BIOACTIVE COMPOUNDS IN DRIED PLUM

ENHANCE OSTEOBLAST ACTIVITY VIA BMP

PATHWAYS AND DECREASE OSTEOCLAST

ACTIVITY BY SUPPRESSING INTRACELLULAR

CALCIUM AND ACTIVATION OF MAPKS

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Abstract: Dietary supplementation with dried plum has been shown to improve bone health in clinical and pre-clinical studies. This is due to the fruit's anabolic and anti-resorptive properties. A crude ethanol polyphenolic extract from dried plum has been found to enhance bone formation and suppress bone resorption *in vitro* and *in vivo*, but the components within this crude extract that exert these anabolic and anti-resorptive effects are not known. These studies were designed to identify the most bioactive components of the total polyphenolic extract of dried plum in enhancing osteoblast activity and reducing osteoclast activity under normal and inflammatory conditions. In addition, mechanisms by which the bioactive components of the polyphenolic extract affect osteoblast and osteoclast function were examined. Extraction with increasing methanol concentration was used to yield six polyphenolic fractions from the total polyphenolic extract of dried plum. It was determined that the two fractions with the lowest organic content enhanced osteoblast activity under normal conditions in primary bone marrow-derived osteoblasts. This was due to increased BMP signaling and *Runx2* expression. DP-FrA and DP-FrB did not have as robust of an effect on osteoblast activity under inflammatory conditions. The fractions with the higher organic content were the most bioactive in suppressing osteoclast differentiation and activity. These fractions downregulated the expression of *Nfatc1* under normal and inflammatory conditions in mono-cultures of bone marrow-derived osteoclasts, as well as under inflammatory conditions in osteoblast and osteoclast co-cultures. The downregulation of *Nfatc1* was due to a suppression of MAPK and calcium signaling. In addition, the fractions downregulated *Rankl* expression in the co-culture system, thereby suppressing the differentiation signal to osteoclast precursors. These studies are the first to identify fractions of the polyphenolic extract of dried plum that are most bioactive in enhancing osteoblast activity and suppressing osteoclast activity in primary cell culture models. These findings provide valuable insight into the mechanisms by which dried plum improves bone health *in vivo*, as well as the types of dietary compounds that should be targeted for use as potential preventative or treatment measures for osteoporosis.

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CHAPTER I

BACKGROUND

Osteoporosis is a major public health threat, with approximately 9 million Americans

diagnosed with the condition and another 48 million considered at risk with low bone

density or osteopenia [1]. It is estimated that 60% of people 50 years and older are either

affected by osteoporosis or are at increased risk for developing osteoporosis [2].

Osteoporotic fractures result in approximately $19 billion in annual health care costs in

the United States, with fracture occurring in postmenopausal women accounting for 76%

of those costs [2]. Aside from the economic burden of osteoporosis, overall health and

quality of life are also significantly affected. Importantly, an increase in all-cause

mortality has been reported in the first year following fracture of the hip. Additionally, an

overall decline in functional status and an individual's ability to live independently

results from fracture of the hip, vertebra, and humerus [2-4]. Treatments to improve

skeletal health exist, but compliance is often low due to cost, potential side effects and

perceived lack of benefit. The greatest impact on osteoporosis prevention is realized with

the achievement of an optimal peak bone mass during the first 2-3 decades of life [5], but

from that point forward the focus shifts to slowing or delaying bone loss. Therefore, the

development of a low cost, effective treatment to slow the rate of bone loss and improve

skeletal health is desirable.

Maintenance of a healthy skeleton in adults requires that bone is periodically remodeled [6]. It has been estimated that this remodeling process results in the replacement of human skeleton every 10 years [7]. Bone remodeling occurs within a basic multicellular unit (BMU) where bone is first resorbed by osteoclasts via acidic degradation of surface mineral and enzymatic digestion of the underlying extracellular matrix [8]. Following resorption, osteoblasts are known to be recruited to the resorption site by products released during matrix degradation, including transforming growth factor-beta (TGF-β), insulin-like growth factor-2 (IGF-2), and platelet derived growth factor (PDGF) [9, 10]. Osteoblasts secrete collagen and non-collagenous extracellular matrix proteins that form osteoid within the resorbed area, providing a surface for hydroxyapatite to bind [11]. In addition, osteoblasts regulate osteoid mineralization by secreting membrane-bound matrix vesicles, inside of which is an optimal environment for initiation of calcium and phosphate crystallization [8, 12]. Hydroxyapatite formation within matrix vesicles is also enhanced by enzymatic elimination of inhibitors of mineralization, such as pyrophosphates and proteoglycans [12]. The duration of the remodeling cycle is approximately 6 months in humans, with the resorptive phase requiring ~1 month and the much longer formation/mineralization phase persisting for an additional 5 months [13]. By comparison, the bone remodeling cycle is much shorter in rodent models such as the mice, requiring 2-4 weeks [14, 15]. Bone loss occurs when the normal cycle of resorption and formation is uncoupled, and the rate of bone resorption exceeds that of bone formation [11]. A persistent increase in the rate of bone remodeling

can result in osteoporotic bone due to the relatively brief period of time required for bone resorption and the substantially longer period of time required for bone formation [16].

Classically, two types of primary osteoporosis are clinically recognized—senile or age-related osteoporosis and postmenopausal osteoporosis [11, 17]. While a reduction in the rate of bone formation is the primary metabolic change associated with aging that leads to bone loss [18], estrogen deficiency initially results in a rapid increase in both bone resorption and, to a lesser extent, bone formation (i.e., bone turnover) [17]. Over time, bone formation and mineralization cannot compensate for the accelerated rate of bone resorption and bone loss occurs. Additionally, following the relatively short-term increase in overall bone turnover, postmenopausal women eventually experience a decline in osteoblast activity and bone formation [19]. This imbalance in bone remodeling is primarily attributed to the direct effects of estrogen deficiency on osteoclast, osteoblast, and osteocyte [20]. Estrogen's effect on bone cells is complex, but in general it alters key regulators of osteoblast and osteoclast differentiation and activity, as well as osteoclast and osteocyte apoptosis [21-24]. Osteoblast differentiation is mediated by estrogen in part via mothers against decapentaplegic homolog 1/5/8 (Smad 1/5/8) and runt-related transcription factor 2 (Runx2) signaling. Estrogen also increases the expression of genes associated with the activity of osteoblasts, including alkaline phosphatase (ALP) and osteocalcin (OCN) [25-27]. Additionally, estrogen decreases osteoclast number by altering key regulators of differentiation expressed by osteoblasts and immune cells, including receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG), as well as by inducing apoptosis of osteoclasts via upregulation of Fas ligand (FasL) and caspases 3 and 8 [28, 29]. Therefore, estrogen deficiency

increases the number of osteoclasts and their lifespan, and decreases osteoblast differentiation and activity. The process through which estrogen affects bone cells will be reviewed in detail in Chapter 2.

In addition to direct effects on bone cells, estrogen indirectly alters bone metabolism by modulating immune responses [30-33]. Estrogen receptors are expressed in a number of different immune cell populations, including monocytes, B cells, and both CD4^{+} and CD8^{+} T cells [32]. Estrogen receptor-alpha (ER-α) is predominantly expressed by CD4^{+} T cells, while ER-β is more highly expressed by B cells [32]. No difference in ER-α and ER-β expression by monocytes has been reported in premenopausal women, but following menopause monocyte expression of ER-α is upregulated [32]. Monocyte production of interleukin (IL)-1, IL-6 and tumor necrosis factor- α (TNF-α), as well as the proliferation of TNF-α- and IL-17-producing CD4^{+} T cells plays a role in the pathophysiology of postmenopausal osteoporosis, suggesting a major role of ER-α signaling in the regulation of the immune response that alters bone metabolism [30, 31, 33, 34].

Dietary components known to have immunomodulating properties have been found to protect against bone loss [51-55]. For instance, green tea polyphenols have been shown to reduce bone loss under estrogen deficiency and inflammatory conditions [55-59]. Blueberries prevented bone loss in ovariectomized (OVX) models of osteoporosis, and decreased the inflammatory response, including inhibition of IL-6 and IL-1β production, in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage cells [53, 60, 61]. Soy consumption improved bone parameters in estrogen deficiency models of osteoporosis [51, 62]. Dried plums, rich in phenolic compounds [63], have been shown

to attenuate and even reverse bone loss due to aging and gonadal hormone deficiency in pre-clinical and clinical studies [52, 64-67]. These effects on BMD and bone microarchitecture result from a temporal suppression of osteoblast activity and sustained suppression of osteoclast activity (i.e., bone turnover) following dried plum consumption [68, 69]. In a clinical study of postmenopausal women, daily consumption of 100 g of dried plum resulted in a higher BMD of the ulna and spine [68]. This improvement in BMD was attributed to a reduction in bone turnover, as evidence by suppression of bone-specific ALP, a marker of bone formation, and tartrate-resistant acid phosphatase 5b (TRAP5b), an indicator of osteoclast activity. In a follow up study, 6 months of 50 g of dried plum daily was as effective as 100g of dried plum in protecting postmenopausal women from bone loss, and this was attributed to a decrease in serum TRAP5b, an indicator of bone resorption [70]. In an animal model of male osteoporosis, dried plum restored trabecular bone in the vertebra and distal femur metaphysis to a similar extent as intermittent parathyroid hormone (PTH) treatment, currently the only FDA-approved anabolic treatment for osteoporosis [52]. Dried plum supplementation altered the systemic immune response in estrogen deficient mice protected from bone loss, including a decrease in splenocyte TNF-α production and a decrease in bone marrow lymphoblast numbers [67].

Efforts to begin to identify the bioactive components in dried plum responsible for these osteoprotective effects have involved both *in vivo* and *in vitro* studies. In a study utilizing aged, osteopenic OVX rats, a crude ethanol extract of the polyphenolic compounds restored bone to a similar extent of that of dried plum (*unpublished data*). This extract has also been shown to increase osteoblast activity and decrease osteoclast

differentiation under normal and inflammatory conditions [71, 72]. Although previous *in vivo* and *in vitro* studies have supported that this crude polyphenolic extract accounts for the majority of the bioactivity of dried plum on bone, studies are needed using a more refined extract (i.e., removal of residual carbohydrates) to determine the types of polyphenolic compound(s) responsible for these effects and their mechanisms of action.

Identifying the bioactive component(s) in dried plum responsible for these beneficial effects on bone is an important step in determining if dietary supplementation could be a component of an osteoporosis prevention or treatment strategy. In addition, understanding the extent to which these bioactive components alter bone metabolism will allow for better understanding of the pathophysiology of osteoporosis and how dietary bioactive components may be used as a part of prevention and treatment strategies.

Purpose

The purpose of this project is to determine the bioactive component(s) in dried plum responsible for the beneficial effects on bone metabolism and the mechanisms through which these components enhance osteoblast activity and suppress osteoclast activity.

Central Hypothesis: Polyphenolic compounds in dried plum favorably affect bone metabolism by suppressing osteoclast activity and increasing osteoblast activity. These effects are mediated by alterations in calcium and MAPK signaling in osteoclasts and BMP signaling in osteoblasts.

Specific Aim 1: To investigate the effects of different fraction(s) of a dried plum polyphenol extract on osteoclasts *in vitro* under normal and inflammatory conditions and how these effects are mediated.

Sub-Aim 1.1: To determine the fraction(s) and dose of the dried plum polyphenol extract that most effectively reduces osteoclastogenesis in a murine immortal cell line.

Sub-Aim 1.2: To examine how the active polyphenolic fraction(s) alter osteoclast differentiation (i.e., quantification of TRAP$^+$ cells) and activity (i.e., resorption pit formation) using primary bone marrow-derived osteoclast cultures.

Sub-Aim 1.3: To examine the extent to which the active polyphenolic fraction(s) alter key regulators of osteoclast differentiation by way of calcium and MAPK signaling pathways.

Sub-Aim 1.4: To examine how the fraction(s) of the dried plum polyphenolic extract alter osteoclast differentiation using murine primary co-cultures, a system that allows osteoblast and osteoclast interaction.

Working Hypothesis 1: The phenolic compounds in dried plum will attenuate the increase in osteoclast differentiation and activity in normal and inflammatory cell culture conditions by suppressing intracellular calcium and MAPK signaling.

Specific Aim 2: To investigate the effects of different fraction(s) of a dried plum polyphenol extract on osteoblasts *in vitro* under normal and inflammatory conditions and how these effects are mediated.

Sub-Aim 2.1: To determine the fraction(s) and dose of a dried plum polyphenol extract that results in the greatest increase in osteoblast activity (i.e., ALP) and function (i.e., formation of mineralized nodules) in a murine immortal cells line.

Sub-Aim 1.2: To examine how the active polyphenolic fraction(s) alter osteoblast activity (i.e., ALP) and function (mineralized nodule formation) using primary osteoblast cultures.

Sub-Aim 2.2: To examine the mechanisms (i.e., BMP and MAPK signaling pathways) by which the most bioactive fraction(s) of the dried plum polyphenolic extract increases osteoblast differentiation and activity.

Working Hypothesis 2: The phenolic compounds in dried plum will attenuate the reduction in osteoblast differentiation and activity by enhancing BMP and MAPK signaling in normal and inflammatory cell culture systems.

CHAPTER II

LITERATURE REVIEW

Introduction

Osteoporosis is a costly and frequently debilitating disease that affects individuals of all ages, but primarily adults over the age of 65 years [2]. Data from the United States Census Bureau indicates that the number of adults aged 62 and older increased by 21% from the year 2000 to the year 2010, the largest increase of any age group [73]. As the age demographic shifts to a higher percentage of older adults, it is expected that the annual incidence of osteoporotic fracture will increase 50% by the year 2025 [2, 74]. Because females have a lower peak bone mass than males [75] and due to accelerated bone loss during the onset of menopause [76], postmenopausal women are likely to experience approximately 75% of these fractures [2].

Coinciding with the increase in fracture incidence, osteoporosis-related health care costs are expected to increase from $16.9 billion in 2005 to an estimated $25.3 billion by the year 2025 [2-4]. Aside from the economic burden of osteoporosis, fractures are associated with a significant increase in all-cause mortality, especially within one year of hip fracture [3, 4]. Whether this increased risk of mortality is due

to complications associated with treatment of fracture (e.g., surgical risks, immobility) or due to comorbidities that contribute to the pathophysiology of fracture (i.e. dementia, kidney disease, neurologic disease, etc.) is not clear [3]. To date, treatment options have not been as successful at reducing the prevalence of osteoporosis as anticipated because of poor adherence due to side effects, lack of perceived benefit and/or cost of treatment [77]. Effective strategies are needed to prevent fracture from occurring in at-risk populations and to improve treatment outcomes, both of which will ultimately reduce healthcare costs and mortality.

Bone Metabolism

To develop better prevention and treatment strategies, the fundamental pathophysiology of bone loss must be understood. Bone remodeling involves bone degradation by osteoclasts coupled with bone formation by osteoblasts and these events occur within a canopy of bone lining cells that provide a physical barrier which forms the BMU [78-80]. This remodeling process is required for the maintenance of a healthy adult skeleton. Bone loss occurs when there is an uncoupling of bone remodeling by osteoblasts and osteoclasts [81]. While signaling from osteocytes, another bone cell population, is now recognized as having a role in the activation of the remodeling process, the focus here will be on the study of the osteoclast and osteoblast in bone resorption and bone formation, respectively. [78, 82, 83].

Bone Resorption

Upon activation of bone remodeling, bone marrow mononuclear monocyte/macrophage osteoclast precursors, derived from hematopoietic stem cells

(HSCs), are recruited by monocyte chemoattractant protein-1 (MCP-1) [84]. MCP-1 is produced by osteoblasts in response to signals from osteocytes or endocrine hormones, such as PTH [84]. The formation of osteoclasts from bone marrow HSCs requires at least two known cytokines, macrophage-colony stimulation factor (M-CSF) and receptor activator of NF- κB ligand (RANKL) [85-87]. Both of these proteins are produced by cells of the osteoblast lineage, as well as some immune cells (e.g., T cell populations). To regulate osteoclast differentiation, osteoblasts secrete osteoprotegerin (OPG), a soluble decoy receptor for RANKL that inhibits HSC differentiation to osteoclasts [11]. Proliferation of osteoclast precursors requires M-CSF binding to its receptor, c-Fms, which results in the interaction and nuclear localization of transcription factors micropthalmia-associated transcription factor (MITF) and PU.1 [86-89]. Treatment of murine HSCs with M-CSF results in translocation of MITF from the cytoplasm to the nucleus [90]. While PU.1 is localized in the nucleus in the absence of M-CSF, it is not present in the promoter regions of target genes related to the resorptive capacity of osteoclasts, such as tartrate resistant acid phosphatase (TRAP), cathepsin K (Ctsk) and carbonic anhydrase II (CAII) [90]. In fact, treatment with M-CSF results in recruitment of both MITF and PU.1 to the promoter regions of target genes. Despite inducing the localization of these transcription factors to the promoter regions of genes essential to the function of osteoclasts, M-CSF alone is not sufficient to induce osteoclastogenesis [90].

Differentiation of osteoclast precursors to mature osteoclasts is initiated by RANKL interaction with the receptor activator of NF- κB (RANK) on HSCs [86, 87, 89]. Interference with RANKL/RANK binding with the treatment of the RANKL antibody, denosumab, has been shown to improve BMD and reduce risk of fracture in

postmenopausal women, and this has been attributed to a decrease in bone resorption [91-93]. The binding of RANKL to RANK activates tumor necrosis factor receptor-associated factor 6 (TRAF6)-mediated signaling cascades, including NF-κB, c-Jun N-terminal kinase (JNK), mitogen activated protein kinase (MAPK) p38, and extracellular signal-regulated kinase (ERK) cascades, that result in the differentiation and activation of osteoclasts (**Figure 1**) [94]. Inhibition of NF-κB, p38 and ERK activation by the phytochemical phenethyl isothiocyanate found in cruciferous vegetables downregulates osteoclast differentiation, demonstrating the necessary role of these signaling cascades in osteoclastogenesis [95].
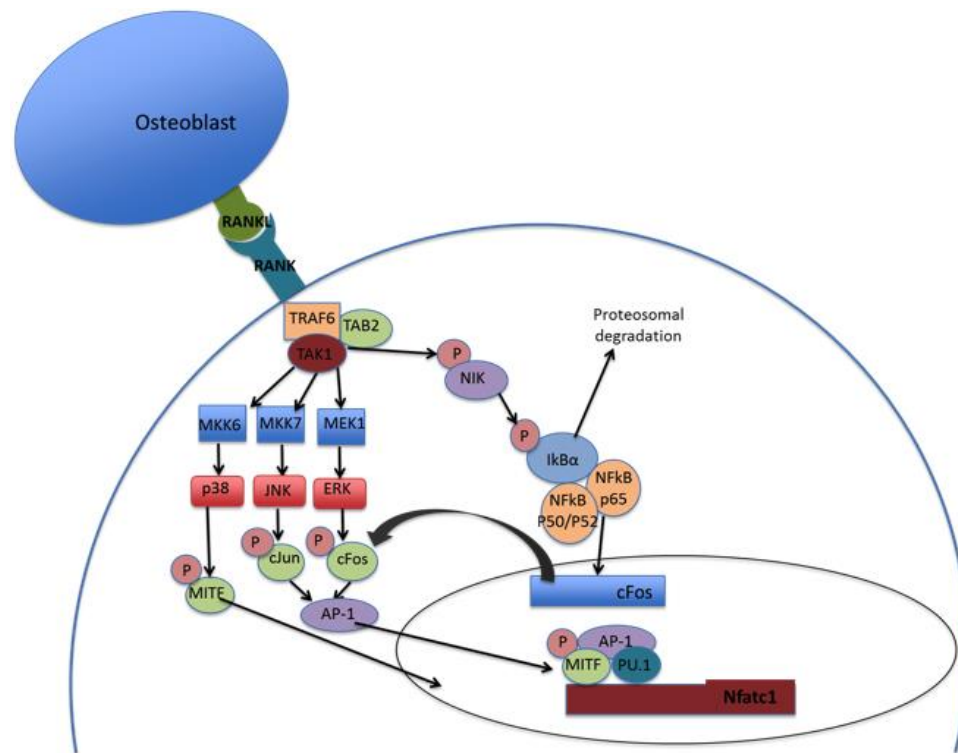
Figure 1. Upon RANKL/RANK binding, TRAF6 forms a complex with TAK1 and TAB2. The TRAF6/TAK1/TAB2 complex phosphorylates NIK. NIK activation leads to the phosphorylation of IκB, marking the NF-κB inhibitor for proteosomal degradation and allowing NF-κB to translocate to the nucleus, where it acts as a transcription factor, inducing *cFos* expression. The TRAF6/TAK1/TAB2 complex also activates p38, JNK and ERK signaling cascades, leading to the activation of transcription factors necessary for the induction of *Nfatc1* expression.

Upon RANKL binding to RANK, TRAF6 forms a complex with the MAP3K TGF-β-activating kinase 1 (TAK1) and the adaptor protein TGF-beta activated kinase 1/MAP3K7 binding protein 2 (TAB2) (**Figure 1**) [96]. The formation of this complex results in the activation of TAK1, which then phosphorylates NF-κB-inducing kinase (NIK) [97]. Activated NIK phosphorylates IκB kinase (IKK) in the cytoplasm, which in turn leads to the phosphorylation of IκB, an NF-κB inhibitor protein. Phosphorylation of IκB induces proteosomal degradation of the protein, allowing for translocation of NF-κB to the nucleus, where it acts as a transcription factor [97].

Translocation of NF-κB to the nucleus induces expression of c-Fos, a major component of the transcription factor activator protein-1 (AP-1) [98]. The AP-1 complex consists of homodimers of c-Fos or heterodimers of c-Fos and c-Jun. Activation of AP-1 occurs via ERK-mediated phosphorylation of c-Fos and JNK-mediated phosphorylation of c-Jun [98]. AP-1 activation is essential for the expression of nuclear factor of activated T-cells, calcineurin-dependent 1 (Nfatc1), a transcription factor necessary for the terminal differentiation of osteoclasts [99-104]. The presence of activated AP-1 at the promoter regions of genes essential to osteoclast function (e.g., TRAP and CtsK) also suggests a role for this transcription factor in the activation of these genes [105]. In fact, activation of TRAP and Ctsk requires the presence of MITF/PU.1 complex, Nfatc1, and phospho-p38 in their promoter regions, indicating a convergence of multiple signaling pathways to induce differentiation and activation of osteoclasts [90].

In addition to initiating NF-κB signaling cascades in preosteoclasts, RANKL also stimulates the TRAF6- and TAK1-mediated activation of JNK, ERK, and p38 MAPK signaling cascades [104, 106]. Phosphorylation of TAK1 induces the p38 MAPK

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signaling cascade, including upregulation and activation of MKK3, MKK6, and p38

within 1 day of soluble RANKL treatment of murine bone marrow monocytes [106].

Activated p38 phosphorylates and activates MITF at the promoter regions of target genes,

including Nfatc1 [90, 101-103]. The activation of MAPK signaling cascades in RANKL-

stimulated RAW 264.7 cell and bone marrow monocyte/macrophages can also be

induced by TRAF6-mediated reactive oxygen species (ROS) production [107]. The

ROS, produced by the plasma membrane bound NADPH oxidase Nox1, initiate JNK,

ERK and p38 MAPK signaling cascades, and treating with an antioxidant has been

shown to inhibit these signaling pathways and the differentiation of osteoclasts [107].

