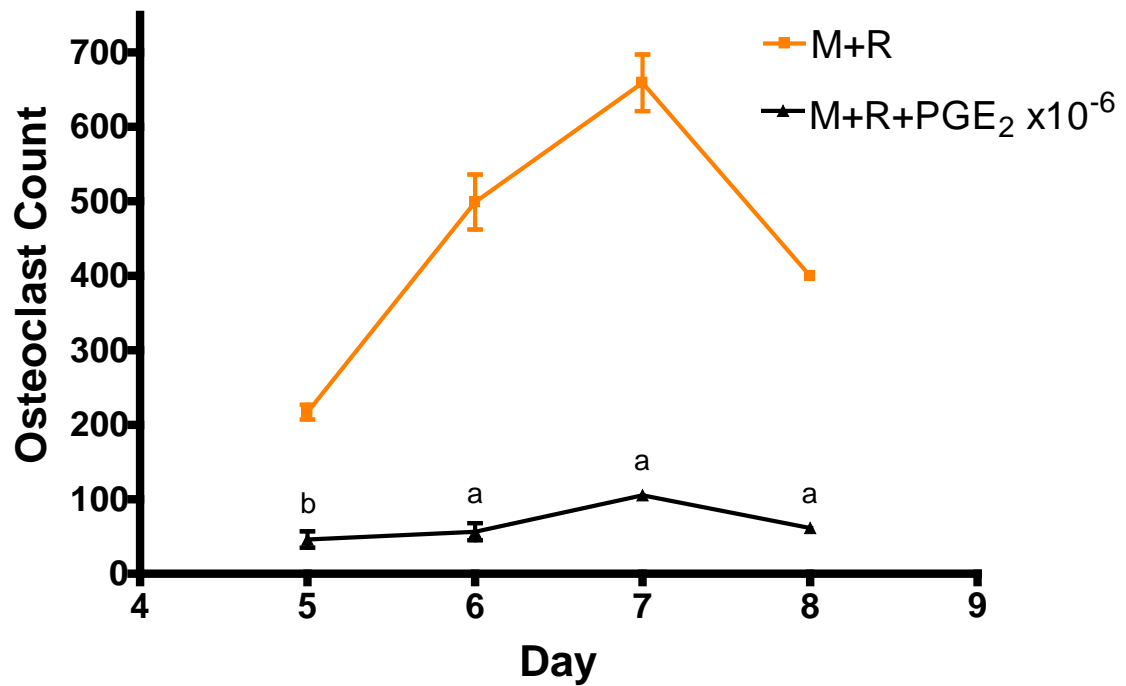


$$R^2 = 0.9981, Y=mX + b, Y=1.139X - 2.137, P < 0.0001$$

**Figure 7:** Correlation of OC counts using the direct and indirect methods. The black squares symbolize the actual OC counts from the direct and indirect quantification methods from each well. The red line symbolizes a 100% correlation between the direct and indirect quantification method, which would yield a slope of 1. A 99.81% correlation was found between the direct and indirect method.