Blocking RANKL-induced JNK, ERK and p38 activation in RAW 264.7 cells with

treatment of caffeic acid 3,4-dihydroxy-phenethyl ester, a compound found in various

medicinal plants, suppressed osteoclast differentiation, demonstrating the necessity of

these signaling cascades in osteoclastogenesis [108]

Also essential to osteoclast differentiation, the binding of RANKL to RANK

stimulates intracellular Ca^{2+} flux (**Figure 2**). A sustained Ca^{2+} oscillation has been

reported approximately 24 hours after soluble RANKL stimulation of

monocyte/macrophage RAW 264.7 cells *in vitro* [109]. These Ca^{2+} oscillations are

essential for sustained activation and auto-amplification of Nfatc1, due to the Ca^{2+}-

dependent calcineurin pathway necessary for Nfatc1 induction [109]. In fact, inhibition

of calcium signaling with treatment of the calcium chelator BAPTA-AM or with the

calcineurin inhibitors FK506 and cyclosporin A, blocks RANKL induced osteoclast

differentiation in bone marrow-derived monocyte/macrophage precursor cells [110].
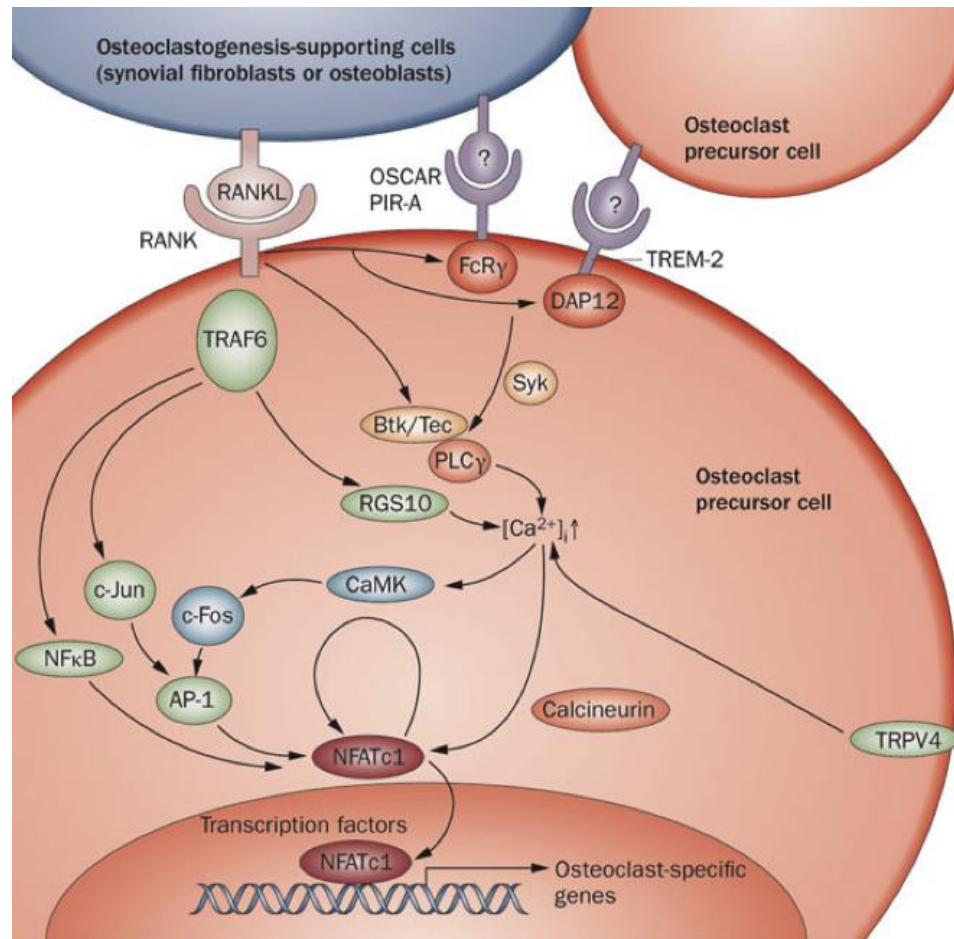
Figure 2. Calcium signaling is initiated upon activation of the DAP12- and FcRγ-associated co-stimulatory receptors OSCAR, PIR-A, TREM2, and SIRPβ1. Activation of DAP12 and FcRγ results in Syk kinase recruitment and subsequent PLCγ activation. PLCγ initiates calcium release from the ER as well as extracellular calcium influx. The increase in intracellular calcium activates CaMK, which phosphorylates ERK and CREB, leading to the induction and activation of cFos. Intracellular calcium also activates calcineurin, which will dephosphorylate Nfatc1, allowing Nfatc1 to translocate to the nucleus to act as a transcription factor [111].

Calcium oscillations are mediated, in part, by the costimulatory receptor osteoclast-associated receptor (OSCAR), which is preferentially expressed on osteoclast precursors, as well as the myeloid receptors paired Ig-like receptor A (PIR-A), triggering receptor expressed on myeloid cells 2 (TREM2), and signaling regulatory protein β1 (SIRPβ1) [112, 113]. Stimulation of membrane-bound OSCAR and PIR-A by binding of

ligands expressed by osteoblasts results in activation of Fc receptor-gamma (FcRγ) [112, 113]. Similarly, stimulation of membrane-bound TREM2 and SIRPβ1 by ligands constitutively expressed on myeloid cells and osteoclast precursors activates the adaptor protein DNAX activation protein of 12 kDa (DAP12) [114]. DAP12 and FcRγ are immunoreceptor tyrosine-adaptation motif (ITAM)-containing adaptor proteins necessary to initiate calcium signaling in myeloid cells [113]. Interfering with these signaling cascades by inhibiting the adaptor proteins or the membrane-bound receptors they associate with impairs osteoclast differentiation and function [113-117]. For example, IL-10 treatment inhibited *Trem2* transcription and downregulated osteoclastogenesis in human osteoclasts *in vitro* [114]. Activation of these ITAM-containing adaptor proteins results in the recruitment of Syk family kinases and subsequent phosphorylation and activation of phospholipase Cγ (PLCγ) in the cytoplasm [113]. Activated PLCγ releases inositol 1,4,5-triphosphate (IP$_3$) from the plasma membrane, which then binds IP$_3$ receptors on the ER membrane, initiating calcium release from the ER [118]. Calcium release from the ER as a result of DAP12 and FcRγ signaling is essential for osteoclast differentiation, as mice deficient in both adaptor proteins fail to produce osteoclasts [117]. Calcium depletion from the ER induces a conformational change in the ER transmembrane protein stromal interaction molecule-1 (STIM1), which allows for oligomerization of the protein [118]. This oligomerization of STIM1 results in activation of calcium release-activated calcium channel 1 (Orai1) at plasma membrane-ER junctions. STIM1 activates Orai1, and the calcium channel opens, resulting in calcium influx through the plasma membrane. This extracellular calcium influx is required for the fusion of osteoclast progenitors into multinucleated osteoclasts [109].

An increase in cytoplasmic calcium concentration activates the calcium binding

protein calmodulin [119]. Calmodulin then binds Ca^{2+}/calmodulin dependent kinases

(CaMK), inducing a conformation change that results in the activation of the CaMK.

Phosphorylation of the activated CaMK is required for maintenance of maximal function

of the kinase. Downstream targets of CaMK in osteoclast differentiation include ERK

and cAMP response element-binding protein (CREB) [120, 121]. Both ERK and CREB

are involved in expression and activation of c-Fos [100]. CREB functions as a

transcription factor necessary for the induction of c-Fos expression while ERK

phosphorylates and activates c-Fos as a component of AP-1 [100, 120]. Due to the role of

c-Fos in Nfatc1 induction, inhibition of either ERK or CREB results in downregulation of

Nfatc1 expression and osteoclast differentiation [120]. Adequate auto-amplification and

upregulation of Nfatc1 occurs after approximately 5 days of RANKL stimulation of

cultured splenic hematopoietic precursor cells [90]. Nfatc1 then binds to the promoter

region of multiple genes necessary for osteoclast differentiation and function, including

genes essential to osteoclast fusion (e.g., ATP6v0d2 and DC-STAMP) [109, 122], as well

as genes essential to the activity of osteoclasts (e.g., TRAP and Ctsk) [90]. Inhibition of

calcium signaling via binding of calmodulin by the polyphenolic compound praeruptorin

A, isolated from the dried root of *Peucedanum praeruptorum* Dunn, resulted in a

decreased activation of CREB and a reduction in auto-amplification of Nfatc1, as well as

a decrease in osteoclast number [123].

Following mature cell formation, the osteoclast attaches to the bone surface via

αvβ3 integrins, which recognize specific amino acid sequences in bone matrix proteins

[124]. Osteoclasts form a sealed microenvironment and begin the resorption process

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[124]. The sealed microenvironment becomes acidified, in part, by the actions of the cytoplasmic and membrane-bound metalloenzyme CAII, which generates protons (H^+) and bicarbonate (HCO_3^-) from water (H_2O) and carbon dioxide (CO_2) [125]. Vacuolar H^+-ATPase pumps localized to the ruffled border of the resorptive surface use energy from the conversion of ATP to ADP to transport the H^+ across the ruffled border [125]. To maintain intracellular pH, the HCO_3^- is exchanged with Cl^- at the basolateral membrane. The Cl^- is then transported through Cl^- channels at the ruffled border to maintain cell polarity [125]. The acidification within the sealed microenvironment results in the mobilization of the mineralized matrix, which exposes the underlying organic matrix consisting largely of type I collagen. The lysosomal enzyme, cathepsin K, the predominant cathepsin expressed by osteoclasts, is responsible for the degradation of type I collagen at resorption sites [126]. In addition to cathepsin K, tartrate-resistant acid phosphatase, matrix metalloproteinase 9 and gelatinase also aid in the osteoclasts' ability to digest the organic matrix of bone [127]. The resorption phase lasts approximately 2 to 4 weeks in humans and approximately 1 week in mice [14, 15]. This phase of bone remodeling concludes when the multinucleated osteoclasts undergo apoptosis [128, 129].

Bone Formation

Following bone resorption, the "reversal phase" of bone remodeling occurs which is characterized by removal of matrix debris and recruitment of osteoblast progenitors, derived from pluripotent mesenchymal stem cells, to the resorbed surface [8, 130]. While it's not entirely clear where the osteoblast progenitor cells in the BMU migrate from, an increase in capillary density around the canopy cells of the BMU is observed during bone resorption [131]. This increase in capillary density coincides with an increase in

proliferating osteoprogenitor cells within the canopy. In addition, there is evidence of cells expressing osteoblast specific genes migrating between the canopy and the bone surface [131]. The signaling molecules involved in this process remain a point of investigation, but it has been suggested that some proteins released from the bone matrix during degradation, such as insulin-like growth factors (IGFs), bone morphogenetic proteins (BMPs), TGF-β, and platelet-derived growth factor (PDGF) signal osteoblasts to begin bone formation [132-134]. However, there is evidence to suggest that TGF-β1, one of 3 isoforms of TGF-β and an abundant cytokines in bone matrix, is also responsible for the chemotaxis of osteoblast precursors to the site of resorption [9]. Osteoprogenitor cell migration has been reported to be significantly inhibited by an antibody specific to TGF-β1 *in vitro*. Additionally, inhibition of IGF-2, one of two isoforms of IGF, and PDGF reduces migration of these cells, but to a lesser degree [9]. Furthermore, antibody inhibition of TGF-β2 or TGF-β3, as well as IGF-1 and BMPs, has no effect on the migration of osteoblast precursors. The influence of TGF-β1 on preosteoblast migration is mediated by TGF-β receptor-1 (TGFBR1) activation of Smad3, which is required for the formation of the lamellipodia-like protrusions essential for cell migration [9]. In addition to TGF-β1-mediated recruitment of preosteoblasts to the site of resorption, there is evidence that osteoclasts signal to osteoblasts via "coupling factors" (i.e., sphingosine 1-phosphate and EphB4/ephrin-B2), which are not associated with any products of resorption [135-137]. While it's not clearly understood and remains topic of further investigation, the reversal phase prepares the bone surface for the next step of bone remodeling, bone formation [130].

The formation of mineralized bone involves proliferation of osteoblast progenitors and matrix maturation and mineralization by differentiating and maturing osteoblasts [138]. Proliferation of pre-osteoblasts provides a pool of precursor cells for the bone formation process. Gene expression of H4 histone, an indicator of DNA synthesis, as well as regulators of cell growth such as c-myc and c-Fos, are upregulated during the proliferation phase [139]. This phase is dependent on ERK phosphorylation and activation [140]. Activation of ERK has been shown to upregulate cyclin D1, a protein that promotes progression from the G1 phase of cell cycle to the S phase [140]. Cells entering the S phase of cell cycle duplicate their DNA and eventually divide into two daughter cells. ERK phosphorylation also activates runt-related transcription factor 2 (Runx2), which will regulate the transition from the proliferative stage to the matrix maturation stage by downregulating cyclin D1 and inhibiting proliferation in favor of maturation of the pre-osteoblasts [141, 142]. Runx2 stimulates the expression of osteoblast-related genes, including type 1 collagen and osteocalcin, by binding to their promoter regions and activating transcription [143]. The layering of collagen signals the termination of proliferation, resulting in a downregulation of histone expression and DNA synthesis [139]. This transition from collagen-producing osteoblast precursor to a mature osteoblast is due, in part, to interactions between collagen and α2-integrins expressed by osteoblast progenitors [144]. Type 1 collagen and α2-integrin interactions result in increased DNA binding activity of Runx2 at promoters of osteoblast-specific genes.

Further differentiation of pre-osteoblasts into mature osteoblasts requires expression of the transcription factor osterix, a downstream target of Runx2 [145, 146].

Osterix is involved in stimulating alkaline phosphatase (ALP) and osteocalcin

production. In fact, the cessation of DNA synthesis by osteoblast progenitors at the end of

the proliferative stage is immediately followed by a ~10-fold increase in ALP mRNA

expression, marking the induction of osteoblast differentiation and the matrix maturation

phase [147]. The MAPK, p38, plays an essential role in the differentiation and activity of

mature osteoblasts, as treatment with a specific p38 inhibitor results in downregulation of

both ALP production and mineralized matrix formation in mouse primary calvarial

osteoblasts [148]. Conversely, treatment with PTH, a known anabolic osteoporosis

treatment, induces p38 activation and ALP production [149].

Activation of p38 in pre-osteoblasts is initiated by BMP receptor signaling at the

plasma membrane [150]. Ligand-activated BMP receptors are serine/threonine kinases

that initiate osteoblast differentiation by phosphorylating Smad 1/5/8 and TAK1 [150,

151]. Activation of TAK1 initiates a signaling cascade that results in the phosphorylation

and activation of p38. Activated p38 in turn phosphorylates Runx2, enhancing the

activity of this osteogenic transcription factor. In addition, TAK1 phosphorylates Smad1

at the same site targeted by the BMP receptor, amplifying the BMP signaling cascade

in differentiating osteoblasts [152]. Activated Smad 1/5/8 induces the expression of the

transcription factor Dlx5, which in turn activates Runx2 (**Figure 3**) [151]. The ability of

Runx2 to induce osteogenic gene expression and therefore osteoblast differentiation

requires activation of both Smad and p38 signaling. Treatment with PTH, currently the

only FDA-approved anabolic osteoporosis treatment, enhances osteoblast differentiation

via the BMP signaling pathway by inducing the expression of BMP-2 in a CREB-

dependent manner [154].
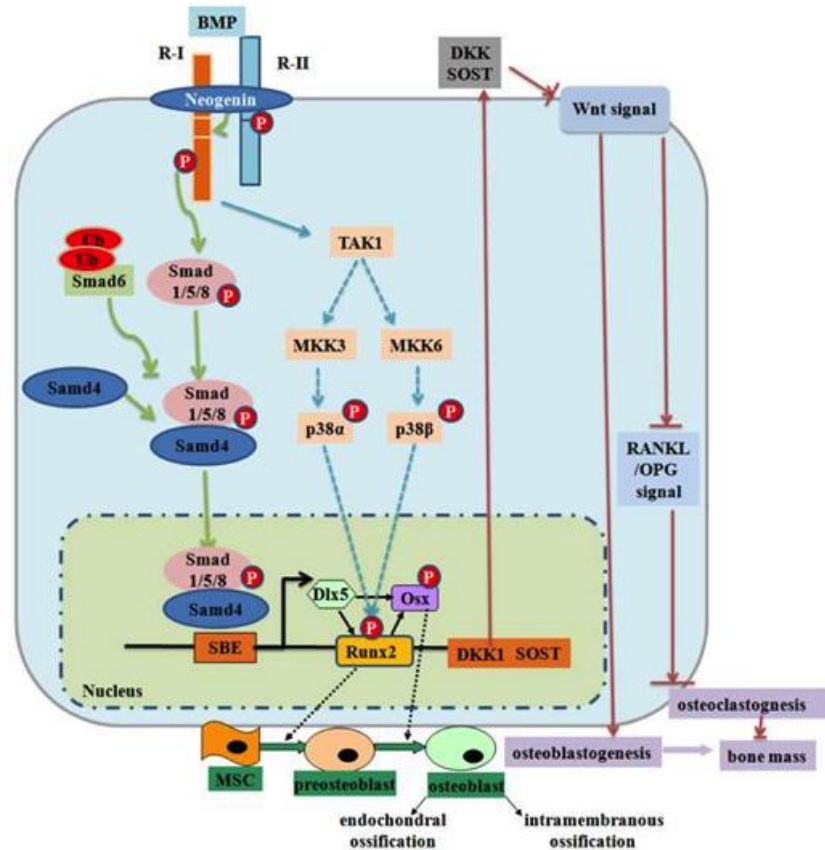
Figure 3. Upon ligand binding, BMP receptors phosphorylate Smad1/5/8 and TAK1. Activation of Smad1/5/8 results in the recruitment of Smad4 and the translocation of the complex to the nucleus, where it acts as a transcription factor. Activation of TAK1 results in the phosphorylation of p38, and activated p38 phosphorylates Runx2, enhancing the activity of the transcription factor. TAK1 can also phosphorylate Smad1 at the same site that targeted by the BMP receptor [153].

In preparation for matrix mineralization, mature osteoblasts deposit non-collagenous proteins such as osteocalcin, bone sialoprotein, and osteopontin, which regulate hydroxyapatite formation [155]. More specifically, osteocalcin, the most abundant non-collagenous protein in the extracellular matrix, is a vitamin K-dependent γ-carboxyglutamic (Gla) acid protein with high affinity for calcium binding [156]. While osteocalcin increases bone mineralization, it limits production of extracellular matrix,

suggesting a major role in regulating bone formation [157]. Bone sialoprotein (BSP) is a phosphoprotein containing an Arginine-Glycine-Aspartic Acid (RGD) motif that interacts with $\alpha_v\beta_3$ integrins on osteoblasts to promote maturation and mineralization of the extracellular matrix [158]. Like BSP, osteopontin also contains an RGD binding domain, as well as serine- and arginine-rich peptide (ASARM) with a high affinity for calcium, causing the protein to bind tightly to hydroxyapatite [159]. The ASARM peptide inhibits hydroxyapatite crystal growth, indicating the protein's role in regulating bone mineralization [160, 161]. However, the protease Phex cleaves the ASARM peptide of osteopontin, which eliminates its ability to inhibit mineralization [161]. Deposition of these non-collagenous matrix proteins by mature osteoblasts prepares the extracellular matrix for mineralization.

The signals that induce the transition to the mineralization phase of bone formation are not clearly understood [139]. Initiation of mineralization occurs within matrix extracellular vesicles produced by osteoblasts [8]. Within these vesicles, calcium and phosphate concentrations increase until formation of hydroxyapatite $(Ca_{10}(PO_4)_6(OH)_2)$ occurs. Availability of inorganic phosphate (Pi) in matrix vesicles has been shown to be a limiting factor in the formation of hydroxyapatite [162, 163]. In fact, *in vitro* data using MC3T3-E1 cells indicates surpassing a threshold of Pi concentration and availability is necessary for the initiation of HA nucleation [164]. Therefore, ALP and Pi transporters are essential to bone mineralization because both increase local Pi concentration. ALP provides Pi via phosphatase activity and improves hydroxyapatite formation by removing pyrophosphates, phosphate-containing inhibitors of crystal growth [8, 165, 166]. Transport of the Pi generated by ALP into the matrix vesicles

requires the BMP2-induced Na^+-Pi cotransporter Pit1 [163]. In fact, evidence suggests

that extracellular Pi concentration is not a limiting factor of mineralization but instead the

ability of cells to transport Pi into the cell is the limiting factor. Much like the initiation of

mineralization, the signals responsible for the cessation of bone mineralization are still

under investigation. Signaling from osteocytes, such as an increased expression of

sclerostin, at least in part, initiates the cessation of bone mineralization [130]. At the end

of the bone remodeling cycle, osteoblasts either undergo apoptosis, become bone lining

cells, or are embedded in the mineralized matrix and become osteocytes [130]

Maintenance of healthy bone requires coupling of bone resorption and bone

formation so that bone is being replaced at the same rate at which it is being resorbed.

Bone integrity is threatened when the balance of bone resorption and bone formation is

disrupted. While there are many factors that can disrupt the balance between the

catabolic and anabolic phases of bone metabolism, osteoporosis most often occurs within

the context of aging and altered gonadal hormone status [167].

Hormonal Regulation of Bone Metabolism

Various hormones play a role in the regulation of bone metabolism, including

gonadal hormones (e.g., estrogen and testosterone), PTH, and pituitary hormones (e.g.,

growth hormone, GH, and follicle-stimulating hormone, FSH) [28, 168-173]. The role of

estrogen in regulating bone metabolism has been extensively studied due to its role in

postmenopausal osteoporosis [23, 28, 174-177]. Estrogen, a steroid hormone, is

produced primarily in the ovaries, but also in the adrenal cortex and some peripheral

tissues (e.g., adipose tissue) [178]. There are 3 forms of naturally occurring estrogen:

17β-estradiol, estrone, and estriol. The most abundant form and the estrogen that has the highest affinity for estrogen receptors (ERs) is 17β-estradiol. Estrone and estriol, which are metabolites of 17β-estradiol, have a much lower affinity for ERs [178]. 17β-estradiol is transported in the serum bound to sex hormone binding protein (SHBP) [36]. It is thought that free estrogen, which is lipophilic, can easily diffuse through the cell membrane of a target cell. Estrogen bound to SHBP is recognized by the receptor megalin and binding of the estrogen/SHBP complex to megalin induces endocytosis of the complex [35]. Once inside the cell, SHBP is degraded in the lysosome while estrogen diffuses through the nuclear membrane, where it interacts with estrogen receptors (ERs).

The presence of ERs has been reported in various cells, including osteoblasts, osteoclasts, and their progenitor cell populations [179-181]. Estrogen directly affects both bone resorption and bone formation by regulating osteoclast and osteoblast differentiation, activity and lifespan [23, 174, 175, 177]. Classical estrogen signaling is mediated via ligand binding to one of two nuclear receptors, ER-α or ER-β [182]. Both ER-α and ER-β are expressed by osteoblasts and osteocytes [183]. Estrogen can increase osteoblast differentiation and activity predominantly via ER-α mediated signaling in bone marrow mesenchymal stem cells and differentiating osteoblasts, respectively [174, 177, 184, 185]. Estrogen also increases bone formation by reducing apoptosis and prolonging the lifespan of mature osteoblasts [174, 176, 177]. Ligand-bound ERs can affect gene transcription directly by binding to estrogen response elements (EREs) in the promoter region of target genes, or indirectly by binding the transcription factors AP-1 and specificity protein 1 (Sp1), at the promoter region of target genes (e.g., Col1a1, TGF-β, OPG, IGF-1) [38, 41, 175, 186-190]. Estrogen also affects osteoclast differentiation and

lifespan [23, 175, 189]. ER-β expression has been reported in osteoclasts, but the presence of ER-α in osteoclasts is controversial [183]. Estrogen primarily regulates osteoclast differentiation by reducing the production of RANKL by osteoblasts, T-cells and B-cells [23] and increasing OPG production by osteoblasts, B cells and bone marrow stromal cells [175]. Estrogen also decreases bone resorption by inducing apoptosis of osteoclasts [28, 189]. Therefore, although accompanied by a relatively short-term increase in overall bone turnover, the reduction in estrogen that occurs with menopause can directly affect bone formation due to a decrease in the differentiation and activity of osteoblasts and an increase in osteoblast apoptosis [19, 174, 177, 184, 185]. Furthermore, estrogen deficiency results in an increase in osteoclast differentiation and lifespan [23, 175, 189].

In addition to a direct effect of estrogen deficiency on bone cells, a decrease in estrogen results in a reduction in endogenous antioxidant capacity and increase in oxidative stress that affects osteoclast and osteoblast differentiation and apoptosis, respectively [191, 192]. Decreasing systemic estrogen levels results in an increase in lipid peroxidation and hydrogen peroxide production in the bone marrow [192]. This increase in oxidative stress results in a reduction in endogenous antioxidants in the bone marrow, such as glutathione peroxidase and superoxide dismutase [192]. Upregulation of reactive oxygen species (ROS) has been shown to induce osteoclast differentiation [193]. Therefore, estrogen deficiency affects bone metabolism directly by influencing osteoblast differentiation and activity and osteoclast differentiation and lifespan, and indirectly via an increase in oxidative stress. As a result of the effects of estrogen on bone metabolism, postmenopausal osteoporosis is characterized by an acceleration of bone loss in the first

4-8 years after estrogen begins to decline, with an estimated 10.5% reduction in BMD of the spine, 7.7% reduction in whole body BMD, and a 5.8% reduction of BMD in the femoral neck [171, 194].

While the acceleration of bone loss due to the decline of estrogen during the peri-menopausal and initial postmenopausal periods results in significantly more women developing osteoporosis, men also are at increased risk of fracture with increasing age [76]. Approximately 30% of all hip fractures occur in men [195]. The primary gonadal hormone in men is testosterone, and there is evidence that testosterone can directly inhibit osteoclast differentiation via androgen receptor signaling on monocyte precursors [170]. However, age-related bone loss in men is primarily due to a decrease in serum 17β-estradiol [196-198]. Serum total and bioavailable 17β-estradiol and estrone are correlated with forearm BMD in young and elderly men, whereas there is no correlation between testosterone and forearm BMD in these populations [199]. Serum 17β-estradiol below the threshold of 40 pmol/L has been associated with a significant increase in the rate of bone loss and bone resorption markers in elderly men [199]. These studies indicate that estrogen plays an important role in regulating bone health in both women and men.

Bone metabolism is highly regulated by direct effects of estrogen on bone cell populations, as well as the indirect effects of estrogen on reducing oxidative stress in the bone marrow. Additionally, immunological alterations associated with estrogen deficiency can also indirectly contribute to bone loss. Understanding how the immune system and bone metabolism are connected is important in developing novel osteoporosis treatments

The Role of the Immune System in the Pathophysiology of Postmenopausal Osteoporosis

A link between the immune system and bone metabolism was first introduced when it was discovered that monocyte/macrophage cells could differentiate into osteoclasts [200, 201]. Following this finding, Pacifici and colleagues provided further evidence of the link between bone metabolism and the immune system by demonstrating that peripheral blood mononuclear cells isolated from osteoporotic men and women produced significantly more IL-1 than PBMCs isolated from age-matched controls[202]. In 1990, Imai and colleagues observed an increase in the ratio of circulating $CD4^+$:$CD8^+$ T cells in osteoporotic, postmenopausal women compared to non-osteoporotic controls [203]. The importance of T cells in estrogen deficiency-related bone loss was first demonstrated when nude mice, which lack T cells, failed to lose bone following OVX [33]. Furthermore, when T cells from wild type mice were transferred to nude mice, bone loss due to OVX occurred. However, if the T cells from TNF-α knockout mice were transferred to nude mice, no OVX-induced bone loss was observed, suggesting a major role of TNF-α [33]. However, these findings are controversial. Lee and colleagues demonstrate that various models of T-cell deficient mice experience OVX-induced trabecular bone loss [10]. Moreover, Choi and colleagues demonstrated that IL-17, produced by $CD4^+$ Th17, was one of the major pro-inflammatory cytokine regulating postmenopausal osteoporosis by stimulating osteoclast differentiation and upregulating TNF-α production by macrophage [204]. Inhibiting IL-17 signaling by deleting its receptor or treating with a blocking antibody specific to IL-17 protects OVX mice from bone loss.

The increase in T cell proliferation and activation associated with estrogen deficiency is due, at least in part, to the upregulation of major histocompatibility complex II (MHCII) expression on macrophages and dendritic cells [30]. Upregulation of MHCII occurs in response to increased production of IFNγ by T-helper 1 cells, which are stimulated by IL-12 and IL-18 production from bone marrow monocytes [30, 205]. IL-12 and IL-18 are induced in a NF-κB- and AP-1-dependent manner, and estrogen directly inhibits the activity of these transcription factors [29, 206]. In estrogen deficiency, the increase in IFNγ can also be attributed to an increase in IL-7, a potent stimulator of B and T cells, and a decrease in the immunosuppressive cytokine, TGF-β [207-210]. Upregulation of IL-7 results in an expansion of RANKL-expressing B cell precursors that contribute to the uncoupling of osteoblast and osteoclast activity [211, 212]. Conversely, estrogen stimulates TGF-β production via ER-mediated activation of the ERE in the promoter region of TGF-β, which prevents bone loss due to a reduction in T cell proliferation and activation [210, 213].

Activation of T cells results in an increase in cytokine production, including but not limited to TNF-α, IL-1β and IL-6 [214, 215]. The increase in TNF-α observed in ovarian hormone deficiency negatively affects both bone formation and bone resorption [216-221]. Osteoclast differentiation is upregulated by TNF-α indirectly by inducing stromal cell and osteoblast production of M-CSF and RANKL, as well as directly by upregulation of RANK and activation of NFκB and AP-1 in osteoclast precursors, thereby enhancing their sensitivity to RANKL stimulation [216, 218, 220, 221]. Additionally, while not as potent as RANKL, TNF-α can induce the differentiation of osteoclasts by binding the p55 TNF receptor on bone marrow monocytes [222, 223].

TNF-α also increases the osteoclast activity by enhancing the ability of mature osteoclasts to form an actin ring [224]. Likewise, osteoblast differentiation is also negatively affected by TNF-α via a reduction in expression and stability of Runx2 [217, 219, 225, 226]. TNF-α-induced NF-κB signaling suppresses BMP signaling by inhibiting the binding of the activating Smad complex to the Runx2 promoter region [226]. In addition, TNF-α upregulates expression of Smad6, a repressor of BMP-induced Smad signaling, as well as Smad ubiquitination regulatory factor 1 (Smurf1), a ubiquitin ligase that marks Smad1 and Runx2 for proteosomal degradation [217, 219, 225]. TNF-α also induces apoptosis of mature osteoblasts in a caspase 8-dependent manner [227, 228]. The net effect of TNF-α on bone metabolism is an increase in osteoclast number and activity and a decrease in osteoblast number. Because TNF-α is a major contributor to the uncoupling of osteoblast and osteoclast activity that occurs in estrogen deficiency, *in vitro* experiments with TNF-α treatment of osteoblast and osteoclast cultures is often used as a model to study estrogen deficiency-induced bone loss.

While TNF-α is a major player in estrogen deficiency-induced bone loss, other cytokines have also been found to play a role. IL-1β signaling has been implicated in the pathophysiology of estrogen deficiency-induced osteoporosis, as OVX IL-1R knockout mice fail to lose bone [215]. While IL-1β cannot directly induce differentiation of osteoclasts in *in vitro* models of osteoclastogenesis, it does enhance the response of osteoclast precursors to TNF-α [229]. Additionally, IL-1β induces stromal cell and osteoblast expression of RANKL, indirectly upregulating osteoclast differentiation [230]. The production of IL-1 by bone marrow stromal cells is induced by IL-6 [231]. Inhibiting IL-6 reduces TNF- and IL-1-induced osteoclast differentiation in human bone

marrow cultures, indicating a role for IL-6 in mediating pro-inflammatory

osteoclastogenesis [231]. It is clear that the immune system plays a major role in the

development of osteoporosis. Potential treatments that can attenuate inflammation or the

response of bone to inflammation are desirable options for osteoporosis treatment.

Current Osteoporosis Treatments

Osteoporosis treatments currently approved by the United States Food and Drug

Administration (FDA) are designed to counter abnormalities in bone metabolism by

reducing bone resorption or increasing bone formation. These drugs include hormone or

estrogen therapy, calcitonin, bisphosphonates, intermittent PTH therapy, denosumab, and

selective estrogen receptor modulators (SERMS). However, bisphosphonates,

intermittent PTH therapy, denosumab, and SERMS are the most commonly prescribed

drugs due to their efficacy and lower risk of side effects, and will be the focus of this

review.

Bisphosphonates, a family of anti-resorptive agents, are currently the most widely

prescribed osteoporosis medication [18]. Bisphosphonates, including alendronate,

risedronate, and zoledronate, function by binding to the hydroxyapatite of bone and

impairing the bone resorption activity of osteoclasts by inducing osteoclast apoptosis

[246-248]. Treatment regimens include the administration of bisphosphonates via daily,

weekly, or monthly oral intake or annual injection. Bisphosphonate treatment results in

increased BMD of the spine and hip within the first year of treatment, but this increase in

BMD in the vertebra is not maintained following cessation of treatment [249, 250]. For

example, women who discontinue bisphosphonate treatment experience a decrease in

BMD of the vertebra and an increased risk of vertebral fracture within 3 to 5 years compared to women who continued with bisphosphonate therapy. Importantly, there are no differences in fracture risk at the femoral neck, total hip or distal radius in this population [249, 250]. These findings indicate that women at high risk for fracture may benefit from continuous bisphosphonate treatment, but long-term use of bisphosphonates is not without risk. The most frequent side effect associated with bisphosphonate treatment is adverse gastrointestinal events, especially since oral treatment should occur in the fasting state due to the poor intestinal absorption of the drug [251]. However, development of a once weekly treatment regimen for both alendronate and risedronate has resulted in improved patient adherence with the same bone response as daily treatment [252, 253]. Additionally, treatment regimens of a once monthly oral preparation or a once yearly infusion of zoledronate have been developed with favorable outcomes on fracture risk [254]. Other side effects associated with bisphosphonate use include esophageal irritation and cancer [255], atypical femur fracture [256], and osteonecrosis of the jaw [257]. Of these, atypical femoral fracture is the only side effect claim substantiated by recent studies, and the benefit of reduction in classical fractures generally outweighs the risks of atypical femoral fracture [258-260]. Today, bisphosphonates continue to be widely used due to their ability to reduce the risk of fracture in a relatively short period of time but long-term use remains a concern.

Currently, the only anabolic medication approved by the FDA is intermittent PTH or a synthetic 1-34 fragment of PTH (teriparatide) [261]. Intermittent PTH treatment increases osteoblast proliferation and differentiation, and reduces pre-osteoblast and osteoblast apoptosis [261-263]. PTH also increases bone resorption via osteoclast

activation, but the net effect of intermittent PTH treatment is anabolic [264, 265].

Teriparatide is prescribed for those with established osteoporosis who are at a particularly

high risk for fragility fractures or have pre-existing fractures [266]. Daily injections of

PTH for up to 18 months results in improved BMD and bone strength much more quickly

and to a greater magnitude than any other osteoporosis treatment [261]. While PTH is an

attractive osteoporosis treatment option, it is cost-prohibitive for many patients and

severe side effects have been reported, including increased risk of gout [266],

hypercalcemia [266], hypercalciuria [267], and potentially osteosarcoma [268].

However, limiting the use of PTH treatment to those who are not at increased risk for

osteosarcoma, as well as the duration of treatment to a maximum of 2 years, has resulted

in a relatively low risk of these more severe side effects [259, 269, 270].

One of the more recently FDA-approved drugs for the treatment of osteoporosis is

the human monoclonal antibody for RANKL, denosumab. Given by injection every 6

months, Denosumab inhibits RANKL from binding to RANK, therefore attenuating

osteoclast differentiation and bone resorption [271]. Denosumab has been found to

increase BMD at the hip and vertebra, reduce risk of fracture, and decrease markers of

osteoclast activity and bone turnover (i.e., serum C-telopeptide or CTX, tartrate-resistant

acid phosphatase-5b or TRAP-5b, and intact N-terminal propeptide of type 1 procollagen

or P1NP) in postmenopausal women [91-93]. Side effects associated with denosumab

use include increased risk of upper respiratory and urinary tract infections [271].

Use of hormone replacement therapy (HRT) solely for the treatment of

postmenopausal osteoporosis is no longer recommended practice due primarily to

findings from the Women's Health Initiative (WHI) Study [272]. In that study, HRT

increased risk for coronary heart disease, stroke, breast cancer, and pulmonary embolism [272]. However, selective estrogen receptor modulators (SERMs), which can act as agonists or antagonists of the estrogen receptor depending on tissue, are a promising alternative to HRT [273]. Raloxifene, an estrogen receptor antagonist in breast tissue and agonist in bone, is effective in reducing vertebral fracture risk in postmenopausal women [274]. However, a comparison of treatment of osteopenic postmenopausal women with raloxifene or the bisphosphonate, alendronate, indicates that alendronate is more effective in increasing BMD of the hip and lumbar spine [275]. Side effects associated with raloxifene use include occurrence of hot flashes and leg cramps, along with the more serious increased risk of venous thromboembolic events and fatal stroke [276, 277]. Recently, the SERM bazedoxifene, in combination with conjugated estrogens, was approved by the FDA for the treatment of postmenopausal symptoms, including severe hot flashes and bone loss [278]. The bazedoxifene/conjugated estrogens combination improves total hip and lumbar BMD in postmenopausal women without the undesirable and dangerous side effects of HRT. However, bazedoxifene/conjugated estrogen use is only approved for women with an intact uterus and only recommended for treatment of osteoporosis in those with significant risk of fracture [278].

Despite a variety of osteoporosis treatment options, lack of adherence to the different treatment regimens and cost are significant problems. Reasons for noncompliance include fear of side effects, perceived lack of benefit, and cost of treatment [279]. Since osteoporosis is frequently asymptomatic until a fracture occurs, complying with often costly preventative treatments that are associated with various side

effects is not desirable. Therefore, more cost-effective treatment options with fewer side effects are needed.

Alternative Osteoporosis Treatment Options

Development of more desirable and efficacious osteoporosis treatments should include more natural options that are also beneficial to overall health. Inflammation is a component of the pathophysiology of most chronic diseases, including osteoporosis. Foods rich in anti-inflammatory phytochemicals may have the capacity to reduce risks associated with various chronic diseases, including diabetes, heart disease, cancer and osteoporosis [280-283]. For these reasons, consuming foods or food components that contain compounds that benefit bone health may be more desirable than complying with a pharmacological regimen.

Prevention of bone loss was demonstrated with dietary supplementation of certain plant-based foods rich in phenolic compounds, including green tea, soy products, blueberries and dried plum [52, 53, 55, 59, 61, 62, 65-67, 284-289]. Isoflavones from soy products, such as daidzein and genistein, as well as the microbial metabolite of these isoflavones, equol, prevented OVX-induced reduction in rat femoral BMD [62, 284, 286]. Green tea consumption also increased whole body and femoral BMD, as well as trabecular bone in the proximal tibia, in various models of bone loss, including bone loss associated with OVX, aging, inflammation, and obesity [55, 59, 62, 284, 286-289]. Diets supplemented with 10% blueberry prevented the OVX-induced decrease in whole body and tibia BMD as well as trabecular bone in rat tibias [53, 61]. The ability of each of these foods to affect bone metabolism has been attributed to their phytochemical content.

Dried plums are a rich source of phytochemicals, especially phenolic acids [63]. Dietary supplementation with dried plum has been shown to improve bone density and bone microarchitecture in osteoporosis models of aging, gonadal hormone deficiency, inflammation and ionizing radiation and the mechanisms involved have been investigated *in vivo* and *in vitro* [52, 65-67, 285, 290-292]. In OVX-induced model of postmenopausal osteoporosis, a diet supplemented with 25% (w/w) dried plum was most effective at preventing bone loss [67, 290]. Dried plum attenuates the increase in bone turnover and inflammation that occurs as a result estrogen deficiency [67]. The suppression of osteoblast activity can be attributed to a downregulation of Runx2 and osteocalcin gene expression, which are indicators of osteoblast differentiation and bone mineralization, respectively. Additionally, dried plum suppresses Nfatc1 gene expression, and therefore osteoclast differentiation and bone resorption. [67]. Furthermore, TNF-α production, a major factor in the pathophysiology of postmenopausal osteoporosis, is suppressed in *ex vivo* cultures of splenocytes from the OVX mice fed 25% dried plum. These studies suggest that dried plum affects bone metabolism directly by altering osteoblast and osteoclast differentiation and activity, and indirectly by modulating the immune response to estrogen deficiency.

Studies of dried plum's effect on age-related bone loss also demonstrate the ability of dried plum supplementation to result in suppression of bone turnover. Histomorphometric and biochemical data demonstrated a suppression of bone formation and osteoclast surface in aged mice supplemented with dried plum at 4 weeks [69]. However, following 12 weeks of dried plum supplementation, bone formation increased by 54% compared to control and urinary pyridinoline was suppressed, indicating a

reduction in bone resorption. These findings indicate dried plum has a biphasic effect on bone formation. Together, these findings in OVX and age-related models of bone loss indicate that dried plum protects bone by suppressing bone turnover, at least initially, in aged mice.

Even more remarkable than preventing bone loss due to aging and ovarian hormone deficiency, is the ability of dried plum supplementation to reverse bone loss. In aged C57Bl/6 male mice, a diet supplemented with 25% dried plum increased trabecular bone volume and cortical thickness [285]. In both male and female models of gonadal hormone deficiency-induced bone loss, dried plum has been compared to intermittent PTH therapy [52, 291]. In osteopenic, orchidectomized (ORX) Sprague Dawley rats, dried plum supplementation restored trabecular bone in the vertebra to a similar extent of that of intermittent PTH treatment [52]. Dried plum also improved cortical bone thickness compared to ORX controls, but the magnitude of response was not as great as that of the PTH treated group. Biomechanical testing of the vertebra indicated that the dried plum had similar effects on bone strength as PTH. In a comparison between dried plum supplementation and PTH treatment in osteopenic, OVX Sprague Dawley rats, it was demonstrated that the bone's physiological response to dried plum and PTH differ [291]. Histomorphometric (i.e., bone formation rate or BFR and mineralizing surface or MS/BS) and biochemical data (i.e., urinary deoxypyridinoline or DPD, and serum P1NP) demonstrates that 6 weeks of dried plum supplementation resulted in an overall suppression of osteoblast and osteoclast activity, although a modest increase in endocortical mineral apposition rate (MAR) was observed [291]. Conversely, intermittent PTH treatment stimulated an increase in bone formation and mineral

apposition rate. These studies indicate that dried plum is an attractive alternative treatment option for osteoporosis due to its ability to benefit bone microarchitecture and strength to a similar extent of that of costly PTH treatment.

To determine whether other commonly consumed dried fruits that were processed similarly to the plum had bone-protective properties comparable to dried plum, diets supplemented with 25% w/w dried apple, dried apricot, dried grape, or dried mango were administered to osteopenic OVX C57BL/6 mice [66]. Dried plum supplementation resulted in a trabecular BV/TV greater than that of all other OVX treatment groups in the vertebra and tibia, and was comparable to that of the sham-operated controls. This study demonstrates that dried plums have a unique composition of nutrients and phytochemicals that exert bone-protective properties in various models of bone loss.

To examine whether the effects of dried plum supplementation seen in animal models translates to humans, clinical trials were conducted in postmenopausal women [64, 68, 70]. Postmenopausal women supplementing their diets with dried plum (100g/day) for one year experienced a significantly greater increase in BMD of the ulna and spine compared to their counterparts supplementing with dried apple [68]. Dried plum consumption resulted in an initial suppression of serum TRAP5b after the first 3 months and a decrease in serum bone-specific ALP (BSAP) and sclerostin, as well as an increase in serum OPG following 12 months of supplementation [68, 293]. More recently, it was found that 6 months of 50 g of dried plum daily was as effective as 100g in protecting postmenopausal women from bone loss, and this was attributed to a decrease in serum TRAP5b levels and bone resorption [70]. Interestingly, an earlier short-term clinical study (90 days) in postmenopausal women found an increase in

BSAP, as well as IGF-1 [64]. The discrepancies in results in these studies may provide further support of dried plum's biphasic effect on bone formation observed in animal studies.

Studies designed to study the effects of dried plum on bone in ovarian hormone deficiency have revealed consistent results. *In vivo* studies and clinical trials have demonstrated that dried plum supplementation improves bone mineral density and trabecular and cortical bone volume [64, 66-68, 70, 291, 293]. A total polyphenolic extract from dried plum increases osteoblast differentiation and mineralization activity and decreases osteoclast differentiation and bone resorption in an inflammatory environment [71, 72]. However, the bioactive components responsible and the exact mechanisms by which they affect bone metabolism remain unclear.

Bioactive Components in Dried Plum

The initial rationale for evaluating the bone protective effects of dried plums was based on their high oxidant radical absorbance capacity (ORAC) score compared to other commonly consumed fruits and vegetables [294]. Certain bioactive components in foods act as antioxidants by donating hydrogen ions to free radicals, thereby neutralizing these potentially damaging compounds [295]. Compounds that contain aromatic rings, such as phenolic acids and flavonoids, effectively donate a hydrogen ion to free radicals while remaining a stable compound [295]. The phenolic compounds rich in dried plums are likely major contributors to its high antioxidant capacity, with chlorogenic acid and its isomers being especially abundant [296]. The most abundant phenolic compounds in dried plum are shown in **Table 1**.

Table 1. The phenolic compounds in greatest abundance in dried plum.

Phenolic Compounds	Common Name	Concentration (mg/100g dried plum)
Flavonols		
Quercetin 3-O-rutinoside	Rutin	2.5
Phenolic acids		
3-Caffeoylquinic acid	Neochlorogenic acid	118.59
4-Caffeoylquinic acid	Cryptochlorogenic acid	31.25
5-Caffeoylquinic acid	Chlorogenic acid	38.79
p-coumaric acid		1.11
Caffeic acid		1.11

While the antioxidant capacity of the phenolic compounds in dried plum make them an attractive component to study, there are other potential bioactive components found in dried plum. Non-digestible carbohydrates (e.g., pectin and hemicellulose) make up approximately 11-12% of the dry matter of dried plum [297]. Non-digestible carbohydrates, such as inulin and fructo-oligosaccharides, have been found to be beneficial in other disease states where inflammation plays a role in the pathophysiology, such as heart disease and diabetes [298, 299]. There is emerging evidence that non-digestible carbohydrates may also be beneficial in other inflammatory disease states, such as osteoporosis [300, 301]. Aside from the functional food components, dried plums are a good source of nutrients that may benefit bone health, including potassium, boron and vitamin K (**Table 2**) [302, 303]. While there are a number of potential bioactive components in dried plum, *in vitro* and *in vivo* data indicate that the phenolic compounds

are in large part responsible for the beneficial alterations in bone metabolism observed with dried plum supplementation [71, 72].

Table 2. Nutrient content of dried plum.

Nutrient	per 100g dried plum
Total kcal	240
Protein (g)	2
Fat (g)	0.4
Carbohydrate (g)	64
Fiber (g)	7
Sugars (g)	38
Calcium (mg)	43
Magnesium (mg)	41
Phosphorus (mg)	69
Potassium (mg)	732
Vitamin K (µg)	60

USDA Nutrient Database For Standard Reference Release 28

The effects of an ethanol polyphenolic extract from dried plum on the differentiation and activity of osteoblasts and osteoclasts in normal and inflammatory environments was examined *in vitro*. It was demonstrated that 10-30 µg/ml of the polyphenolic extract attenuated the LPS-induced increase in TNF-α and nitric oxide in the murine monocyte/macrophage cells, RAW 264.7, during osteoclast differentiation [72]. These anti-inflammatory effects coincide with the polyphenolic extract attenuating the LPS-induced increase in osteoclast differentiation and activity in both RAW 264.7 cells and primary bone marrow monocyte/macrophages [72]. Furthermore, the phenolic extract attenuates osteoclast differentiation in cells under oxidative stress, treated with hydrogen peroxide, in a dose-dependent manner [72]. Gene expression analyses reveal

that the phenolic extract decreases osteoclast differentiation and activity at least in part by downregulating expression of Nfatc1. These findings indicate that a crude phenolic extract from dried plum is able to directly reduce osteoclast differentiation and activity under both normal and inflammatory conditions.