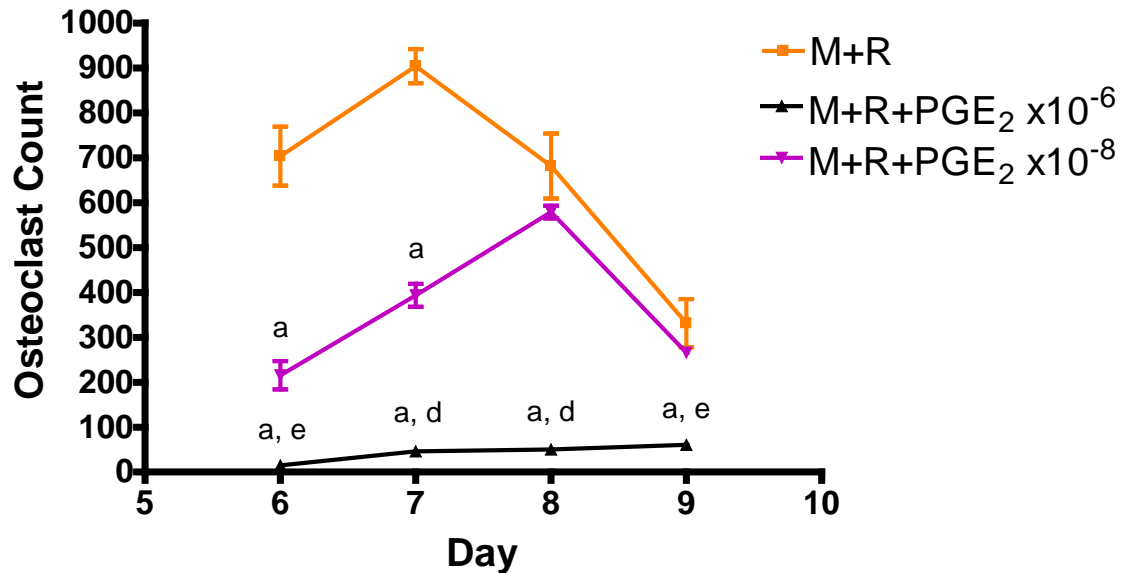


## Experiment 1



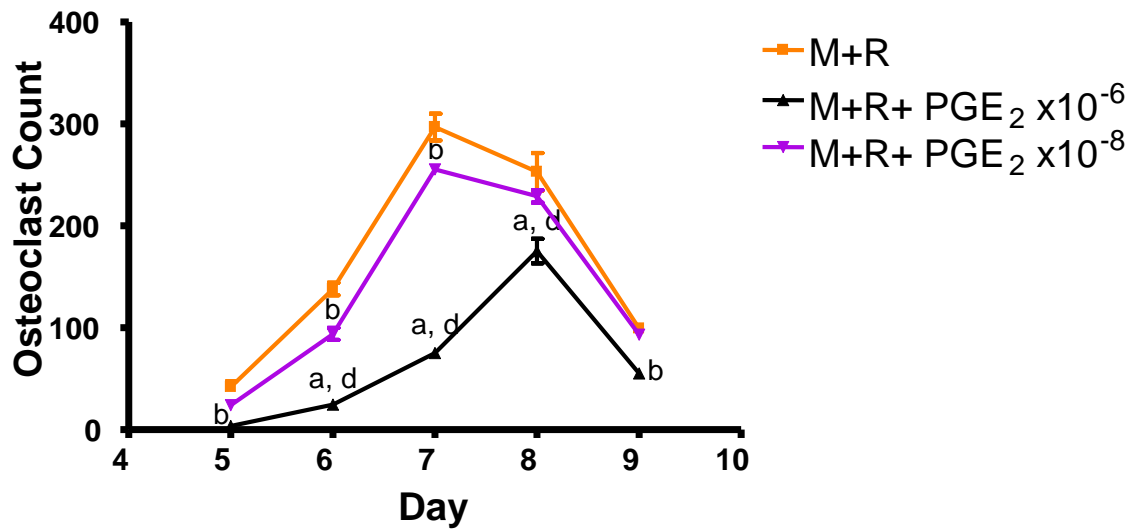
**Figure 8:** Experiment 1: Effect of PGE<sub>2</sub> on OC formation in BMMs cultured in 48 well dishes treated with M-CSF (M, 30 ng/ml), RANKL (R, 100 ng/ml), and in the presence or absence of PGE<sub>2</sub> (10<sup>-6</sup> M) for 5-8 days. Cultures were TRAP stained, and TRAP positive MNC cells were counted using the digital photography method. No TRAP positive MNCs were observed in cultures without RANKL. Symbols are means ±SEM for n=3 wells. <sup>a</sup> Significant effect of PGE<sub>2</sub>, p<0.001; <sup>b</sup> p<0.01