In addition to suppressing osteoclast differentiation and activity, the crude extract of dried plum polyphenolics also increased ALP production and mineralized nodule formation in murine pre-osteoblastic MC3T3-E1 cells under normal and inflammatory conditions [71]. The polyphenolic extract increased the gene expression of Runx2 and Osterix, which play important roles in osteoblast differentiation [71]. These findings suggest that the polyphenols from dried plum can also protect differentiating osteoblasts from the detrimental effects of TNF-α in an osteoblast cell culture model.

In addition to *in vitro* evidence of the efficacy of phenolic compounds, a dietary supplement of a total polyphenolic extract of dried plum also improved bone parameters *in vivo*. Dietary supplementation with the total polyphenolic extract resulted in ~82% increase in vertebral trabecular bone volume in aged, osteopenic OVX Sprague Dawley rats (*unpublished data*). Coinciding with the increase in trabecular bone volume, consumption of the total polyphenolic extract also resulted in an ~18-fold increase in total force necessary to induce fracture in the vertebra. Supplementation with a combination of potential bioactive components of dried plum, including the total polyphenolic extract, vitamin K and potassium, further improved vertebral trabecular bone volume. In fact, consumption of the combination of bioactive components resulted in restoration of trabecular bone in the vertebra comparable to that of dried plum. Interestingly, only the polyphenol extract, and not the combination of bioactive

components, results in greater femoral cortical bone thickness. This restoration of bone can at least partially be attributed to a reduction in the bone resorption marker, urinary DPD. The polyphenolic-supplemented group has lower urinary DPD than the OVX control, but not to the level of the dried plum group. These findings indicate the phenolic compounds in dried plum account for the majority of the bioactivity of the fruit, and that dietary supplementation of these compounds can improve bone health.

Evidence from *in vitro* and *in vivo* studies indicate that the polyphenols in dried plum are the primary bioactive components. Current *in vitro* evidence indicates that the polyphenols from dried plum directly benefits bone metabolism by increasing osteoblast differentiation and activity and decreasing osteoclast differentiation and activity [71, 72]. The total polyphenolic extract also restored trabecular and cortical bone volume *in vivo (unpublished data)*. While there is evidence to support that phenolic compounds are absorbed and transported to the bone microenvironment where they can directly interact with osteoblasts and osteoclasts, the bioavailability of some of these compounds may be limited. Thus it stands to reason that another potential site where their biological effects could be exerted that could ultimately affect bone metabolism is within the local immune system of the gut.

Conclusion

The role of the immune system in postmenopausal bone loss has been appreciated for many years. Despite advances in the field of osteoporosis, there is still a need for treatments that are lower in cost and have fewer side effects. Dried plum supplementation has been shown to be an effective treatment option, but the bioactive

components of dried plum and the physiological mechanisms by which it improves bone

health are unknown. Both *in vitro* and *in vivo* data indicate that the phenolic compounds

in dried plum are primarily responsible for the beneficial alterations in bone metabolism.

However, it is unclear whether a combination of phenolic compounds is necessary or

whether there is a single phenolic compound that provides the bioactivity of dried plum.

Determining which compounds in dried plum are responsible for altering bone

metabolism, and determining the mechanisms by which this occurs, is necessary to

develop an efficacious and desirable osteoporosis treatment option.

CHAPTER III

IDENTIFICATION OF POLYPHENOLIC FRACTIONS OF A TOTAL POLYPHENOLIC

EXTRACT OF DRIED PLUM WHICH ATTENUATE OSTEOCLAST DIFFERENTIATION

BY DOWNREGULATING NFATC1

Authors: Jennifer L. Graef and Brenda J. Smith

Abstract:
Clinical and pre-clinical studies have shown that dietary supplementation with dried

plums improves bone health. These osteoprotective effects are a result, in part, of the

fruit's anti-resorptive properties, which are mediated by its polyphenolic compounds.

This study was designed to determine if certain types of polyphenols are responsible for

anti-resorptive effects of dried plum and their mechanisms of action. Extraction with

increasing methanol concentration was used to yield six semi-purified polyphenol

fractions from the total polyphenolic extract of dried plum. Initial screening, using an

immortalized cell line, revealed that the two fractions with the highest organic content

had most marked capacity to downregulate osteoclast differentiation under normal and

inflammatory. This decrease in osteoclastogenesis and osteoclast activity was confirmed

in primary bone marrow-derived osteoclast cultures. Calcium signaling, which is

essential to the auto-amplification of $Nfatc1$ and therefore the differentiation of

osteoclasts, was suppressed in the primary bone marrow-derived osteoclasts under

both normal and inflammatory conditions. Intracellular calcium concentrations, as well as mRNA expression of co-stimulatory receptors involved in calcium signaling, including *Oscar*, *Sirpb1*, and *Trem2*, were suppressed under normal conditions, while only *Sirpb1* was downregulated under inflammatory conditions. In addition to calcium signaling, phosphorylation of Erk and p38 MAPK signaling, involved in the expression and activation of Nfatc1, was also suppressed by the polyphenolic fractions. Additionally, the polyphenolic fractions reduced osteoclast number in an osteoblast and osteoclast co-culture system under both normal and inflammatory conditions. This study shows that polyphenolic fractions of dried plum suppressed osteoclast differentiation by downregulating calcium and MAPK signaling, resulting in suppression of *Nfatc1* expression. Therefore, the polyphenolic fractions were able to suppress bone resorption in these cell culture models.

Introduction

Osteoporosis is a major public health threat, with an estimated 60% of people over the age of 50 diagnosed are at an increased risk (i.e., osteopenic) [1]. Of the $19 billion in annual healthcare costs associated with osteoporotic fracture, 76% of those costs are incurred by postmenopausal women [1]. Estrogen deficiency results in an uncoupling of the activity of bone resorbing osteoclasts and bone forming osteoblasts, resulting in a net effect of bone loss [1, 2]. The prominent metabolic shift in bone remodeling that leads to estrogen deficiency-induced bone loss is an increase in osteoclast differentiation and activity [3, 4]. This is due, in part, to the increase in immune cell activation, including proliferation of osteoclast precursors and a systemic

increase in the pro-inflammatory cytokines TNF-α, IL-1, IL, and IL-17 [5-7]. These same cytokines are also known to play a role in bone loss that occurs in inflammatory conditions such as rheumatoid arthritis and periodontal disease [8, 9].

Enhanced osteoclast differentiation is observed in inflammatory environments [10]. For example, TNF-α increases the sensitivity of osteoclast precursors to receptor activator of nuclear factor-kappaB ligand (RANKL) stimulation by inducing upregulation of RANK and activating nuclear factor-kappaB (NF-κB) [11, 12]. The differentiation of osteoclasts requires the activation and auto-amplification of the transcription factor nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (Nfatc1) [13]. Upon RANKL stimulation of osteoclast precursors, expression of Nfatc1 is regulated by TNF receptor-activated factor 6 (TRAF6)-mediated signaling cascades, as well as by calcium-regulated signaling pathways [13, 14]. Activation of TRAF6 initiates various signaling cascades that are essential to the initial upregulation of Nfatc1 expression, including mitogen activated protein kinase (MAPK) and NF-κB signaling cascades [14]. The auto-amplification of Nfatc1 necessary for late stage osteoclast differentiation is further modulated by calcium-regulated signaling pathways [13]. Co-stimulatory receptors, such as osteoclast-associated receptor (OSCAR), triggering receptor expressed on myeloid cells 2 (TREM2), and signaling regulatory protein β1 (SIRPβ1), regulate calcium oscillations in the differentiating osteoclast [15, 16]. Inhibition of TRAF6-mediated or calcium-regulated signaling pathways interferes with osteoclast differentiation [16-20].

Reducing bone resorption is essential to attenuating the loss of bone, especially in postmenopausal osteoporosis. Clinical trials, as well as pre-clinical studies in animal

models, have demonstrated the anti-resorptive effects of supplementing the diet with dried plum in order to prevent or reverse bone loss due to estrogen deficiency [21, 22]. Postmenopausal women consuming dried plum (50 g) per day for 6 months had increased bone mineral density (BMD) of the spine and ulna, and this was attributed to a decline in serum TRAP5b, which is secreted by osteoclasts during the resorption phase of bone remodeling [21]. In an ovariectomized mouse model, dietary supplementation with dried plum attenuated bone loss and this was attributed, in part, to a decrease in the systemic immune activation due to estrogen deficiency, including a suppression of splenocyte production of TNF-α and alterations in bone marrow immune cell populations [22]. Dried plum supplementation prevented the ovariectomy-induced decrease in granulocyte and committed monocyte (i.e. CD115^{+} or M-CSFR^{+}) numbers. In addition, dried plum supplementation reduced the number of lymphoblasts in the bone marrow [22]. Perhaps even more intriguing than dried plum's ability to prevent bone loss is the finding that dried plum supplementation can reverse bone loss due to estrogen deficiency and age [23-27]. Dried plum has been shown to suppress bone resorption and restore trabecular bone volume *in vivo* in an aged, osteopenic model of osteoporosis [28]. In osteopenic OVX Sprague Dawley rats, dried plum supplementation improved whole body bone mineral density, vertebral trabecular bone volume and cortical thickness [27]. These improvements were attributed to a reduction in bone resorption, as indicated by downregulation of *Nfatc1* expression in the distal femur metaphysis and decreased urinary deoxypyridinoline levels, as well as in increase in bone formation, as indicated by upregulated mRNA expression of *Bmp4* and *Igf1* in the distal femur metaphysis and histomorphometric evidence of increased tibial endocortical mineral apposition rate.

However, the bioactive components in dried plum that are responsible for improved bone metabolism remain unclear.

Much of the focus of investigating the bioactive components in dried plums has been on the polyphenols. Dried plums are a rich source of polyphenolic compounds that have potent antioxidant and anti-inflammatory properties [29]. The anti-resorptive properties of these compounds extracted from dried plum have been demonstrated *in vitro* and *in vivo.* For instance, an ethanol extract of dried plum's polyphenols attenuated osteoclast differentiation and resorption activity under inflammatory conditions in the murine monocyte/macrophage cells, RAW 264.7, as well as in primary bone marrow cells [30]. This decrease in osteoclast differentiation and activity was attributed to, at least in part, a downregulation of *Nfatc1* gene expression. *In vivo*, this crude polyphenol extract was also able to restore BMD and reverse vertebral trabecular bone loss in aged, osteopenic OVX Sprague Dawley rats to a similar degree as that of dried plum, a response coinciding with a decrease in the biomarker of bone resorption, urinary deoxypyridinoline (*unpublished data*). While the ability of this polyphenolic extract to restore bone to a similar extent as the whole fruit is a promising advancement in determining the bioactive components, it remains unclear if there are certain types of polyphenolic compounds that are responsible for the decrease in osteoclast activity. Moreover, elucidating the mechanisms by which these compounds suppress the differentiation of osteoclasts and their activity is necessary in fully understanding its potential as a therapeutic option for osteoporosis.

The purpose of this study was to determine the types of polyphenols within the ethanol polyphenolic extract that suppress osteoclast activity using primary cell culture

systems. Furthermore, because bone loss is often the result of inflammation, the mechanisms responsible for the downregulation of osteoclast activity were examined under normal and inflammatory conditions.

Methods

Reagents

The dried plum powder from which the extracts were derived was supplied by the California Dried Plum Board, the same source of dried plum used in previous *in vivo* experiments [22-24, 27, 28, 30, 31]. Murine macrophage/monocyte cells, RAW 264.7, were purchased from American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's modified Eagle medium (DMEM), Minimum Essential Medium, alpha-modification (α-MEM), penicillin/streptomycin (P/S), L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), receptor activator of NF-κB ligand (RANKL), macrophage colony stimulating factor (M-CSF), lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY). Collagenase was purchased from Worthington (Lakewood, New Jersey).

Animal procedures for primary-derived cells for mono-cultures and co-cultures

All procedures involving the use of animals to derive primary bone marrow cells or neonatal calvarial cells were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Isolation polyphenolic fractions from dried plum

Chromatography using HP-20 resin (Sigma-Aldrich, St. Louis, MO) was utilized to extract semi-purified polyphenolic fractions from dried plum powder. First, a crude polyphenol extract was derived from sonicating 500 g dried plum powder in 80% methanol under pulsated nitrogen gas two times. The liquid phase was subjected to column chromatography using 300 g of HP-20 resin. The column was then rinsed 5 times with 200 ml of deionized water to yield a water soluble carbohydrate-rich extract. Following the water rinses, the column was washed with 100% methanol to yield a water insoluble total polyphenolic-rich extract which was subjected to additional column chromatography using 200 g HP-20 resin. Six semi-purified polyphenol fractions with similar solubility properties were eluted from the column with increasing concentration of methanol (i.e. 0, 20, 40, 60, 80 and 100% methanol). These fractions were lyophilized and the weight of each fraction was as follows: DP-FrA=17.85 g; DP-FrB=4.42 g; DP-FrC=3.27 g; DP-FrD=4.14 g; DP-FrE=2.16 g; and DP-FrF=1.39 g.

Screening of the polyphenolic fractions

RAW 264.7 cells, immortalized monocyte/macrophages, that can be differentiated into osteoclasts with RANKL treatment, were used to screen the capacity of the fractions to reduce osteoclast differentiation. Cells were maintained in DMEM supplemented with 10% FBS and 1% P/S. The effects of the polyphenolic fraction on cell growth were assessed in doses ranging from 0.005 μg/ml – 20 μg/ml using the MTT assay.

Based on the results of the MTT assay, the capacity of the fractions to reduce osteoclastogenesis at doses of 0.1, 1, and 10 μg/ml were examined. Cells were plated at a

density of 2 x 10^3 cells/well in a 96-well plate and treated with RANKL (30 ng/ml) to induce osteoclast differentiation. The cells were treated with polyphenolic fractions (0, 0.1, 1, or 10 μg/ml) beginning 24 hours after RANKL treatment. Following 5 days of RANKL treatment, the cells were washed with PBS and fixed in a 1:1 mixture of ethanol and acetone for 5 minutes. After 5 minutes of fixation, the cells were rinsed with deionized water and the osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, St. Louis, MO) expression. Large, multi-nucleated, TRAP⁺ cells were quantified by counting the number of cells per well using an inverted light microscope (Nikon Instruments Inc, Melville, NY).

In order to confirm the fractions and doses of polyphenols that reduced osteoclast differentiation under normal conditions would have similar effects under inflammatory conditions, a second set of experiments were performed. Briefly, RAW 264.7 cells were plated and cultured as described above and then treated with 0 or 1 ng/ml of LPS on day 4 of RANKL treatment. The cells were then fixed, TRAP stained and the number of osteoclast quantified per well.

All screening experiments with RAW 264.7 cells were repeated a minimum of 2-3 times to confirm results. The mean number of osteoclasts per treatment was expressed relative to the mean of the control group in each experiment (i.e., no polyphenol or LPS).

Polyphenolic fractions and primary osteoclast differentiation

The fractions with the greatest bioactivity in suppressing osteoclast differentiation were further examined in primary bone marrow-derived osteoclast cultures. To obtain primary osteoclasts, bone marrow was flushed from long bones of 4-6 week old C57Bl/6

mice (Charles River, Wilmington, MA). Collected cells were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% P/S for 2 days prior to collecting the non-adherent, hematopoietic bone marrow cells for experiments.

To examine the ability of the polyphenolic fractions to reduce osteoclast differentiation and activity in murine primary bone marrow-derived osteoclasts under normal and inflammatory conditions, non-adherent bone marrow cells were plated (1 x 10^5 cells/well) in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1% P/S and 30 ng/ml M-CSF in 96-well plates. Following 3 days of MCSF treatment, the media was replaced and supplemented with 50 ng/ml RANKL. On the 4th day of RANKL treatment, the cells were treated with 0, 1 or 10 μg/ml polyphenolic fractions with or without TNF-α (1 ng/ml). The following day, the cells were stained for TRAP and multinucleated, TRAP$^+$ osteoclasts were quantified.

Polyphenolic fractions alter osteoclast activity

The effect of the polyphenolic fractions on osteoclast activity was assessed using a resorption pit assay. Dentin discs were soaked in culture media in a 96-well plate for 1 hour prior to the experiment. Non-adherent bone marrow cells were plated in α-MEM supplemented with 10% FBS, 2 mM glutamine, 1% penicillin/streptomycin, and MCSF (30 ng/ml) at a density of 2.5 x 10^5 cells/well. Following 3 days of culture, the media was replaced and RANKL (50 ng/ml) was added. On day 4 of RANKL treatment, the cells were treated with the polyphenolic fractions identified as having the greatest effect on osteoclastogenesis (i.e., DP-FrE and DP-FrF) at doses of 0, 1, or 10 μg/ml, with or without TNF-α (1 ng/ml). After 7 days of treatment, the cells were removed from the

dentin discs by incubating in 10% bleach in PBS for 30 minutes at room temperature. The

discs were then washed 3 times with DI water and stained with 1% toluidine blue in 0.5%

sodium tetraborate for 3 minutes. The excess stain was removed and the discs were rinsed

in DI water until no excess stain remained. Resorption pit area was determined by

evaluating light microscopy images using ImageJ software (NIH, Bethesda, MD) and the

data was expressed as a percentage of the total area of the dentin disc.

Intracellular calcium in primary bone marrow-derived osteoclasts

For intracellular calcium measurement, bone marrow cells were plated in 96 well

plates and osteoclasts were generated as described above. On day 4 of RANKL treatment,

the cells were incubated in Fluo-4 dye (ThermoFisher, Waltham, MA) for 1 hour prior to

treatment with 0 or 10 μg/ml of polyphenolic fraction, DP-FrE or DP-FrF, with or

without 1 ng/ml TNF-α. To assess changes over time, intracellular calcium was

determined at 5, 30 and 60 minutes post-treatment with polyphenolic fractions by

measuring fluorescence at excitation wavelength of 494 nm and emission at 516 nm.

Analysis of gene expression related to osteoclast differentiation and calcium signaling

For gene expression analyses, non-adherent bone marrow cells were plated in 24-

well plates at a density of 6×10^5 cells/well and treated with M-CSF as describe above.

Following 3 days of culture, the media was replaced and additionally supplemented with

RANKL. On day 4 of RANKL treatment, the cells were treated with polyphenolic

fractions, DP-FrE or DP-FrF (0 or 10 μg/ml) with TNF-α (0 or 1 ng/ml). Total RNA was

extracted from cells following 1 hour of treatment using Trizol reagent (Life

Technologies, Carlsbad, CA) according to the manufacturer's protocol. The concentration and quality of the RNA was assessed via optical density determination at 260 and 280 nm, as well as agarose gel electrophoresis. The relative abundance of mRNA for genes related to osteoclast differentiation and calcium signaling (**Table 1**) was assessed using SYBR-Green technologies (Life Technologies, Carlsbad, CA) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). All mRNA expression levels were evaluated via the comparative cycle threshold (C_T) method (User Bulletin #2, Applied Biosystems, Foster City, CA) using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as an invariant control.

Protein expression analyses

For protein expression analyses, bone marrow cells were plated in 24-well plates and osteoclasts were generated as described above in the gene expression analyses experiments. On day 4 of RANKL treatment, the cells were treated with 0 or 10 μg/ml of DP-FrE or DP-FrF with and without 1 ng/ml TNF-α. Total protein was harvested from the cells following 30 minutes and 1 hour of treatment by first aspirating the media and washing the cells with sterile PBS. Radioimmunoprecipitation assay (RIPA, Cell Signaling, Danvers, MA) buffer was then added to each well and the cells were collected. The lysate was sonicated 6 x 15 sec and centrifuged at 16,000 x g to remove cellular debris. Total protein (40 μg) was denatured in Laemmli sample buffer at 95°C for 5 minutes. Protein was separated on a 4-20% gradient polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidine fluoride (PVDF) membrane. Ponceau S staining confirmed equal

transfer of all samples. The PVDF membranes were then blocked in 5% nonfat milk or bovine serum albumin (BSA) in tris buffered saline (TBS) and 0.1% Tween-20. Next, the membranes were incubated with either p-p44/42 (pERK), p44/42 (ERK), p-p38, or p38 antibodies (Cell Signaling Technology) with gentle shaking overnight at 4°C. Actin (Santa Cruz Biotech) was used as a loading control. After overnight incubation with primary antibody, the membranes were washed and incubated with secondary antibody for 1 hour prior to signal detection using SuperSignal West (ThermoFisher) chemiluminescent substrate. The blots were exposed using the ProteinSimple Fluorchem R (San Jose, CA). Density of the bands was assessed using UN-SCAN-IT gel analysis software (Silk Scientific Inc, Orem, UT).

Polyphenolic fractions on osteoclast differentiation in a co-culture system

To determine the most bioactive extract or extracts in reducing osteoclast differentiation in an environment that more closely mimics the coupled activity of osteoblasts and osteoclasts *in vivo*, murine primary co-cultures were used. Osteoblasts were isolated from the calvaria of 3-5 day old C57BL/6 neonates using sequential collagenase digestion. Briefly, the calvaria were rinsed in 0.05% EDTA (pH 7.4) for 20-30 minutes followed by six 12-minute incubations in 0.6 mg/ml collagenase in α-MEM at 37°C on a shaker. Following the first collagenase digestion, the media was discarded. The cells liberated during the 2nd-6th rounds of collagenase digestion were collected and centrifuged at 2000 rpm for 6 minutes at 25°C. The cells were cultured in α-MEM supplemented with 10% FBS, 1% P/S, and 2 mM L-glutamine until used for co-culture

experiments. Non-adherent, hematopoietic bone marrow cells were collected as described above for the generation of osteoclasts.

The ability of DP-FrE and DP-FrF to reduce osteoclast differentiation in murine primary co-cultures was examined. Calvarial-derived osteoblasts were plated in α-MEM supplemented with 10% FBS, 1% P/S, 2 mM L-glutamine, and 10 nM 1,25-dihydroxyvitamin D_3 at a density of 5 x 10^4 cells on 6.5 mm polycarbonate inserts (1 x 10^8 0.4-μm pores; Corning, Tewksbury, MA) and bone marrow cells were plated at a density of 1.0 x 10^6 cells/well in a 24-well plate. Osteoblasts were treated with 0 or 10 μg/ml of polyphenolic fractions DP-FrE and DP-FrF with or without TNF-α (1 ng/ml) for 10 days and then as described previously TRAP staining of the bone marrow cells was performed. The number of TRAP+, multinucleated large osteoclasts was quantified per well.

To assess gene expression in primary co-culture experiments, calvarial osteoblasts and non-adherent bone marrow cells were cultured together at a density of 5.0 x 10^5 cells and 1.5 x 10^6 cells, respectively, in a 24-well plate in α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, and 10 nM 1,25-dihydroxyvitamin D_3. Following 6 days of treatment 0 or 10 μg/ml of DP-FrE or DP-FrF under normal or inflammatory (1 ng/ml TNF-α) conditions, RNA was extracted and qRT-PCR completed as described above.

Identifying phytochemicals present in DP-FrE nd DP-FrF

The fractions were assessed for the presence of 14 phytochemicals known to be present in dried plum (**Table 3**) [32]. Liquid chromotagraphy (LC) and mass

spectrometry (MS) were used to determine the presence of the phytochemicals using methods previously described [33]. Briefly, known standards of the phytochemicals were used to identify the presence of any of the compounds in the fractions. Quantification of the detected compounds was done using a standard curve derived from the pure standard of the compound.

Statistical Analyses

Statistical analyses were performed using SAS Version 9.3 (SAS Institute, NC). If the data were not normally distributed, log transformation was completed prior to statistical analysis. For the screening assays, doses within a given fraction were compared using ANOVA with Bonferroni adjustment, due to the number of comparisons, when the overall ANOVA is $p < 0.05$. For all other assays, the effect of polyphenolic fraction treatment at a given dose was analyzed using ANOVA and Fisher's least significant difference (LSD) post hoc analyses. Each experiment was repeated a minimum of 2-3 times. Values are expressed as means ± standard error (SE) unless otherwise indicated.

Results

Screening of the polyphenolic fractions in RAW 264.7 cells

To determine the most bioactive polyphenolic fraction(s) in reducing osteoclast differentiation, a dose response study was performed using RAW 264.7 cells treated with RANKL. The polyphenolic fractions that reduced osteoclast differentiation compared to control under normal conditions were DP-FrA, DP-FrE, and DP-FrF. DP-FrA reduced

osteoclast number at the 10 μg/ml dose, while DP-FrE and DP-FrF reduced osteoclast

numbers at treatment doses of 0.1 μg/ml, 1 μg/ml, and 10 μg/ml (**Table 2**). To assess

whether these most bioactive fractions could also suppress osteoclast differentiation

under inflammatory conditions, the cells were treated with LPS 24 hours prior to TRAP

staining. Under inflammatory conditions, each of the three fractions significantly reduced

osteoclast differentiation at treatment doses of 1 μg/ml and 10 μg/ml, while DP-FrA and

DP-FrF also reduced osteoclast differentiation at a dose of 0.1 μg/ml compared to that of

the LPS-treated control (**Table 2**). From these assays, it was determined that DP-FrA,

DP-FrE and DP-FrF were able to reduce osteoclast differentiation and that this response

occurred under normal as well as inflammatory conditions. Based on these results, the

bioactivity of these three fractions was further assessed in subsequent primary osteoclasts

experiments.

DP fractions on primary osteoclast differentiation and activity under normal conditions

Next, the capacity of DP-FrA, DP-FrE and DP-FrF to alter osteoclast

differentiation in primary bone marrow-derived osteoclasts was assessed. Both the 1

μg/ml ($p = 0.0033$) and 10 μg/ml dose ($p = 0.0010$) of DP-FrE and DP-FrF reduced the

number of multinucleated TRAP$^+$ osteoclasts (**Figure 1a**). DP-FrA was not able to

significantly decrease osteoclastogenesis in the primary cell cultures and as a result of

this observation, all subsequent experiments focused on DP-FrE and DP-FrF fractions.

To determine if the decrease in osteoclast number observed with polyphenolic

fraction treatment resulted in a decrease in the activity of osteoclasts, primary bone

marrow-derived osteoclasts were cultured on dentin discs to assess resorption pit area.

DP-FrE and DP-FrF at both doses (i.e., 1 and 10 µg/ml) significantly reduced resorption pit area compared to the control (**Figure 1b**).

Effects of DP fractions on osteoclast differentiation and activity under inflammatory conditions

The ability of DP-FrE and DP-FrF to reduce osteoclast differentiation and activity in an inflammatory environment was also assessed. Osteoclast differentiation was significantly upregulated in TNF-α stimulated cultures, and both DP-FrE and DP-FrF at 1 and 10 µg/ml attenuated this response (**Figure 2a**). In fact, osteoclast differentiation was suppressed by 36% by the higher dose of DP-FrE and by 43% by DP-FrF in TNF-α-treated cultures. These responses resulted in the number of osteoclasts being reduced to the level of the control cells cultured under normal conditions.

To determine whether DP-FrE and DP-FrF could also reduce osteoclast activity in an inflammatory environment, primary bone marrow-derived osteoclasts were cultured on dentin discs and treated with the polyphenolic fractions and TNF-α. TNF-α increased osteoclast activity as indicated by resorption pit area by ~4-fold ($p = 0.0009$) compared to the untreated control cells (**Figure 2b**). Treatment with DP-FrE at the 1 µg/ml attenuated the TNF-α-induced increase ($p = 0.0028$) in resorption pit area, although not to the same magnitude as that of DP-FrF. Treatment with DP-FrF at 1 µg/ml of resulted in a 73% decrease ($p = 0.0004$) in resorption pit area compared to TNF-α-treated control, and this reduction in resorption resulted in a resorbed area similar to that of the control not stimulated with TNF-α. At a treatment dose of 10 µg/ml, both DP-FrE and DP-FrF attenuated TNF-α –induced bone resorption to the level of the control not stimulated with

TNF-α. Because a dose of 10 μg/ml of DP-FrE was more effective than a dose of 1

μg/ml, all subsequent experiments examining mechanisms by which the polyphenolic

fractions downregulate osteoclast differentiation are treated with 10 μg/ml of DP-FrE or

DP-FrF.

DP fractions alter gene and protein expression related to osteoclast differentiation under

normal conditions

To examine alterations in regulators of osteoclast differentiation, mRNA

expression of genes essential to the differentiation of osteoclasts was assessed. The

relative abundance of *Nfatc1,* a key transcription factor that regulates osteoclastogenesis

was also suppressed by DP-FrE and DP-FrF (**Figure 3a**). Upstream of *Nfatc1*, expression

of *Traf6*, which upon RANKL binding to RANK initiates signaling cascades (e.g.,

MAPK and NF-κB) essential to osteoclast differentiation was not altered with either DP

fraction (**Figure 3b**). However, *cFos*, which heterodimerizes with cJun to form the

transcription factor AP-1 that induces *Nfatc1*, was downregulated by both DP-FrE and

DP-FrF (**Figure 3c**).

Protein analyses were performed to determine if RANKL-stimulated MAPK

activation was suppressed with DP fraction treatment and representative blots are shown

in **Figure 3d**. Following 30 minutes of treatment with the polyphenolic fractions,

phosphorylation of p38 was not significantly altered (**Figure 3e**) and phosphorylated

Erk1/2 tended ($p = 0.0712$) to be suppressed (**Figure 3f**). After 1 hour of treatment, DP-

FrF tended ($p = 0.0737$) to suppress p38 phosphorylation (**Figure 3g**). Phosphorylation of

Erk1/2 was decreased by DP-FrE and to a lesser extent by DP-FrF, compared to control

(**Figure 3h**). This data indicates that DP-FrE and DP-FrF downregulated *Nfatc1*, and this is in part due to suppression of *cFos* upstream of *Nfatc1*, as well as reduced activation of p38 and Erk1/2.

DP fractions alter gene and protein expression related to osteoclast differentiation under inflammatory conditions

To determine the effects of DP-FrE and DP-FrF on regulators of osteoclast differentiation in an inflammatory environment, the relative abundance of mRNA for genes encoding for proteins that are essential to the differentiation of osteoclasts was assessed following 1 hour of treatment with DP-FrE or DP-FrF with or without TNF-α. Expression of *Nfatc1* was upregulated by TNF-α treatment, but DP-FrF was able to attenuate this response (**Figure 4a**). Upstream of *Nfatc1*, the expression of *Traf6* was upregulated by TNF-α, but both DP-FrE and DP-FrF were able to attenuate this response (**Figure 4b**). In fact, mRNA expression of *Traf6* in DP-FrE and DP-FrF treated cultures stimulated with TNF-α was similar to that of the control, under normal conditions. In addition, *cFos* expression, which is induced by TRAF6 and NF-κB signaling and results in the induction of *Nfatc1*, was not altered by either TNF-α or DP phenolic fraction treatment at (**Figure 4c**). Expression of *Ikbkb,* a kinase that is upregulated during inflammation and activates NF-κB signaling, was suppressed by DP-FrF in TNF-α stimulated cultures to a level lower than that of the untreated control and the TNF-α treated control (**Figure 4d**). In summary, gene expression indicative of osteoclast differentiation was suppressed by treatment with the polyphenolic fractions under inflammatory conditions. Next, protein analyses were performed to determine if

RANKL-stimulated MAPK activation was suppressed with DP fraction treatment under inflammatory conditions and representative blots are shown in **Figure 4e**. Phosphorylation of p38 was not significantly altered by TNF-α or either of the fractions following 30 minutes of treatment (**Figure 4f**). Phosphorylation of Erk1/2 was significantly upregulated by TNF-α following 30 minutes of treatment, and both DP-FrE and DP-FrF attenuated this response (**Figure 4g**). Likewise, after 1 hour of treatment p38 was not significantly altered by TNF-α or the fractions, although there was a strong trend ($p = 0.0512$) for the upregulation of phosphorylated p38 with TNF-α and a suppression of this response with both fractions (**Figure 4h**). Both DP-FrE and DP-FrF suppressed phosphorylation of Erk1/2 following 1 hour of treatment compared to the TNF-treated control (**Figure 4i**). This data indicates that under inflammatory conditions, DP-FrE and DP-FrF downregulated *Nfatc1*, and this is in part due to suppression of *Traf6* and *cFos* upstream of *Nfatc1*, as well as reduced activation of p38 and Erk1/2, which is similar to observations under normal conditions.

Calcium signaling in primary bone marrow-derived osteoclasts under normal conditions

Differentiation of osteoclasts requires calcium oscillations regulated by co-stimulatory membrane-bound receptors, including OSCAR, SIRPβ1, and TREM2. Treatment of primary bone marrow-derived osteoclasts with DP-FrF for 5 minutes and 1 hour resulted in a significant decrease in intracellular calcium concentration compared to control following 5 minutes and one hour, with DP-FrE treatment having an intermediate effect (**Figure 5a**). DP-FrF treatment resulted in a ~2-fold downregulation of *Oscar* (**Figure 5c**)*,* an osteoclast-specific receptor that is transcriptionally regulated by Nfatc1

and activated by collagen, and is important in the auto-amplification of Nfatc1 via its role in activating phospholipase Cγ and calcium signaling. Additionally, DP-Fr treatment resulted in a decreased mRNA expression of *Sirpb1*, a co-stimulatory receptor expressed by myeloid cells that is known to regulate calcium signaling (**Figure 5d**). Furthermore, treatment with both DP-FrE and DP-FrF resulted in an ~2-fold downregulation of *Trem2*, which regulates calcium signaling by activating phosphoinositide 3-kinase and subsequently calcium release from the endoplasmic reticulum (**Figure 5e**). This data shows that the DP-FrE and DP-FrF reduce osteoclast differentiation at least in part by inhibiting calcium signaling in the differentiating osteoclast.

Calcium signaling in primary bone marrow-derived osteoclasts under inflammatory conditions

　　　　Similar to the response under normal conditions, intracellular calcium was suppressed by the treatment with the fractions in an inflammatory environment. Following 30 minutes of treatment with TNF-α and the fracitons, DP-FrE significantly reduced intracellular calcium (**Figure 5b**). Additionally, intracellular calcium was robustly upregulated following 2 hours of treatment with TNF-α, but DP-FrF attenuated this response. Examination of mRNA expression of the co-stimulatory receptors that initiate calcium signaling in osteoclasts indicated that *Sirpb1* was suppressed by both DP-FrE and DP-FrF in TNF-α stimulated cultures compared to the TNF-α treated control (**Figure 5g**). In fact, DP-FrF suppressed *Sirpb1* to a level even lower than that of the untreated control. Unlike in the osteoclast cultures under normal conditions (i.e., no TNF-

α), treatment with the DP-phenolic fractions did not alter the expression of *Oscar* (**Figure 5f**) or *Trem2* (**Figure 5h**).

Effects of DP fractions on osteoclast differentiation in primary co-cultures

To assess the bioactivity of the fractions in a system that allows for osteoblast and osteoclast interaction, primary osteoblasts derived from fetal murine calvaria and primary bone marrow-derived osteoclasts were co-cultured. Under normal conditions, osteoclast differentiation tended to be suppressed ($p = 0.0530$) with treatment with the DP fractions (**Figure 6a**). Regulators of osteoclast differentiation are produced by the osteoblasts in the co-culture system in response to stimulation by 1,25-dihydroxyvitamin D_3 and TNF-α. Therefore, the effect of the DP fractions on osteoblast expression of signaling molecules that affect osteoclast differentiation was assessed. The relative mRNA abundance of *Opg* and *Rankl* was not altered by either DP-FrE or DP-FrF in the co-cultures (**Figure 6b and 6c**). The relative mRNA abundance of regulators of osteoclast differentiation was also assessed. Expression of *Nfatc1* was downregulated by both DP-FrE and DP-FrF following 6 days of co-culture and treatment with the polyphenolic fractions (**Figure 6d**). However, *Traf6* and *cFos* were not altered by treatment with DP fractions in the osteoblast and osteoclast co-cultures in normal conditions at this time point (**Figure 6e and 6f**).

In an inflammatory environment, TNF-α upregulated osteoclast differentiation in the co-cultures and treatment with DP-FrF reduced the number of multinucleated TRAP+ cells compared to the TNF-α control ($p = 0.0001$; **Figure 7a**). In fact, the number of multinucleated TRAP+ osteoclasts in the DP-FrF-treated cultures was similar to that of

the control (i.e., no TNF-α). While mRNA expression of *Rankl* was not altered in the co-cultures under normal conditions, both DP-FrE and DP-FrF downregulated *Rankl* expression in TNF-α stimulated co-cultures (**Figure 7b**). The expression of *Opg* was not altered by DP fractions or TNF-α (**Figure 7c**). Further explanation for the reduction in osteoclast number was the expression of *Nfatc1*, which was upregulated by TNF-α, and both DP-FrE and DP-FrF were able to attenuate this response (**Figure 7d).** Similar to the co-cultures under normal conditions, *Traf6* expression was not altered by DP fractions or TNF-α. However, *cFos* expression was upregulated by TNF-α, and both DP-FrE and DP-FrF attenuated this response (**Figure 7f**). In fact, expression of both *cFos* and *Nfatc1* were suppressed in co-cultures treated with TNF-α and DP-FrE or DP-FrF to the level of the untreated control. These findings suggest that the polyphenolic fractions may both directly and indirectly reduce osteoclast differentiation in a co-culture model, in which osteoblast and osteoclast activity are coupled. The suppression of *Rankl* expression suggests the polyphenolic fractions can downregulate osteoclast differentiation by reducing the production of stimulatory molecules by osteoblasts, while *Nfatc1* expression data from the mono-cultures and these co-cultures suggest that the DP fractions can also directly downregulate differentiation pathways in osteoclast precursors.

Characterization of polyphenolic compounds in DP-FrE and DP-FrF

Dried plums are rich in polyphenols, and especially phenolic acids. The presence of 14 phytochemicals known to be in dried plums was assessed in DP-FrE and DP-FrF (**Table 3**). Interestingly, of the 14 compounds, only cryptochlorogenic acid, neochlorogenic acid, and rutin were detected in each fraction. Each of the chlorogenic

acid isormers was more abundant in the fraction with the highest content of organics, DP-FrF, than in DP-FrE.

Discussion

This study is the first to examine the fractions of a polyphenolic extract of dried plum that reduce osteoclast differentiation and activity. The fractions of a total polyphenolic extract from dried plum with the highest organics content downregulate osteoclast differentiation and activity in primary bone marrow-derived osteoclasts, and this is due to suppression of *Nfatc1* expression, the master regulator of osteoclast differentiation. These results are similar to those previously reported in RAW 264.7 cells treated with an ethanol extract of dried plum's polyphenols [30]. The expression of *Nfatc1* is regulated by TRAF6-mediated and calcium-dependent signaling pathways. TRAF6-mediated NF-κB activation results in the induction of *cFos* expression, a major component of the transcription factor activator protein-1 (AP-1) that regulates *Nfatc1* expression [14]. In the current study, the fractions downregulated expression of *cFos* under normal and inflammatory conditions. The fractions also suppressed the TNF-α-induced upregulation of *Traf6* expression.

The induction of *Nfatc1* expression is additionally regulated by TRAF6-mediated MAPK signaling, including the activation of p38 and Erk [14]. Activation of AP-1 by Erk-mediated phosphorylation induces *Nfatc1* mRNA expression [34]. Furthermore, the presence of p38 at the promoter region of *Nfatc1* is required for its transcription [35]. Inhibition of any of these pathways can result in the downregulation of *Nfatc1*. Plant-derived polyphenolic compounds have been demonstrated to inhibit MAPK signaling and

subsequent osteoclast differentiation *in vitro* [19, 20]. For example, the polyphenol phenethyl isothiocyanate found in cruciferous vegetables downregulates NF-κB, Erk and p38 activation and results in a decrease in osteoclast differentiation in RAW 264.7 cells [19]. In addition, the polyphenolic compound caffeic acid 3,4-dihydroxy-phenethyl ester, found in various medicinal plants, can inhibit the activation of Erk and p38 in bone marrow monocytes and RAW 264.7 cells, resulting in a suppression of osteoclast differentiation [20]. In our study, treatment with DP-FrE and, to a lesser degree DP-FrF, both of which contain the phenolic compounds neochlorogenic acid and crytpochlorogenic acid, as well as the flavanol rutin, attenuated the RANKL-stimulated phosphorylation of Erk and tended to reduce the phosphorylation of p38 under normal and inflammatory conditions. The inhibition of MAPK activation and *cFos* expression can, at least in part, provide explanation for the suppression of *Nfatc1* mRNA levels and osteoclast numbers.

While the initial expression of *Nfatc1* requires TRAF6-mediated signaling cascades, sustained intracellular calcium oscillations are essential for the calcium-dependent auto-amplification of *Nfatc1*, and subsequently osteoclast differentiation [13]. Co-stimulatory receptors expressed by osteoclast precursors, including OSCAR, TREM2, and SIRPβ1, as well as calcium stores in the endoplasmic reticulum, mediate calcium oscillations in the differentiating osteoclast [15, 16]. An increase in intracellular calcium is required to activate calcium-dependent enzymes that regulate the dephosphorylation and subsequent auto-amplification of *Nfatc1* [36]. Therefore, it was important to investigate the role of intracellular calcium in the observed suppression of *Nfatc1* with treatment with the fractions, and how this response may be mediated by co-stimulatory

receptors. Under normal conditions Treatment with DP-FrF significantly reduced mRNA expression of *Oscar*, *Trem2*, and *Sirpb1*, and, subsequently, intracellular calcium concentration, in differentiating osteoclasts derived from primary bone marrow cells. Treatment with DP-FrE also downregulated expression of these co-stimulatory receptors and intracellular calcium concentration, but to a lesser extent as DP-FrF. Likewise, the fractions attenuated the TNF-α-induced increase in intracellular calcium, and this was due, in part, to a downregulation of *Sirpb1*. Ligands expressed by osteoblasts and myeloid cells activate these co-stimulatory receptors (e.g. collagen and semaphorin 6D), which are associated with immunoreceptor tyrosine-adaptation motif (ITAM)-containing adaptor proteins Fc receptor-gamma (FcRγ) and DNAX activation protein of 12 kDa (DAP12) [15, 16, 18]. The activation of DAP12 and FcRγ initiates a signaling cascade that results in calcium release from the endoplasmic reticulum [37]. Depletion of endoplasmic reticulum calcium stores induces extracellular calcium influx through calcium channels on the plasma membrane [13, 37]. The increase in cytoplasmic calcium concentration is essential to the function of the calcium-dependent calcineurin pathway necessary for Nfatc1 auto-amplification [13]. Osteoclast differentiation is impaired if the adaptor proteins or the membrane-bound co-stimulatory receptors they associate with are inhibited [16-18, 38, 39]. Therefore, inhibition of calcium signaling may provide further explanation for the suppression of *Nfatc1* expression and reduction in osteoclast number.

Osteoclast differentiation and activity is enhanced in an inflammatory environment. The inflammatory cytokine TNF-α, the production of which is increased with estrogen deficiency and in inflammatory diseases such as rheumatoid arthritis, is known to upregulate osteoclast differentiation and activity [3, 6, 8]. Treatment with the

fractions attenuated the TNF-α-induced increase is osteoclast differentiation and activity.

This enhanced differentiation is due, in part, to an increased expression of RANK by

osteoclast precursors [40]. Additionally, TNF-α increases the activation of NF-κB and

AP-1 in osteoclast precursors, enhancing their sensitivity to RANKL stimulation [3, 11,

12, 41]. The TNF-α-induced increase in osteoclast number was suppressed by both DP-

FrE and DP-FrF. The expression of *Traf6* was also significantly downregulated by both

DP-FrE and DP-FrF, suggesting the treatments may attenuate the TNF-α-enhanced

sensitivity of osteoclast precursors to RANKL stimulation. Furthermore, treatment with

DP-FrF significantly reduced the mRNA expression of *Ikbkb,* the enzyme that activates

NF-κB, allowing for its translocation to the nucleus where it induces the expression of the

AP-1 component c-Fos. Surprisingly, *cFos* expression was not significantly altered by

either TNF-α or treatment with DP-FrF. However, *Nfatc1* expression was significantly

suppressed with treatment of DP-FrF, and this may be due, in part, to downregulation of

NF-κB activation.

 To further examine mechanisms by which *Nfatc1* expression may be suppressed,

intracellular calcium and the expression of co-simulatory receptors was examined in the

presence of TNF-α. The fractions attenuated the increase in intracellular calcium that

resulted from TNF-α treatment. However, the expression of costimulatory receptors

involved in calcium signaling was not altered to the same magnitude by DP-FrE and DP-

FrF in the inflammatory conditions compared to the normal conditions. In fact, only

Sirpb1 was significantly downregulated by the polyphenolic fractions in the TNF-α

treated cultures. Both SIRPβ1 and TREM2 are DAP12-associated receptors, while

OSCAR is linked with the adaptor protein FcRγ [16]. There is evidence to suggest that

DAP12 is a predominant regulator of calcium signaling over FcRγ in osteoclasts, and so it is possible that downregulation of *SIRPβ1* results in a suppression of DAP12 signaling of enough magnitude to significantly reduce calcium signaling [42].

While assessing how the polyphenolic fractions alter signaling pathways in a bone marrow-derived osteoclast population provides valuable insight into the mechanisms involved in the anti-resorptive capacity of dried plum, supra-physiological doses of RANKL are traditionally used to stimulate osteoclast differentiation. Furthermore, the mono-culture system does not allow for the study of coupled osteoblast and osteoclast activity that occurs *in vivo*. Therefore, the ability of the polyphenolic fractions to reduce osteoclast differentiation in osteoblast and osteoclast co-cultures was assessed. There was a trend for reduced osteoclast differentiation with polyphenolic fraction treatment under normal conditions, and this can be attributed, at least in part, to a significant downregulation of *Nfatc1* by both DP-FrE and DP-FrF. Under inflammatory conditions, the magnitude of response to DP-FrF was greater than that of DP-FrE in downregulating osteoclast differentiation. In this co-culture system, it is likely that osteoclast differentiation was upregulated by TNF-α by both direct and indirect effects, including induced stromal cell and osteoblast production of M-CSF and RANKL, as well as increased sensitivity of osteoclast precursors to RANKL stimulation [5, 11, 12, 41]. Both DP-FrE and DP-FrF reduced *Rankl* expression in the co-culture system. Furthermore, both fractions downregulated *cFos*, and subsequently, *Nfatc1* expression. Therefore, it is not clear why only DP-FrF significantly reduced osteoclast numbers, and indicates that other signaling mechanisms are likely involved.

In summary, this study is the first to examine fractions of a polyphenolic extract from dried plum that downregulate osteoclast differentiation and activity in murine primary cells under normal and inflammatory conditions. While both fractions were able to suppress osteoclast differentiation and activity, DP-FrF appeared to have a more robust effect, especially under inflammatory conditions. Of the polyphenols analyzed, these fractions are rich in neochlorogenic acid, crytpocholorogenic acid, and rutin. We have demonstrated for the first time that these fractions downregulate *Nfatc1* expression at least in part, by suppressing MAPK and calcium signaling pathways. We have also demonstrated that at least DP-FrF can downregulate osteoclast differentiation in the osteoblast and osteoclast co-culture system. However, the mechanisms involved in attenuating osteoclast differentiation in the co-culture system remain unclear. Further investigation is needed to determine how these fractions of the polyphenolic extract of dried plum affect bone metabolism *in vivo*. Because osteoclasts are derived from monocytes, and activated immune cells use similar MAPK and calcium signaling mechanisms, it is possible that these fractions can attenuate bone loss by both directly affecting osteoclast differentiation as well as indirectly downregulating inflammatory immune responses that induce bone resorption. Further characterization of the bioactive compounds within the fractions is needed if a treatment option for osteoporosis is to be developed from these fractions.

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Table 1. Primer sequences for qRT-PCR.