## Experiment 2



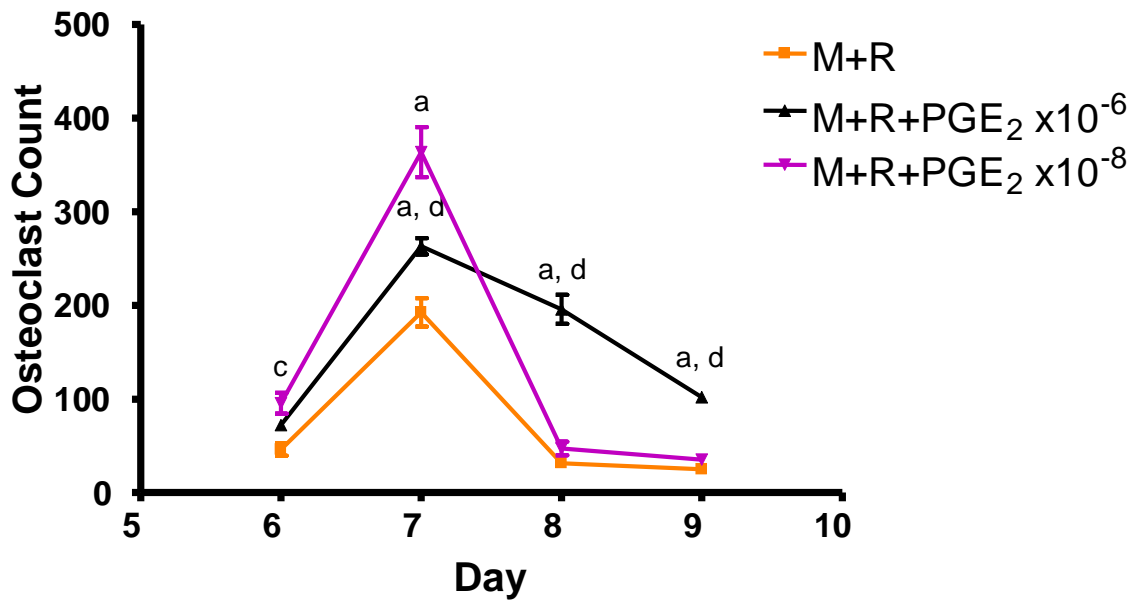
**Figure 9:** Experiment 2: Effect of PGE<sub>2</sub> on OC formation in BMMs cultured in 48 well dishes treated with M-CSF (M, 30 ng/ml), RANKL (R, 100 ng/ml), and in the presence or absence of PGE<sub>2</sub> (10<sup>-6</sup> M or 10<sup>-8</sup> M) for 6-9 days. Cultures were TRAP stained, and TRAP positive MNC cells were counted using the digital photography method. No TRAP positive MNCs were observed in cultures without RANKL. Symbols are means  $\pm$ SEM for n=3 wells. <sup>a</sup> Significant effect of PGE<sub>2</sub> p<.001. <sup>d</sup> Significantly different from PGE<sub>2</sub> x 10<sup>-8</sup>, p<.001; <sup>e</sup> p<.01.

### Experiment 3



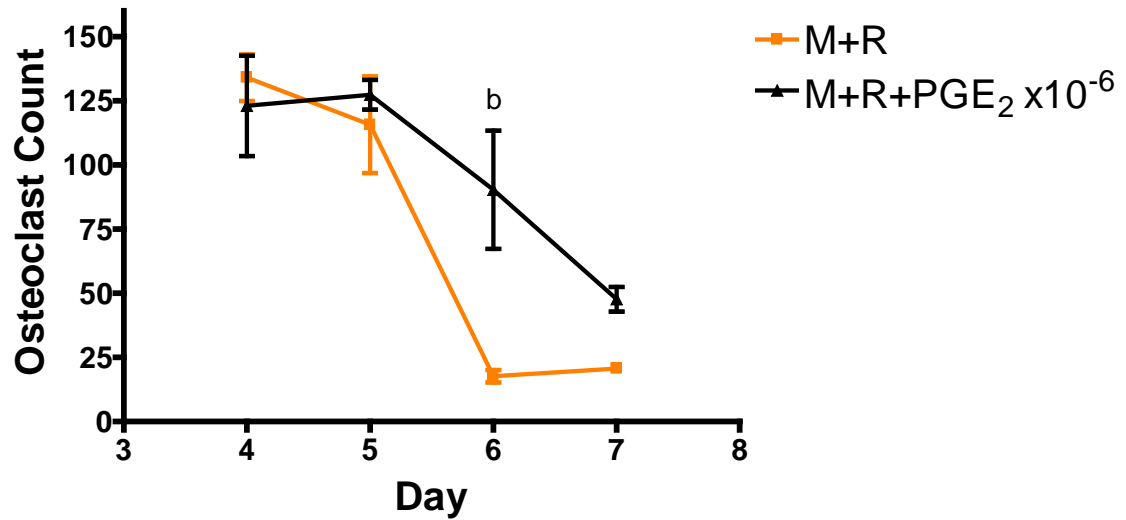
**Figure 10:** Experiment 3: Effect of PGE<sub>2</sub> on OC formation in BMMs cultured in 48 well dishes treated with M-CSF (M, 30 ng/ml), RANKL (R, 100 ng/ml), and in the presence or absence of PGE<sub>2</sub> (10<sup>-6</sup> M or 10<sup>-8</sup> M) for 5-9 days. Cultures were TRAP stained, and TRAP positive MNC cells were counted using the digital photography method. No TRAP positive MNCs were observed in cultures without RANKL. Symbols are means ±SEM for n=3 wells. <sup>a</sup> Significant effect of PGE<sub>2</sub>, p<.001, <sup>b</sup> p<.01. <sup>d</sup> Significantly different from PGE<sub>2</sub> x 10<sup>-8</sup>, p<.001.