Transcript	Sequence (5'-3')
Traf6	F: CAG CAG TGT AAC GGG ATC TAC
	R: CTG TGT AGA ATC CAG GGC TAT G
cFos	F: GGA CAG CCT TTC CTA CTA CCA TTC C
	R: AAA GTT GGC ACT AGA GAC GGA CAG A
Nfatc1	F: GCG AAG CCC AAG TCT CTT TCC
	R: GTA TGG ACC AGA ATG TGA
Oscar	F: CGT GCT GAC TTC ACA CCA ACA
	R: CAC AGC GCA GGC TTA CGT T
Sirpb1	F: GTC ACT CCT GCT GAT TCG G
	R: GTC ACT GTC TGC TGA GGG AC
Trem2	F: TCC CAA GCC CTC AAC ACC A
	R: TTC CAG CAA GGG TGT CAT CTG CGA
Ikbkb	F: GCG AGC AGC CAT GAT GAG T
	R: GGA GGC CAT GGC GTT CT
Opg	F: TCC CGA GGA CCA CAA TGA AC
	R: TGG GTT GTC CAT TCA ATG ATG T
Rankl	F: TCT GCA GCA TCG CTC TGT TC
	R: AGC AGT GAG TGC TGT CTT CTG ATA TT

Table 2. Screening of the polyphenolic fractions on osteoclast differentiation using RAW 264.7 cells

Treatment dose:	CON	LPS CON	0.1 µg/ml	1 µg/ml	10 µg/ml	*p-value*
Normal						
DP-FrA	100.0 ± 7.0^a		97.1 ± 11.0^a	82.3 ± 4.6^{ab}	72.3 ± 6.0^b	0.0487
DP-FrB	100.0 ± 7.0		91.6 ± 7.3	83.8 ± 3.8	75.6 ± 6.2	0.0991
DP-FrC	100.0 ± 7.0		80.4 ± 3.1	89.7 ± 6.2	81.8 ± 8.0	0.1612
DP-FrD	100.0 ± 7.0		89.0 ± 6.2	85.5 ± 4.5	73.6 ± 5.3	0.0506
DP-FrE	100.0 ± 7.0^a		78.5 ± 3.7^b	77.6 ± 6.0^b	73.6 ± 5.4^b	0.0343
DP-FrF	100.0 ± 7.0^a		79.3 ± 1.9^b	74.1 ± 3.8^{bc}	65.5 ± 2.4^c	0.0007
Inflammatory						
DP-FrA	100.0 ± 4.3^b	134.6 ± 6.7^a	106.3 ± 8.7^b	97.0 ± 3.4^b	92.6 ± 2.8^b	0.0008
DP-FrE	100.0 ± 4.3^b	134.6 ± 6.7^a	127.3 ± 2.4^a	103.2 ± 4.7^b	99.3 ± 3.2^b	<.0001
DP-FrF	100.0 ± 4.3^b	134.6 ± 6.7^a	102.2 ± 4.6^b	100.2 ± 5.8^b	87.7 ± 3.8^b	0.0002

Data representative of 2-3 repeated experiments and expressed relative control and presented as mean $\pm$ SE (n = 6). Statistically significant differences are indicated by difference in superscript letters, p < 0.05.

Table 3. The most bioactive fractions contained a combination of phytochemicals.

	mg/100 g total phenolic extract		% of total compounds analyzed	
	DP-FrE	DP-FrF	DP-FrE	DP-FrF
Chlorogenic acid	n.d.	n.d.		
Cryptochlorogenic acid	8.69	22.02	55.5	49.7
Neochlorogenic acid	5.96	21.45	38.1	48.4
Caffeic acid	n.d.	n.d.		
Quinic acid	n.d.	n.d.		
o-Coumaric acid	n.d.	n.d.		
m-Coumaric acid	n.d.	n.d.		
Ferulic acid	n.d.	n.d.		
Cyanidin 3-rutinoside	n.d.	n.d.		
Cyanidin 3-glucoside	n.d.	n.d.		
Quercetin	n.d.	n.d.		
Rutin	1.01	0.85	6.4	1.9
Sorbic acid	n.d.	n.d.		
5-Hydroxymethyl-2-furaldehyde	n.d.	n.d.		

Figure Legends

Figure 1. DP-FrE and DP-FrF, but not DP-FrA reduced osteoclast differentiation in primary bone marrow-derived osteoclasts. Primary non-adherent bone marrow cells were treated with 30 ng/ml MCSF and 50 ng/ml RANKL for 5 days. On day 4 of differentiation, the cells were treated with 1 or 10 µg/ml of the fractions. (a) On day 5 the cells were stained and the multi-nucleated, TRAP-positive osteoclasts were quantified. (b) On day 10, resorption pits were stained and resorbed area was quantified. (c) Large, multinucleated osteoclasts are indicated with arrows. (d) Resorption pits are indicated by arrows. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 2. DP-FrE and DP-FrF reduced osteoclast differentiation and osteoclast activity under inflammatory conditions. On day 4 of differentiation, the cells were treated with TNF-α (1 ng/ml) and then 1 or 10 µg/ml of the fractions. (a) On day 5, cells were stained and the multi-nucleated, TRAP-positive osteoclasts were quantified. (b) On day 10, resorption pits were stained and resorption pit area was quantified. (c) Large, multinucleated osteoclasts are indicated with arrows. (d) Resorption pits are indicated by arrows. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 3. DP-FrE and DP-FrF downregulated expression of genes essential to osteoclast differentiation, as well as MAPK signaling. On day 4 of differentiation, RNA was extracted from osteoclasts treated for 1 hr with DP polyphenolic fractions (10 µg/ml). Relative mRNA expression of (a) *Nfatc1*, (b) *Traf6* and (c) *cFos* was assessed with qRT-PCR using *Gapdh* as an invariant control. Additionally, on day 4 of differentiation, protein was extracted from osteoclasts treated for 30 min or 1 hr with DP polyphenolic fractions (10 µg/ml). Relative abundance of phosphorylated p38 and ERK was examined via immunoblotting and (d) shows representative blots. Western blot results for relative phosphorylation of p38 are quantified following (e) 30 min and (g) 1 hr of treatment with the fractions. Western blot results for relative phosphorylation of pErk1/2 are quantified following (f) 30 min and (h) 1 hr of treatment with the fractions. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 4. DP-FrE and DP-FrF downregulated expression of genes essential to osteoclast differentiation. On day 4 of differentiation, the cells were treated with TNF-α (1 ng/ml) and then the DP polyphenolic fractions (1 or 10 µg/ml). RNA and protein was extracted from osteoclasts treated for 1 hr with 1ng/ml TNF-α and 10 µg/ml of DP polyphenolic fractions. Relative mRNA expression of (a) *Nfatc1*, (b) *Traf6*, (c) *cFos*, and (d) *Ikbkb* was assessed with qRT-PCR using *Gapdh* as a control. Additionally, on day 4 of differentiation, protein was extracted from osteoclasts treated for 30 min and 1 hr with

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TNF-α (1 ng/ml) and DP polyphenolic fractions (10 µg/ml). Relative abundance of phosphorylated p38 and ERK was examined via immunoblotting and (e) shows representative blots. Western blot results for relative phosphorylation of p38 are quantified following (f) 30 min and (h) 1 hr of treatment with the fractions. Western blot results for relative phosphorylation of pErk1/2 are quantified following (g) 30 min and (i) 1 hr of treatment with the fractions. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 5. Treatment with DP polyphenolic fractions altered calcium transport in primary bone marrow-derived osteoclasts. On day 4 of differentiation, the cells were incubated in Fluo-4 dye for 1 hour prior to treatment with DP-FrE or DP-FrF (10 µg/ml). Intracellular calcium was assessed under (a) normal and (b) inflammatory conditions. Additionally, RNA was extracted following treatment with the DP fractions (10 µg/ml) for 1 hr. Relative mRNA abundance of (c) *Oscar*, (d) *Sirbp1*, and (e) *Trem2* under normal conditions, and (f) *Oscar*, (g) *Sirbp1*, and (h) *Trem2* under inflammatory conditions, was assessed with qRT-PCR using *Gapdh* as an invariant control. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 6. Alterations in osteoclast differentiation in co-cultures treated with DP polyphenolic fractions under normal conditions. Primary calvarial osteoblasts were treated with 1,25-hydroxy vitamin D3 (10 nM) and DP polyphenolic fractions (0 or 10 µg/ml) and co-cultured with non-adherent bone marrow cells. (a) Cells were stained and the multinucleated TRAP+ osteoclasts were quantified following 10 days of co-culture. RNA was extracted from co-cultures following 6 days of treatment with DP polyphenolic fractions. Relative mRNA abundance of (b) *Rankl*, (c) *Opg* , (d) *Nfatc1*, (e) *Traf6* and (f) *cFos*, was assessed with qRT-PCR using *Gapdh* as a control. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 7. Treatment with DP-FrF suppressed osteoclast differentiation in TNF-α stimulated co-cultures. Primary calvarial osteoblasts were treated with 1,25-hydroxy vitamin D3 (10 nM) and DP polyphenolic fractions (10 µg/ml) and TNF-α or vehicle and co-cultured with non-adherent bone marrow cells. (a) Cells were stained and the multinucleated TRAP+ osteoclasts were quantified following 10 days of co-culture. RNA was extracted from co-cultures following 6 days of treatment with DP polyphenolic fractions and TNF-α. Relative mRNA abundance of (b) *Rankl*, (c) *Opg*, (d) *Nfatc1*, (e) *Traf6*, and (f) *cFos* was assessed with qRT-PCR using *Gapdh* as a control. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 1.

a.

b.

c.

RANKL	+	+	+	+	+	+	+
DP-FrA (μg/ml)	-	1	10	-	-	-	-
DP-FrE (μg/ml)	-	-	-	1	10	-	-
DP-FrF (μg/ml)	-	-	-	-	-	1	10

d.

RANKL	+	+	+	+	+
DP-FrE (μg/ml)	-	1	10	-	-
DP-FrF (μg/ml)	-	-	-	1	10

Figure 2.

a.

Osteoclast Number

160
140
120
100
80
60
40
20
0

a
b
b
b
a
b
b
b

1 µg/ml   10 µg/ml
TNF-α - + + +   - + + +
DP-Fr - - + +   - - + +

■ CON
□ TNF-α CON
□ DP-FrE
■ DP-FrF

b.

Resorption pit area (% total area)

40
35
30
25
20
15
10
5
0

a
b
c
c
a
b
b
b

1 µg/ml   10 µg/ml
TNF-α - + + +   - + + +
DP-Fr - - + +   - - + +

■ CON
□ TNF CON
□ DP-FrE
■ DP-FrF

c.

4x

20x

RANKL	+	+	+	+	+	+
TNF-α	-	+	+	+	+	+
DP-FrE (µg/ml)	-	-	1	10	-	-
DP-FrF (µg/ml)	-	-	-	-	1	10

d.

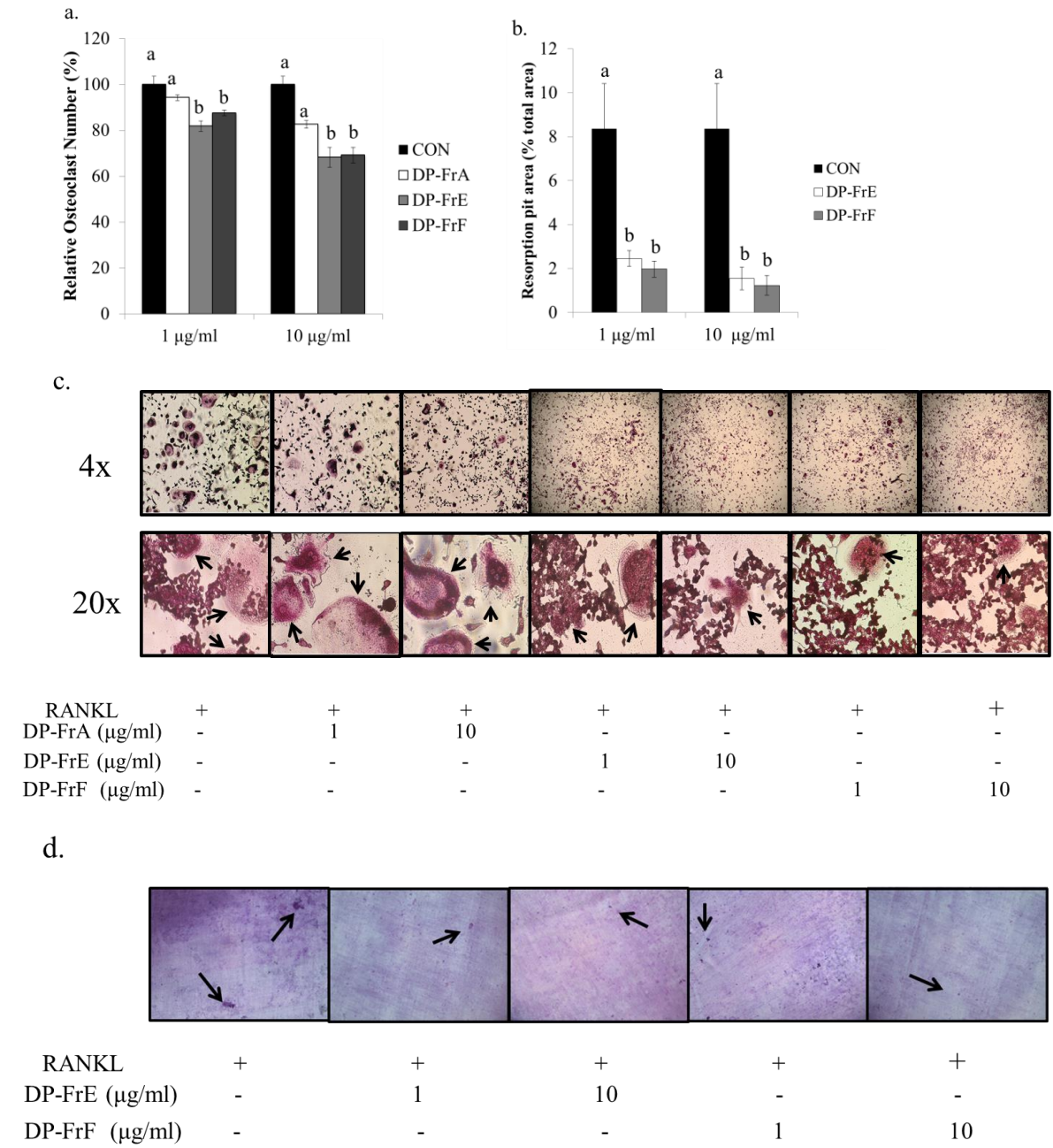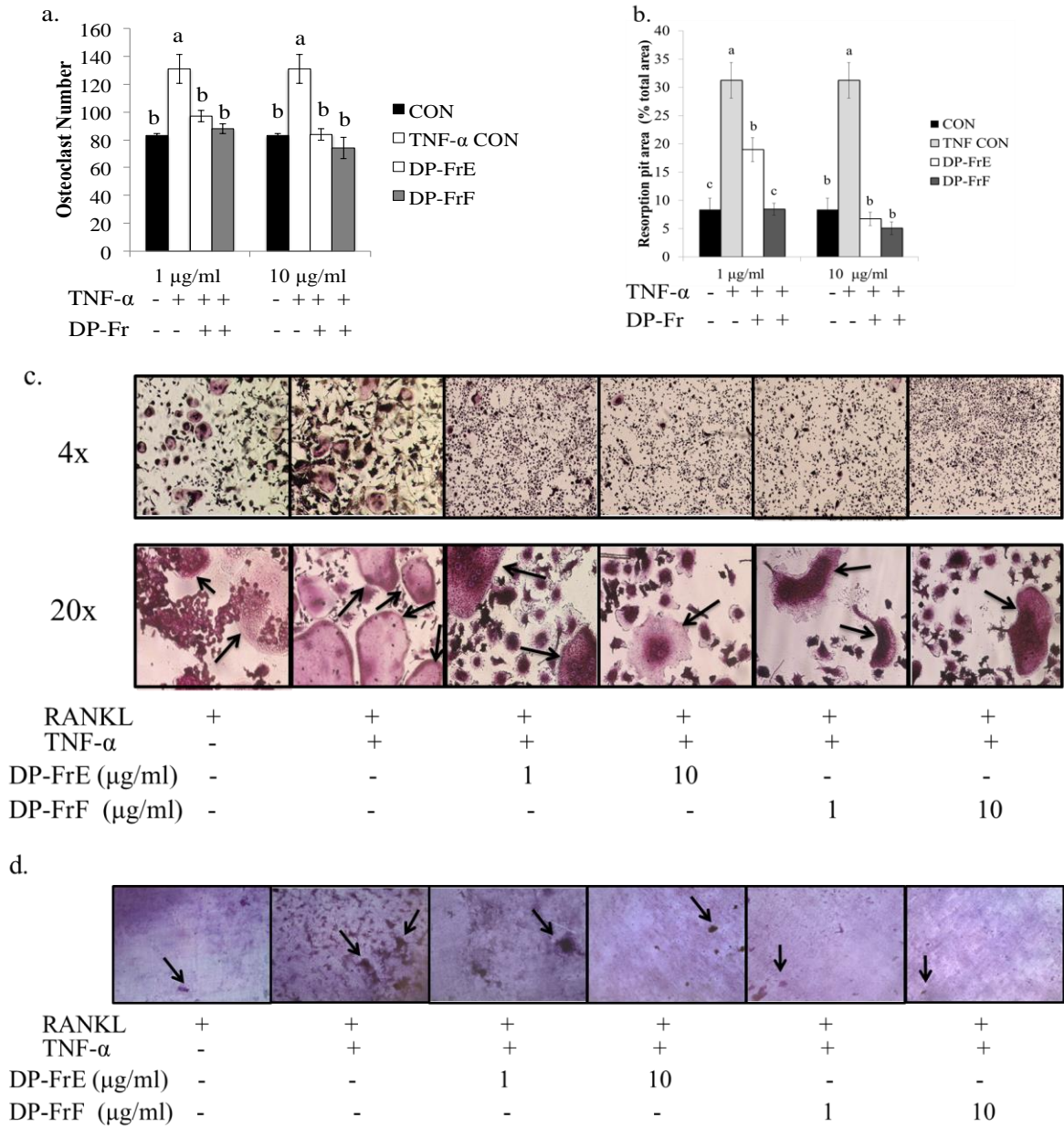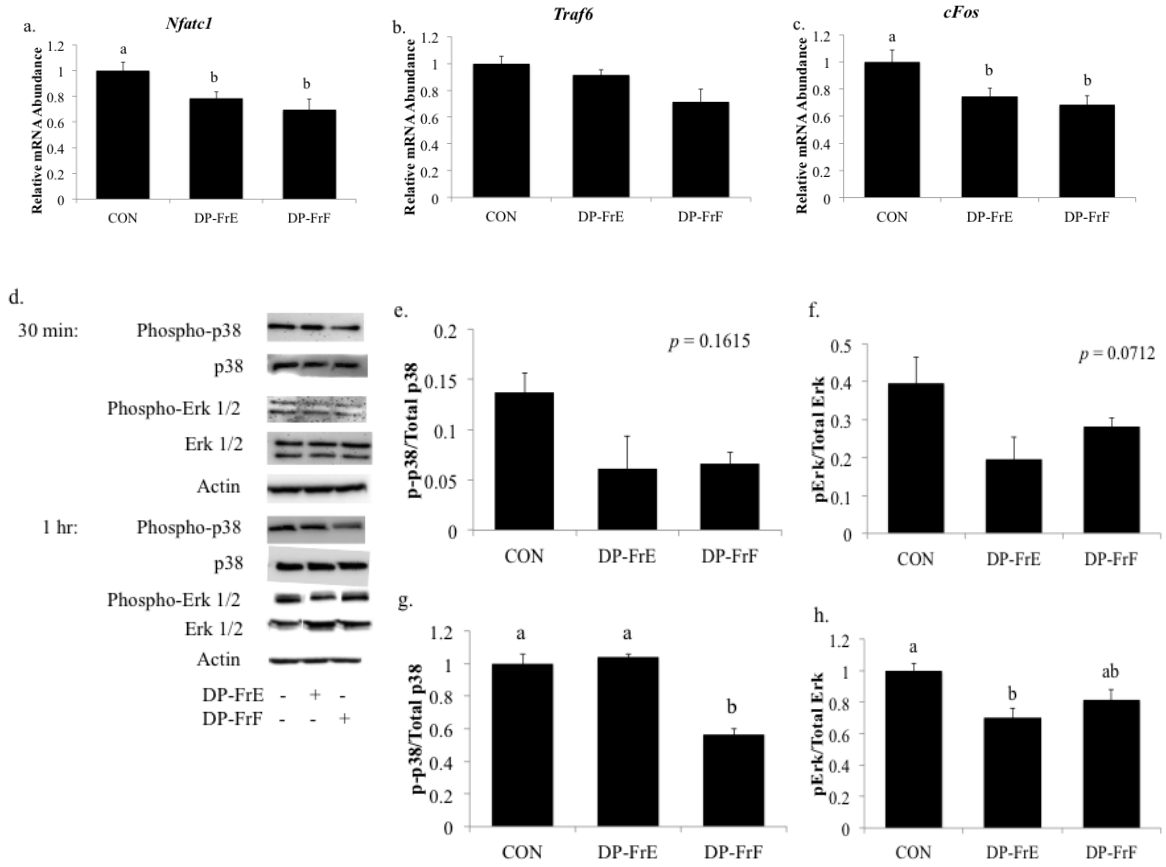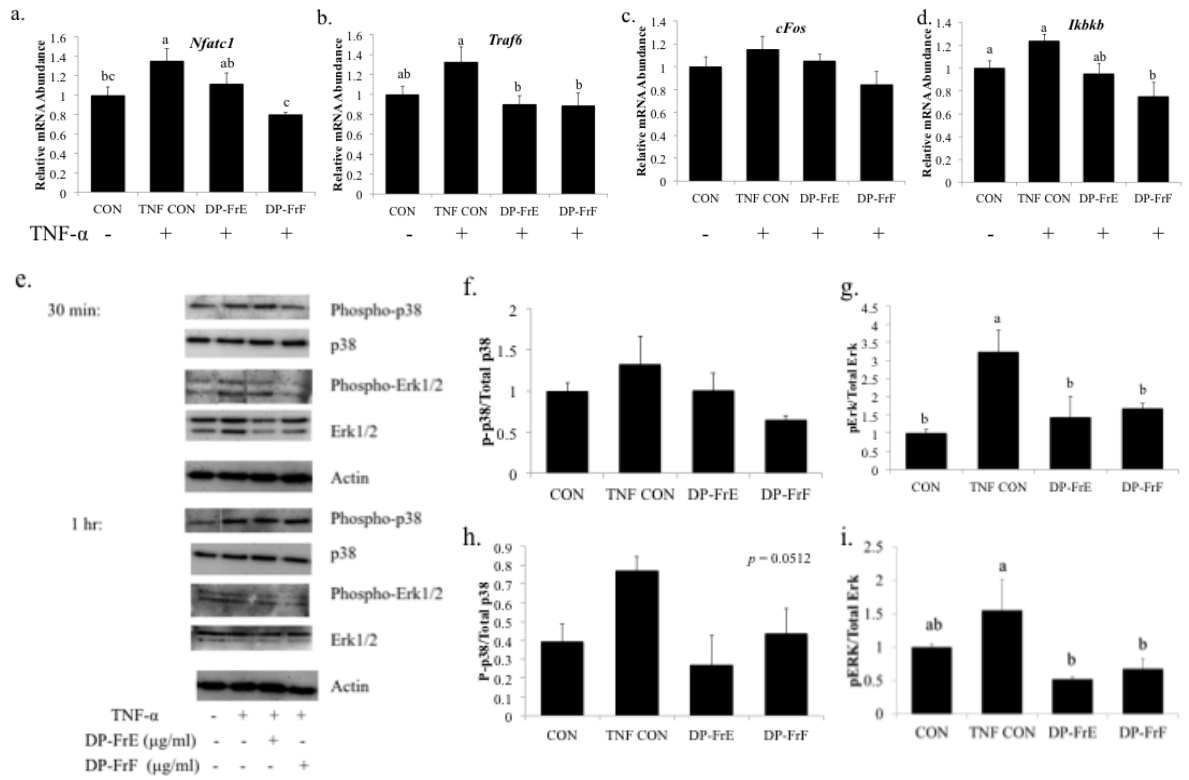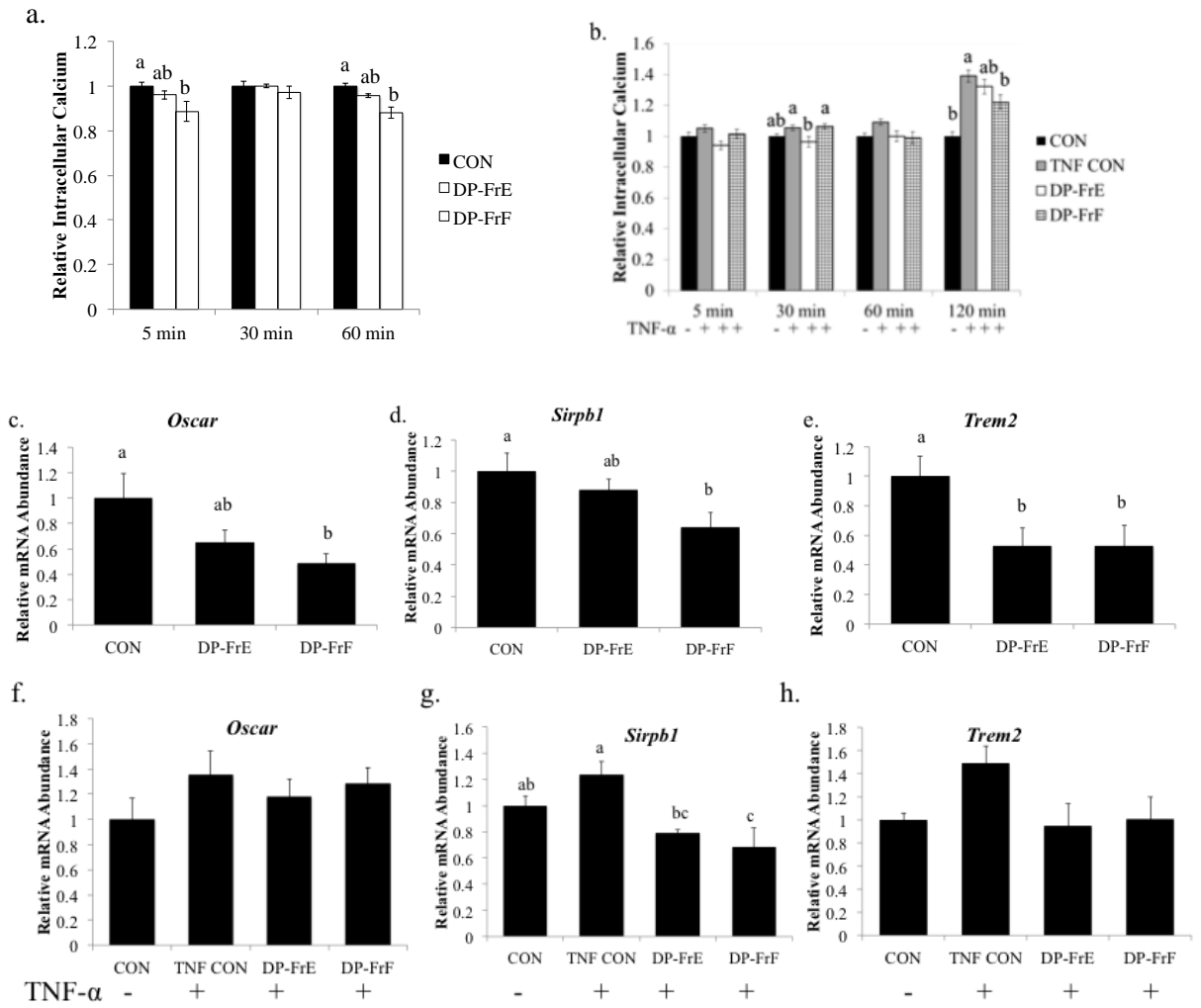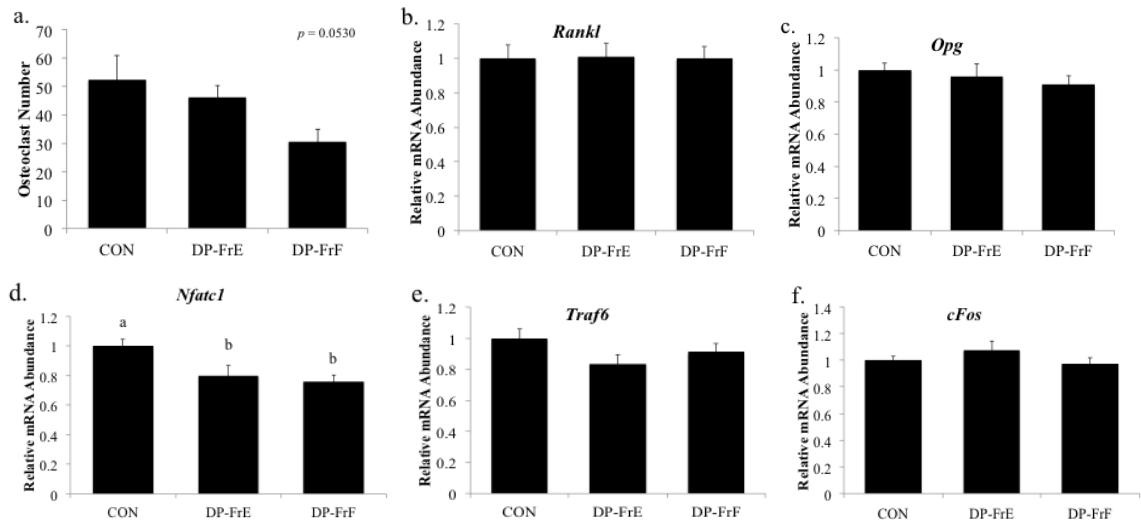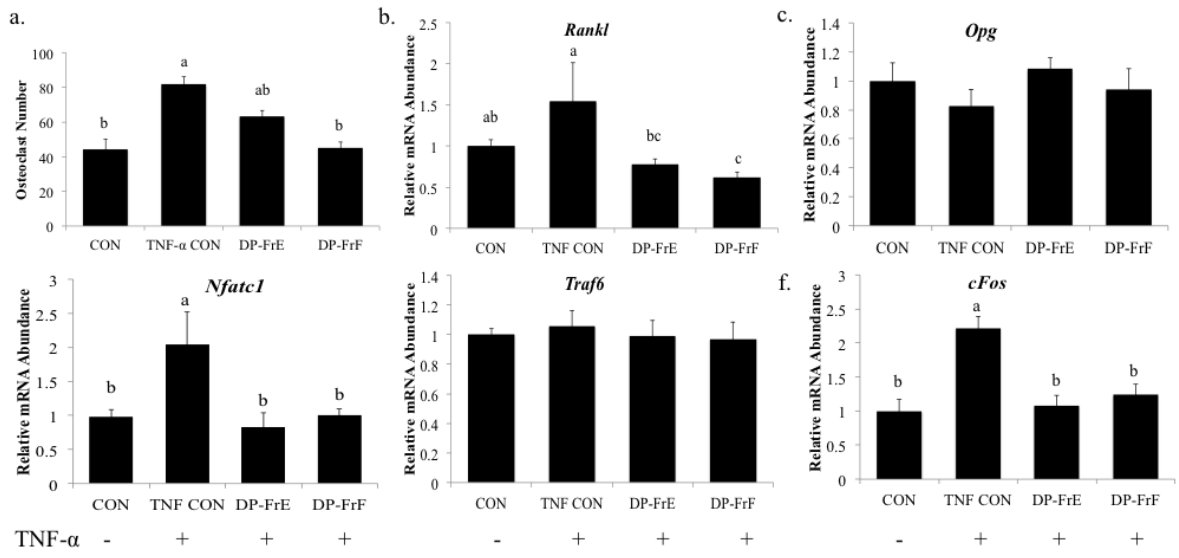| RANKL | + | + | + | + | + | + |
TNF-α	-	+	+	+	+	+
DP-FrE (µg/ml)	-	-	1	10	-	-
DP-FrF (µg/ml)	-	-	-	-	1	10

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Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

CHAPTER IV

FRACTIONS OF A POLYPHENOLIC EXTRACT OF DRIED PLUM ENHANCE BMP
SIGNALING AND MINERALIZATION ACITIVITY OF OSTEOBLASTS

Authors: Jennifer L. Graef and Brenda J. Smith

Abstract:

Dried plum supplementation has been shown to increase bone formation *in vivo* and in
clinical studies. A crude ethanol polyphenolic extract from dried plum has been found to
enhance bone formation, but it is unclear if certain types of polyphenolic compounds are
responsible for stimulating osteoblast activity. This study was designed to examine the
most bioactive components of the total polyphenolic extract of dried plum in enhancing
osteoblast activity and function, and to examine mechanisms involved. Increasing
methanol concentration was used to yield six polyphenolic fractions from the crude
polyphenolic extract of dried plum. Initially, the fractions were screened based on their
capacity to increase ALP activity and mineralized nodule formation in MC3T3-E1 cells,
followed by a series of experiments using primary bone marrow-derived osteoblasts to
confirm this bioactivity and assess mechanisms of action. The fractions with the lowest
organics content (DP-FrA and DP-FrB) enhanced osteoblast activity and function under
normal conditions as indicated by an increase in extracellular ALP activity and
mineralized nodule formation, but to a lesser extent under inflammatory conditions.
Under normal conditions, both fractions increased *Bmp2* gene expression. DP-FrB was

most effective at upregulating *Tak1* and *Smad1*, as well as protein levels of phospho-p38. Under inflammatory conditions, TNF-α upregulated the gene expression of *Smad6,* resulting in a suppression of BMP signaling, and neither fraction was able to attenuate this response. In conclusion, this study identifies fractions of the polyphenolic extract of dried plum that upregulate osteoblast activity and this response results from the upregulation of BMP signaling and *Runx2* expression. Treatment with DP-FrA and DP-FrB increased mRNA expression of *Bmp2*. Furthermore, DP-FrB, and to a lesser extent DP-FrA, upregulated expression of *Tak1* and *Smad1*, as well as p38 activation, within the BMP signaling cascade. In conclusion, this study identifies fractions of the polyphenolic extract of dried plum that upregulate osteoblast activity in primary bone marrow-derived osteoblasts under normal conditions, and to a less extent, under inflammatory conditions. Osteoblast activity is enhanced by these fractions via upregulation of BMP signaling and *Runx2* expression. Since osteoporosis often occurs as a result of inflammation, further investigation is needed to determine whether a higher dose of DP-FrA or DP-FrB, or perhaps a combination of polyphenolic fractions, is able to significantly improve osteoblast activity and function under inflammatory conditions.

Introduction

Maintenance of a healthy skeleton requires the continual remodeling of bone throughout adult life, with the removal of bone tissue by osteoclasts and the formation and mineralization of new bone matrix by osteoblasts [1]. Bone loss occurs when there is an imbalance in the rate of bone formation compared to the rate of bone resorption. Currently, most of the FDA-approved osteoporosis treatments demonstrate primarily anti-resorptive activity by decreasing the differentiation of osteoclasts or inducing osteoclast

apoptosis [2]. In contrast, strontium ranelate, a drug with anabolic and anti-catabolic properties is currently approved for use in European countries by not the United States [3]. Furthermore, the FDA-approved treatment teraperatide, a synthetic fragment of parathyroid hormone, is known to promote bone formation and result in increases in bone mineral density and strength [4]. The relatively prohibitive cost and unknown long-term efficacy and safety of these pharamacological agents results in a need for development of other anabolic therapeutic options.

Typically, an increase in bone formation results from an increase in osteoblast number or their activity. Osteoblast differentiation is initiated by bone morphogenetic protein (BMP) signaling [5]. The activation of BMP type I receptors by the binding of osteogenic BMPs (e.g., BMP2 and BMP4) induces osteoblast differentiation by activating the Smad 1/5/8 complex and the MAP3K TGF-β-activating kinase 1 (TAK1) [5-7]. Activated Smad1/5/8 induces the expression of runt-related transcription factor 2 (Runx2), an essential osteogenic transcription factor that induces the expression of osteoblast-related genes, such as type I collagen and osteocalcin [6, 8]. Activation of TAK1 initiates a MAPK signaling cascade that results in the activation of p38, which then phosphorylates and enhances the activity of Runx2 [7]. Runx2 induces the expression of genes related to osteoblast differentiation and bone formation, including type 1 collagen and osteocalcin, by binding to their promoter regions and activating transcription [8]. The ability of Runx2 to induce osteogenic gene expression requires both activated Smad and p38 signaling. Conversely, inhibition of extracellular regulated kinase (Erk) promotes osteoblast differentiation and matrix mineralization, indicating phase specific roles for MAPK proteins [9].

Osteoblast differentiation and activity is suppressed in diseases of inflammation that lead to bone loss. Inflammation such as that occurring from periodic increases in TNF-α observed early in estrogen deficiency or with flares in rheumatoid arthritis, negatively affects bone formation [10-15]. Differentiation of osteoblasts is inhibited by TNF-α due to a reduction of the expression and stability of Runx2 [11, 13-15]. This is due, in part, to TNF-α induced inhibition of BMP signaling via activation of NF-κB and upregulated expression of BMP-inhibitory Smad6, both of which result in inhibition of BMP-induced Runx2 expression [14, 15]. In addition to inhibiting the differentiation of osteoblasts, TNF-α also induces apoptosis of mature osteoblasts in a caspase 8-dependent manner [10, 12]. Therefore, TNF-α negatively regulates bone formation by inhibiting the differentiation of osteoblasts, as well as by inducing apoptosis of mature osteoblasts.

Consumption of dried plums has been found to improve bone health in aged models of osteoporosis as well as in gonadal hormone deficient models of osteoporosis, in which bone loss is attributed, at least in part, to an increase immune cell activation or inflammation [16-18]. Dried plum supplementation prevented from bone loss in estrogen-deficient C57BL/6 mice, and this was partially attributed to a reduction in OVX-induced immune cell activation, as evidence by attenuated TNF-α production *ex vivo* from stimulated splenocytes [19]. Restoration of trabecular bone in osteopenic, OVX Sprague Dawley rats was observed with dried plum consumption, and histomorphometic analyses indicated a slight increase in endocortical mineral apposition rate (MAR) [18]. Furthermore, in aged, osteopenic C57Bl/6 male mice, dietary supplementation with dried plum increased trabecular bone volume and cortical thickness, and this was attributed to a biphasic response to dried plum, in which initially (4 weeks) both bone resorption and

bone formation was suppressed, but following 12 weeks of supplementation, bone formation rebounded [17]. In a model of gonadal hormone deficiency in males, dried plum restored trabecular bone to a similar extent as that of intermittent PTH in osteopenic, orchidectomized (ORX) Sprague Dawley rats [16]. These studies suggest that dried plum supplementation may improve bone health by enhancing bone formation in addition to suppressing bone resorption.

Currently, the bioactive components of dried plum responsible for the anabolic effects observed in bone are not known. Compositional analyses reveal that dried plums are a good source of potassium, Vitamin K and boron, as well as non-digestible carbohydrates [20-22]. Dried plums are also a rich source of polyphenolic compounds, especially chlorogenic acid isomers, and much of the focus on dried plum's bioactive components has focused on these compounds [23]. A recent study in aged, ovarian hormone deficient Sprague Dawley rats showed that dietary supplementation with a crude ethanol extract of dried plum's polyphenols resulted in restoration of BMD and vertebral trabecular bone to a similar level as that of dried plum supplementation (*unpublished data*). Furthermore, *in vitro* studies demonstrated that this extract induced osteoblast activity as indicated by ALP production and increased mineralization in the murine calvarial pre-osteoblast MC3T3-E1 cells [24]. The polyphenolic extract also attenuated the negative effects of TNF-α on osteoblast differentiation and activity. These studies suggest that polyphenolic compounds within dried plum can improve bone formation, even in an inflammatory environment. However, whether there are certain types of compounds within the polyphenolic extract that are responsible for these effects

and the mechanisms by which they enhance osteoblast activity and mineralization capacity are not known.

The bioactivity of the polyphenolic compounds found in plants has largely been attributed to antioxidant and anti-inflammatory capabilities [23, 25-27]. Green tea polyphenols have been found to protect against bone loss in *in vivo* models of chronic inflammation [28, 29]. The phenolic compound, oleuropein, in olive oil and the flavonoid, phlorizin, rich in apples, have each been shown to prevent inflammation-induced bone loss in estrogen deficiency models [30-32]. In addition to their anti-inflammatory properties, plant-derived polyphenolic compounds have been reported to affect signaling pathways that induce osteoblast differentiation and activity [33, 34]. The flavanone hesperitin, in citrus fruits, induced differentiation of rat primary osteoblasts *in vitro* by stimulating the BMP signaling pathway [34]. Myricetin, a flavonol commonly found in various edible plants, including walnuts, onions, berries and red grapes, upregulated ALP activity and type 1 collagen and osteocalcin protein, resulting in increased mineralization, in *in vitro* models of human osteoblasts [33]. This stimulation of osteoblast differentiation and activity was attributed to upregulation of the BMP signaling pathway and activation of Smad 1/5/8 and the mitogen-activated protein kinase (MAPK) p38 [33]. The ability of these polyphenolic compounds to enhance osteoblast differentiation activity suggests that foods rich in these compounds may be able to enhance bone formation.

Dried plum is rich in polyphenols, including the phenolic acids chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, caffeic acid and *p*-coumaric acid, as well as the flavonol glycoside rutin [23]. Based on the evidence from previous animal and in

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vitro studies, it is possible that polyphenolic compounds in dried plum improve osteoblast activity by stimulating BMP signaling and enhancing mineralization activity. The purpose of this study was to begin to examine which polyphenolic compounds in dried plum are the most bioactive in increasing osteoblast differentiation and function and to assess whether BMP signaling pathways are altered in response to treatment with these phenolic compounds. Due to the role of the inflammatory response in the inhibition of bone formation, a series of studies was designed to investigate the osteoblast response under normal and inflammatory conditions.

Methods

Isolation of phenolic fractions from dried plum

Fractions of polyphenolic compounds were derived from a crude polyphenolic extract of dried plum powder (California Dried Plum Board) based on solubility. In short, dried plum powder (500 g) was dissolved in 80% methanol and sonicated under nitrogen gas flow for 20 minutes to derive a total polyphenolic extract, rich in both polyphenols and carbohydrates. Following filtration, the powder was rinsed from the filter and 80% methanol was added and the sonication repeated. The total polyphenolic extract was subjected to column chromatography using 300 g of HP-20 resin. The HP-20 resin was washed five times with 200 ml of deionized (DI) H_2O to remove carbohydrates. Next, the HP-20 resin was washed five times with 200 ml of methanol to elute a polyphenol-rich extract, and this extract was again subjected to column chromotagraphy using 200 g of HP-20 resin. The weights of six semi-purified polyphenolic fractions eluted from the resin with increasing concentrations of methanol (0 – 100%) are as follows: DP-FrA,

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17.85 g; DP-FrB, 4.42 g; DP-FrC, 3.22 g; DP-FrD, 4.14 g; DP-FrE, 2.16 g; and DP-FrF, 1.39 g.

Screening the bioactivity of the polyphenolic fractions in enhancing osteoblast activity and function

A commercially available pre-osteoblastic cell line (MC3T3-E1; RIKEN, Japan) was used to screen the effects of the fractions on osteoblast activity and function. Murine calvarial pre-osteoblastic cells were seeded at a density of 2.5 x 10^5 in a 24-well plate in alpha-modified minimum essential medium (α-MEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 2 mM L-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich). To assess dosage limitations, proliferation assays (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) utilizing a dose response of the polyphenolic fractions were completed to determine doses that did not negatively affect cell growth (*data not shown*).

The bioactivity of the fractions in enhancing osteoblast activity was screened by measuring extracellular ALP production and mineralization capacity in MC3T3-E1 cells. The cells were plated at a density of 2.5 x 10^5 in a 24-well plate in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. When the cells reached ~95% confluence, 25 μg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO) were added to the complete α-MEM described above to induce osteogenesis. The media was replaced every 2-3 days until the designated endpoints. To examine the dose-dependent effects of the phenolic fractions on osteoblast activity and function, extracellular ALP and nodule formation were assessed under

normal conditions. Extracellular ALP was analyzed in the media using a fluorometric

assay kit (Biovision, Milpitas, CA) following 7 days of MC3T3-E1 treatment with

osteogenic media and phenolic fractions. Briefly, the conditioned media was incubated

with a 4-methylumbelliferyl phosphate disodium salt (MUP) substrate, and ALP within

the media cleaves a phosphate group on the MUP substrate, resulting in a fluorescent

signal measured at an excitation wavelength of 360 nm and an emission wavelength of

440 nm. The 7-day time point was selected because ALP production has been reported to

peak in MC3T3-E1 cells between days 7-14 of treatment with ascorbate [35].

Extracellular ALP was expressed relative to the control cells not treated with phenolic

compounds. Mineralization was assessed as an indicator of osteoblast function in

response to the different fractions of the dried plum extract. Alizarin red S staining, in

which alizarin red S forms a chelate with calcium cations, was used to assess

mineralization activity of MC3T3 cells following 28 days of culture in osteogenic media

and treatment with phenolic fractions. Briefly, the media was removed and cells were

fixed with 10% neutral buffered formalin (NBF) for 15 minutes and then incubated in 40

mM of alizarin red S (pH 4.2) for 20 minutes at room temperature. Excess stain was

washed from the wells with DI water and the alizarin red S stain was eluted from the

mineralized nodules with 10% acetic acid and heated to 85°C for 10 minutes. Samples

were kept on ice prior to centrifugation to remove cellular debris. The pH of the samples

was neutralized with the addition of 150 μl of 10% ammonium hydroxide, and alizarin

red S was quantified in the supernatant by assessing the absorbance at a wavelength of

405 nm and using a standard curve. The fractions and doses that resulted in the greatest

increase in ALP activity and nodule mineralization were identified from these screening

assays (10 µg/mL of DP-FrA and DP-FrB), which were used in all subsequent experiments with primary osteoblasts.

ALP activity and mineralization capacity in primary osteoblasts

To prepare primary osteoblast cultures, bone marrow was flushed from 4-week-old C57Bl/6 female mice. All animal procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee. Bone marrow cells were cultured in α-MEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 1% penicillin/streptoymycin in 75 mm flasks for two days. The media was then removed and adherent cells were trypsinized with 0.25% Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) and collected. The cells were seeded at a density of 5×10^5 in a 24-well plate and maintained in the supplemented α-MEM until reaching ~95% confluence after which 50 µg/ml ascorbic acid and 3 mM β-glycerophosphate was added to the α-MEM to induce osteoblastic differentiation. Media was replaced every 2-3 days until designated study end points. Cells were allowed to differentiate in osteogenic media for 7 days prior to any treatment with DP-FrA or DP-FrB (10 µg/ml) with or without TNF-α (1 ng/ml) to study the osteoblast response to treatments under normal or inflammatory conditions.