## Experiment 4



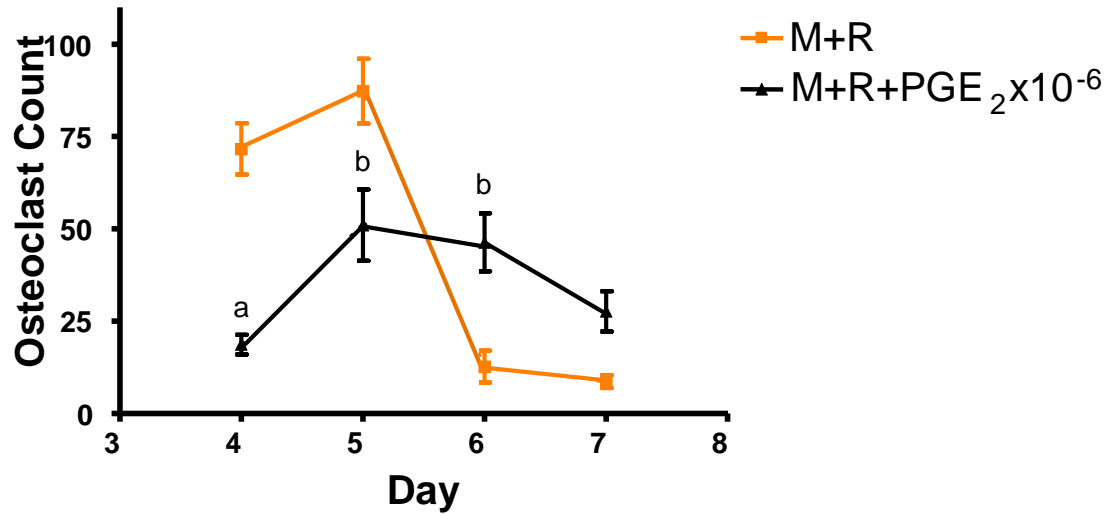
**Figure 11:** Experiment 4: Effect of PGE<sub>2</sub> on OC formation in BMMs cultured in 96 well dishes treated with M-CSF (M, 30 ng/ml), RANKL (R, 60 ng/ml), and in the presence or absence of PGE<sub>2</sub> (10<sup>-6</sup> M or 10<sup>-8</sup> M) for 6-9 days. Cultures were TRAP stained, and TRAP positive MNC cells were counted using the digital photography method. No TRAP positive MNCs were observed in cultures without RANKL. Symbols are means  $\pm$ SEM for n=3 wells. <sup>a</sup> Significant effect of PGE<sub>2</sub>, p<.001; <sup>c</sup> p<.05. <sup>d</sup> Significantly different from PGE<sub>2</sub> x 10<sup>-8</sup>, p<.001.

## Experiment 5



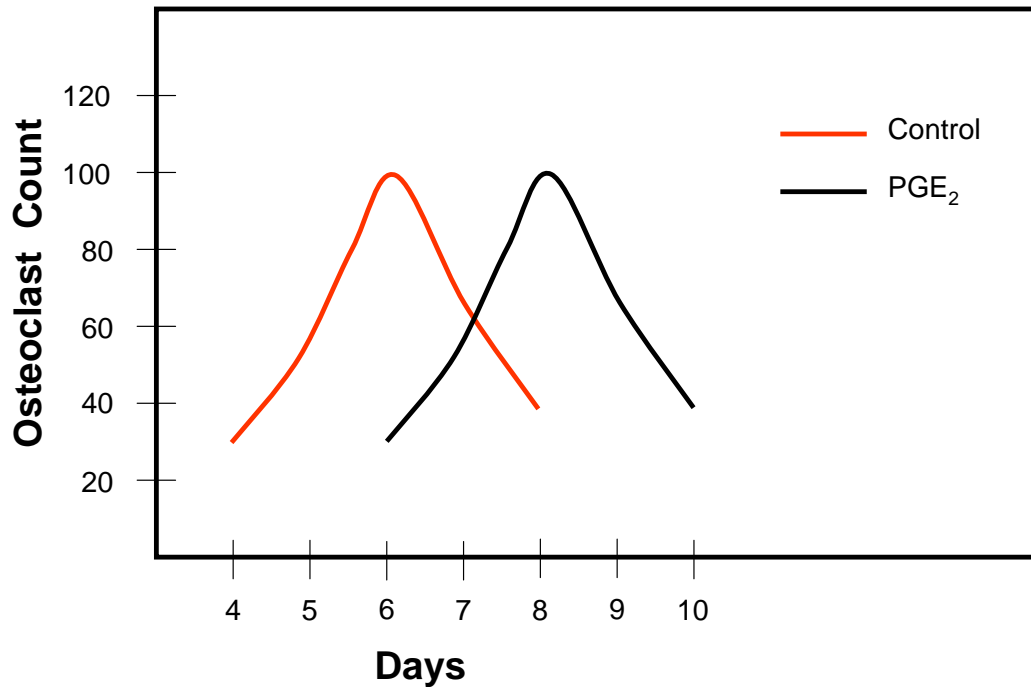
**Figure 12:** Experiment 5: Effect of PGE<sub>2</sub> on OC formation in BMMs cultured in 96 well dishes treated with M-CSF (M, 30 ng/ml), RANKL (R, 100 ng/ml), and in the presence or absence of PGE<sub>2</sub> (10<sup>-6</sup> M) for 4-7 days. Cultures were TRAP stained, and TRAP positive MNC cells were counted using the digital photography method. No TRAP positive MNCs were observed in cultures without RANKL. Symbols are means ±SEM for n=3 wells. <sup>b</sup> Significant effect of PGE<sub>2</sub>, p<.01.

## Experiment 6



**Figure 13:** Experiment 6: Effect of PGE<sub>2</sub> on OC formation in BMMs cultured in 96 well dishes treated with M-CSF (M, 30 ng/ml), RANKL (R, 100 ng/ml), and in the presence or absence of PGE<sub>2</sub> (10<sup>-6</sup> M) for 4-7 days. Cultures were TRAP stained, and TRAP positive MNC cells were counted using the digital photography method. No TRAP positive MNCs were observed in cultures without RANKL. Symbols are means ±SEM for n=3 wells. <sup>a</sup> Significant effect of PGE<sub>2</sub>, p<.001; <sup>b</sup> p<.01.

## Hypothetical PGE<sub>2</sub> Experiment



**Figure 14:** Hypothetical PGE<sub>2</sub> experiment showing phase shift of OC counts and OC production. This hypothetical situation illustrates that there is no difference in OC cycle or the long term effects on OC counts from PGE<sub>2</sub>, rather, just a shift in the OC production phase. Note, OC counts at days 4, 5, and 6 would indicate PGE<sub>2</sub> inhibits OC formation, at day 7 there is no significant difference, while at days 8, 9, and 10 PGE<sub>2</sub> stimulates OC formation.

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