To determine the effect of treatment on osteoblast activity and function, extracellular ALP and calcified nodule formation was assessed. Following 3 and 7 days of treatment with DP-FrA or DP-FrB with 0 or 1 ng/ml TNF-α, extracellular ALP was analyzed in the media using a fluorometric assay kit (Biovision, Milpitas, CA), as described above. To assess nodule formation of primary osteoblasts following 14 days of treatment with DP-FrA or DP-FrB (10 µg/ml) with or without TNF-α (1 ng/ml), von

Kossa staining was utilized. Due to non-specific staining known to occur with von Kossa in MC3T3-E1 cultures, alizarin red S staining was used to assess mineralization in those cultures [36]. However, von Kossa staining, in which silver ions bind the anions of calcium salts (e.g. phosphate, carbonate, or sulfate), is an accepted measurement of calcified nodules in osteogenic primary cultures, and was therefore used in these experiments. Briefly, cells were fixed for 5 minutes at room temperature in 10% NBF prior to incubating in 5% silver nitrate for 20 minutes under an ultraviolet light. The cells were then washed and incubated in 5% sodium thiosulfate for 3 minutes, followed by counterstaining with Nuclear Fast Red solution for 5 minutes. The cells were then rinsed and allowed to dry at room temperature prior to imaging using CellSens software (Olympus Life Science, Center Valley, PA). Mineralization area was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Western blotting

Primary bone marrow-derived osteoblasts were cultured as described previously. Because of the more diverse and uncommitted cell populations in the bone marrow cultures compared to the more purified and committed pre-osteoblastic MC3T3-E1 cells, the cells were differentiated in osteogenic media for 7 days prior to treatment with DP-FrA or DP-FrB. Total protein was harvested after 15 minutes, 1 hour, or 4 hours of treatment by washing the cells in sterile PBS and adding radioimmunoprecipitation assay (RIPA) buffer (Life Technologies, Carlsbad, CA) with protease inhibitors (Cell Signaling Technology, Danvers, MA) directly to the wells. Cells were dislodged from the well surface with a rubber cell scraper prior to collection. Sonication and vortexing were

alternately done in 5 minute increments for a total of 60 minutes to ensure proteins were

liberated from cells. Following the final vortex, the cells were centrifuged at 16,000 x g

for 10 minutes to remove cellular debris and the supernatant was collected. Protein

concentration was determined using the bicinchoninic acid assay (BCA) assay.

Expression of proteins of interest were analyzed by separating 20 µg of total protein on a

denaturing sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then

transferring to a polyvinylidine fluoride (PVDF) membrane. Equal transfer of samples

was confirmed with Ponceau staining prior to blocking the membrane with either 5%

nonfat milk or 5% bovine serum albumin (BSA) for one hour. The membrane was then

incubated at 4°C overnight with antibodies (Cell Signaling Technology, Danvers, MA) to

p38, phospho-p38, p44/42 (Erk 1/2), or phospho-p44/42 (phospho-ERK1/2). Actin (Santa

Cruz) was utilized as a loading control. Following an overnight incubation with primary

antibody, the membranes were washed and incubated with secondary antibody for 1 hour

prior to signal detection using SuperSignal West (ThermoFisher) chemiluminescent

substrate. The blots were developed using the ProteinSimple Fluorchem R (San Jose, CA)

and the density of the bands was assessed using UN-SCAN-IT gel analysis software (Silk

Scientific Inc, Orem, UT).

Gene expression analyses

For gene expression analyses, primary bone marrow derived osteoblasts were

cultured for 7 days in osteogenic media and then treated with DP-FrA or DP-FrB, with or

without TNF-α. Total RNA was harvested after 15 minutes, 1 hour, or 24 hours of

treatment by washing the cells in sterile PBS, adding Trizol (Life Technologies) to the

well and then following the extraction protocol provided by the manufacturer. To ensure complete collection of cells from the well, a rubber cell scraper was used to scrape the cells. The concentration of the RNA was obtained via spectrophotometry (NanoDrop) and the quality of the 28S and 18S rRNA bands was confirmed via agarose gel electrophoresis. Reverse transcription of cDNA was performed using 2 μg of RNA and the relative abundance of mRNA of interest (**Table 1**) via real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR; Applied Biosystems, Foster City, CA) using SYBR Green technologies (Life Technologies, Carlsbad, CA). The comparative cycle threshold (C_T) method (User Bulletin #2, Applied Biosystems, Foster City, CA) was used to evaluate mRNA expression levels, using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as an endogenous control.

Identifying phytochemicals present in DP-FrA nd DP-FrB

The presence of 14 phytochemicals known to be present in dried plum (**Table 2**) were was determined in DP-FrA and DP-FrB using liquid chromatography-mass spectrometry (LC/MS) methods previously described [31][32]. Briefly, compounds detected via MS in the fractions were compared to known standards of the phytochemicals to identify the presence of any of the compounds in the fractions. Quantification of the detected compounds was done using a standard curve derived from the pure standard of the compound.

Statistical analyses

All statistical analyses were performed using SAS Version 9.3 (SAS Institute, NC). For data not normally distributed, log transformation was completed prior to

statistical analysis. For the screening assays with MC3T3-E1 cells, polyphenolic fraction treatments, including an untreated control, were compared using ANOVA with Bonferroni adjustment, due to the number of comparisons, when the overall ANOVA is p < 0.05. For all other assays, the effect of polyphenolic fraction treatment at a given dose was analyzed using ANOVA and Fisher's least significant difference (LSD) post hoc analyses. Each experiment was repeated 2-3 times. Values are expressed as means ± standard error (SE).

Results

Identifying the fractions that improve osteoblast activity in MC3T3-E1 cells

To identify the fractions and doses of the dried plum phenolic fractions with the greatest effect on osteoblast activity, extracellular ALP activity was assessed in the media of MC3T3-E1 cells. Extracellular ALP activity was upregulated by DP-FrB ($p < 0.05$) ~2-fold compared to control with treatment at both the 5 μg/ml and 10 μg/ml dose (**Figure 1a**). Treatment with DP-FrA resulted in a 19.5% increase in ALP activity with a treatment dose of 10 μg/ml, but did not differ statistically from control. No other phenolic fractions improved extracellular ALP production compared to control.

Based on the results of the ALP assay in which DP-FrA and DP-FrB appear to enhance ALP activity most at the 10 μg/ml dose, the effects of 10 μg/ml of the polyphenolic fractions on MC3T3-E1 cell function was evaluated based on Alizarin red staining. Following 28 days of culture in osteogenic media, treatment with both DP-FrA and DP-FrB increased mineralized nodule formation ($p < 0.05$) compared to the control. The magnitude of response was greater in DP-FrA than DP-FrB treated cultures (**Figure**

1b). DP-FrE treatment significantly decreased mineralized nodule formation compared to control and no other treatments altered mineralization.

The results of these screening assays using MC3T3-E1 cells revealed that DP-FrA and DP-FrB at a dose of 10 µg/ml had the greatest potential for increasing osteoblast activity and function. Therefore, the focus of all subsequent experiments was on understanding how DP-FrA and DP-FrB altered primary osteoblasts under normal and inflammatory conditions and the mechanism through which these responses were mediated.

DP-FrA and DP-FrB enhance ALP activity and mineralization in murine primary bone marrow-derived osteoblasts

The ability of DP-FrA and DP-FrB to enhance osteoblast activity was assessed after 3 and 7 days of treatment. At day 3, ALP activity was significantly increased (i.e., ~2-fold) by both DP-FrA and DP-FrB compared to the control (**Figure 2a**). ALP continued to be elevated in response to DP-FrA and DP-FrB at day 7 compared to the control, but to a lesser extent (i.e., ~1.5-fold). To determine if the increase in ALP translated to improved, mineralized nodule formation, Von Kossa staining was performed following 14 days of treatment. Representative images of von Kossa staining are shown in **Figure 2b**. Both DP-FrA and DP-FrB significantly increased mineralized nodule formation in bone marrow-derived osteoblasts (**Figure 2c**).

Gene expression analyses were performed to assess alterations in the relative abundance of genes that are involved in osteoblast differentiation and mineralization in response to the polyphenolic fractions. Following 1 hour of treatment, DP-FrA and DP-FrB both increased *Bmp2* mRNA expression, which stimulates osteoblast differentiation

by initiating the BMP/Smad signaling cascade, compared to control (**Figure 2d**). By 24 hours post-treatment with the phenolic fractions, *Bmp2* expression normalized to that of the control. Likewise, expression of *Runx2,* a target of BMP/Smad signaling and an essential transcription factor for activation of genes related to osteoblast differentiation was upregulated by both DP-FrA and DP-FrB compared to control following 1 hour of treatment, and normalized by 24 hours post-phenolic fraction treatment (**Figure 2e**). In addition, *Bsp*, a phosphoprotein that promotes the mineralization of the extracellular matrix by interacting $\alpha_v\beta_3$ integrins on osteoblasts, was upregulated by DP-FrA following 1 hour of treatment and by DP-FrB following 24 hours of treatment (**Figure 2f**). Similarly, *Phex*, which promotes matrix mineralization by cleaving the inhibitory serine- and arginine-rich peptide of osteopontin that binds tightly to calcium, was also upregulated by DP-FrA after 1 hour of treatment and by DP-FrB after 24 hours of treatment (**Figure 2g**). These data show that the dried plum phenolic fractions can increase bone formation by inducing the gene expression of regulators of differentiation and mineralization activity of osteoblasts.

BMP and MAPK signaling is enhanced by phenolic fractions

Next, gene expression was assessed to examine whether activation of BMP signaling in primary bone marrow-derived osteoblasts could explain the upregulation of *Runx2* and osteoblast activity. Following 15 minutes of treatment with the phenolic fractions, gene expression of *Tak1*, a kinase that activates MAPK signaling cascades and also phosphorylates Smad1 protein, was upregulated by DP-FrB compared to control (**Figure 3a**). Expression of *Smad1*, a transcription factor that induces osteoblast

differentiation, was also upregulated by DP-FrB compared to control (**Figure 3b**).

Treatment with Dp-FrA resulted an upregulation of *Smad1* that did not reach the

magnitude of response of that of DP-FrB. *Smad5* gene expression was not altered by

treatment with either dried plum phenolic fraction (**Figure 3c**).

To assess whether proteins within the osteogenic BMP signaling cascade were

altered with treatment with DP-FrA and DP-FrB, phosphorylation of p38, Erk, and

Smad1/5 were examined over time. Representative blots are shown in **Figure 3d**. While

15 minutes of treatment with DP-FrB did not alter activation of p38, which stimulates

ALP production and matrix mineralization, 1 hour of treatment increased

phosphorylation of p38 (**Figure 3e**). Similarly, treatment with DP-FrA did not alter p38

phosphorylation following 15 minutes of treatment, but enhance phosphorylation of p38

after 1 hour of treatment, although not to the same degree as DP-FrB. By 4 hours post-

treatment, p38 phosphorylation was not different in cells treated with DP-FrA or DP-FrB

compared to control. Interestingly, activation of Erk1/2, which is essential for pre-

osteoblast proliferation and osteoblast differentiation, but negatively regulates bone

mineralization, was decreased after 1 hour of treatment with both DP-FrA and DP-FrB

(**Figure 3f**). Similar to the phospho-p38 response, phosphorylation of Erk1/2 was not

altered by DP-FrA or DP-FrB after 15 minutes or 4 hours of treatment. Finally, the

upregulation of phosphorylation of Smad1/5 was observed following 15 minutes of

treatment with both DP-FrA and DP-FrB (**Figure 3g**). These findings demonstrate that

BMP signaling is enhanced with treatment of the polyphenolic fractions.

Effects of phenolic fractions on TNF-α-treated primary bone marrow-derived osteoblasts

To examine whether DP-FrA and DP-FrB could induce bone formation in osteoblast under inflammatory conditions, cells were treated with the phenolic fractions in the presence of TNF-α. Following 3 and 7 days of treatment, TNF-α suppressed extracellular ALP production compared to an untreated control (**Figure 4a**). Treatment with dried plum phenolic fractions did not improve extracellular ALP compared to TNF-α treated control. Furthermore, treatment with TNF-α suppressed mineralization and, neither DP-FrA nor DP-FrB did not significantly reverse this suppression as indicated by the representative von Kossa staining (**Figure 4b)**. Quantification of mineralization area showed a trend ($p = 0.0674$) for increased mineralization with polyphenolic fraction treatment, but neither DP-FrA or DP-FrB were able to significantly improve mineralization in the presences of TNF-α (**Figure 4c**). This data demonstrates that while the phenolic fractions were not able to rescue osteoblast ALP activity from the detrimental effects of TNF-α at the dose studied, there was a trend for improved osteoblast function.

To explore why DP-FrA and DP-FrB did not significantly improve osteoblast activity and function under inflammatory conditions, regulators of BMP signaling were examined in primary bone marrow-derived osteoblasts treated with TNF-α and the polyphenolic fractions. Treatment with TNF-α for 1 hour did not affect transcription of *Tak1*, *Smad1*, or *Smad5* (**Figure 4d-f**). However, mRNA expression of *Smad6*, an inhibitor of BMP/Smad signaling, was upregulated by TNF-α, and neither DP-FrA or DP-FrB were able to attenuate this response (**Figure 4g**). Furthermore, *Bmp2* expression was suppressed by TNF-α, and DP-FrA and DP-FrB could not reverse this suppression

(**Figure 4h**). *Runx2* expression was unaltered following 1 hour of treatment with TNF-α and the dried plum phenolic fractions (**Figure 4i**).

Identification of phenolic acids present in DP-FrA and DP-FrB

Both DP-FrA and DP-FrB were analyzed for the presence of some of the polyphenols previously reported in dried plum extracts. DP-FrA and DP-FrB both contained cryptochlorogenic acid and neochlorogenic acid (**Table 2**) and both fractions contained a greater percentage of neochlorogenic acid than cryptochlorogenic acid (**Figure 5**). Chlorogenic acid, caffeic acid, quinic acid, o-coumaric acid, m-coumaric acid, ferulic acid cyanidin 3-rutinoside, cyanidin 3-glucoside, quercetin, rutin, sorbic acid, and 5-hydroxymethyl-2-furaldehyde were not detected in either fraction. This data indicates that neochlorogenic acid and cryptochlorogenic acid may be, at least in part, responsible for the beneficial effects of dried plum on osteoblast activity and function.

Discussion

Previously, it was demonstrated that a crude ethanol extract of polyphenols from dried plum enhanced activity of osteoblasts derived from MC3T3-E1 cells by upregulating the expression of *Runx2* [24]. In the current study, we identify fractions of the polyphenolic extract from dried plum that induce upregulation of *Runx2* expression in differentiating primary bone marrow-derived osteoblasts. The upregulation of Runx2 occurs as a result of BMP signaling [5]. The BMP receptors, activated in pre-osteoblasts by binding of osteogenic BMPs including BMP2 and BMP4, are serine/threonine kinases that initiate osteoblast differentiation by phosphorylating Smad 1/5/8 and TAK1 [5, 7].

The phosphorylation of Smad1/5/8 induces nuclear translocation of the protein complex, where it acts to induce *Runx2* expression [5]. An upregulation of *Bmp4* expression has been demonstrated in OVX Sprague Dawley rats in response to dried plum supplementation [18]. Furthermore, activation of TAK1 initiates a signaling cascade that results in the phosphorylation and activation of p38 [37]. Activated p38 enhances osteoblast differentiation by phosphorylating Smad1 at the same site targeted by the BMP receptor, thereby amplifying the BMP signaling cascade. Additionally, p38 phosphorylates and activates Runx2 protein, enhancing the activity of this osteogenic transcription factor [5, 37]. The activation of p38 is essential to inducing osteoblast activity, as treatment with a p38 inhibitor results in downregulation of ALP production and mineralized matrix formation in mouse primary calvarial osteoblasts [38]. The upregulation of BMP signaling and the activation of p38 by plant-based polyphenolic compounds have been demonstrated *in vitro* [34, 39-41]. The current study demonstrates an increase in *Bmp2, Tak1* and *Smad1* gene expression, as well as an upregulation in p38 and Smad1/5 phosphorylation, which suggests the polyphenolic fractions enhanced BMP signaling in differentiating bone marrow-derived osteoblasts. Conversely, while the activation of Erk is important in the proliferation of osteoblast precursor cells [42], phosphorylated Erk negatively regulates bone mineralization *in vitro* [43]. In the current study, treatment with both DP-FrA and DP-FrB resulted in a suppression of Erk phosphorylation, indicating that the polyphenolic fractions increase osteoblast mineralization activity, at least in part, by inhibiting Erk.

The differentiation and maturation of osteoblasts results in an increase in mineralized bone formation [44]. In aged, osteopenic male C57Bl/6 mice, a 54% increase

in bone formation rate in trabecular bone of the distal femur metaphysis was observed following 12 weeks of dried plum consumption [45]. In addition, dried plum supplementation resulted in an increase in endocortical mineral apposition rate in OVX Sprague Dawley rats, providing further evidence that dried plum can enhance bone formation *in vivo* [18]. Mature osteoblasts produce collagen, creating an extracellular matrix, as well as non-collagenous proteins, such as bone sialoprotein and osteopontin, in preparation of matrix mineralization [46]. Bone sialoprotein (Bsp) promotes maturation and mineralization of the extracellular matrix via the interaction of an Arginine-Glycine-Aspartic Acid (RGD) motif with $\alpha_v\beta_3$ integrins on osteoblasts [44]. Likewise, osteopontin also contains an RGD binding domain, as well as serine- and arginine-rich peptide (ASARM) with a high affinity for calcium, resulting in tight binding of the protein to hydroxyapatite [47]. The binding of the ASARM peptide of osteopontin to hydroxyapatite functions to inhibit crystal growth, indicating the protein's role in regulating bone mineralization [48, 49]. However, the protease Phex cleaves the ASARM peptide of osteopontin, which eliminates its ability to inhibit mineralization [49]. In the current study, both DP-FrA and DP-FrB upregulated the expression of *Bsp* and *Phex*, suggesting a mechanism for the observed increased mineralization capacity of osteoblasts treated with these polyphenolic fractions.

A decline in osteoblast activity, and therefore bone formation, is observed in conditions of inflammation, including that which occurs with estrogen deficiency and rheumatoid arthritis [11, 14, 15]. The inflammatory cytokine, TNF-α, is known to suppress osteoblast differentiation by reducing the expression and stability of Runx2 [11, 13-15]. TNF-α-upregulates NF-κB signaling, which in turn suppresses BMP signaling by

inhibiting the binding of Smad1/5/8 to the Runx2 promoter region [15]. Furthermore, TNF-α upregulates expression of Smad6, a repressor of BMP-induced Smad signaling, as well as Smad ubiquitination regulatory factor 1 (Smurf1), a ubiquitin ligase that marks Smad1 and Runx2 for proteosomal degradation [11, 13, 14]. Previously, it was demonstrated that a crude ethanol extract of dried plum polyphenols could attenuate the TNF-α-induced decline in osteoblast activity and function using the MC3T3-E1 cell line [24]. Likewise, the crude polyphenolic extract from dried plum reversed bone loss in osteopenic, OVX Sprague Dawley rats (*unpublished data*). In the current study, fractions of the polyphenol extract from dried plum were not able to significantly alter the TNF-α-induced reduction in ALP activity and mineralization capacity in primary bone marrow-derived osteoblasts, although there was a trend for improved mineralization with treatment with the fractions. This is due, at least in part, to the upregulation of inhibitory Smad6 with TNF-α treatment, which neither DP-FrA or DP-FrB was able to attenuate. It's possible that a higher dose of the fractions or a combination of the fractions is needed to protect the differentiating osteoblasts from the detrimental effects of TNF-α. Conversely, the dose of fractions used in this study may be able to protect osteoblasts from a lower dose of TNF-α.

In conclusion, this study is the first to identify fractions of a polyphenolic extract of dried plum that enhance osteoblast activity and function in murine primary bone marrow-derived osteoblasts under normal conditions. The effects are mediated, at least in part, by enhanced BMP signaling and an upregulation of *Runx2*, as well as an increase in gene expression related to mineralization activity of osteoblasts. However, these fractions were not able to significantly alter the TNF-α-induced suppression of ALP and

mineralization activity of differentiating osteoblasts, and this is due, in part, to a suppression of BMP signaling by Smad6. Further investigation is needed to determine whether a higher dose of the fractions would enhance osteoblast activity and function under inflammatory conditions. In addition, because these fractions may contain more than just neochlorogenic acid and cryptochlorogenic acid, further characterization of the components of DP-FrA and DP-FrB is needed to elucidate the bioactive compounds within dried plum. Furthermore, the capacity of these fractions to enhance bone formation *in vivo* is a necessary step in developing a potential treatment option for osteoporosis.

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Table 1. Primer sequences for qRT-PCR.

Transcript	Sequence (5'-3')
Bmp2	F: GGA CAT CCG CTC CAC AAA
	R: GGC GCT TCC GCT GTT T
Runx2	F: TCT ACA GGC CCT GGT TCT
	R: ATG TTC CAC TCT CCT CTT CTC TTG
Bsp	F: ACA CCC CAA GCA CAG ACT TTT G
	R: TCC TCG TCG CTT TCC TTC ACT
Phex	F: GGC ATG ACT GCT GTA AGA TCA GAT
	R: AGC TCC ATT GAC ATA AGG CAC T
Tak1	F: CGT CTT CTG CCA GTG AGA TG
	R: ATC TTT TGC TCT CCA CTT AGC TT
Smad1	F: GCC CAT GGA CAC GAA CAT G
	R: TGA ACA TCT CCT CTG CTG ATT TCA
Smad5	F: AGT GAC AGC AGC ATC TTT GTT CA
	R: GTG GGA TGG AAG CCA TGG T
Smad6	F: CTG TCC GAT TCT ACA TTG TCT TAC ACT
	R: CAT GCT GGC ATC TGA GAA TTC A

Table 2. Assessment of phytochemical content in DP-FrA and DP-FrB.

	mg/100 g total phenolic extract		% of total compounds analyzed	
	DP-FrA	DP-FrB	DP-FrA	DP-FrB
Chlorogenic acid	n.d.	n.d.		
Cryptochlorogenic acid	16.9	86.9	24.0	40.9
Neochlorogenic acid	53.6	125.7	76.0	59.1
Caffeic acid	n.d.	n.d.		
Quinic acid	n.d.	n.d.		
o-Coumaric acid	n.d.	n.d.		
m-Coumaric acid	n.d.	n.d.		
Ferulic acid	n.d.	n.d.		
Cyanidin 3-rutinoside	n.d.	n.d.		
Cyanidin 3-glucoside	n.d.	n.d.		
Quercetin	n.d.	n.d.		
Rutin	n.d.	n.d.		
Sorbic acid	n.d.	n.d.		
5-Hydroxymethyl-2-furaldehyde	n.d.	n.d.		

Figure legends

Figure 1. Dried plum phenolic fractions DP-FrA and DP-FrB improve osteoblast activity and function in MC3T3-E1 cells. Cells were treated with osteogenic media (alpha-MEM + 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, 25 µg/ml ascorbic acid and 10 mM β-glycerophosphate). a) Extracellular ALP was assessed following 7 days of treatment with DP-FrA and DP-FrB (n=6). b) Calcified nodules were stained with alizarin red following 28 days of treatment with osteogenic media and DP fractions (10 µg/ml) and then the stain was eluted and quantified (n=6). Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 2. DP-FrA and DP-FrB induce ALP activity and mineralization in primary bone marrow-derived osteoblasts. Primary bone marrow stromal cells were treated with osteogenic media (alpha-MEM + 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, 50 µg/ml ascorbic acid and 3 mM β-glycerophosphate) and allowed to differentiate for 7 days. Cells were then treated with the polyphenolic fractions on day 7 of differentiation. Extracellular ALP a) was assessed in the media following 3 or 7 days of polyphenolic treatment (10 µg/ml). Mineralized nodules were stained using Von Kossa staining following 14 days of treatment with of DP-FrA and DP-FrB (10 µg/ml). b) Representative wells treated with DP-FrA and DP-FrB show increased von Kossa staining and c) increased the percentage of mineralized area (n=6). RNA was extracted following treatment with DP fractions for one hour or 24 hours. Relative mRNA expression of d) *Bmp2*, e) *Runx2*, f) *Bsp*, and g) *Phex* was assessed using qRT-PCR with *Gapdh* as a control (n=6). Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 3. DP-FrA and DP-FrB upregulate BMP signaling in primary bone marrow-derived osteoblasts. Primary bone marrow stromal cells were treated with osteogenic media and allowed to differentiate for 7 days. Cells were then treated with the DP fractions and RNA was extracted after 15 minutes. Relative mRNA expression of a) *Tak1*, b) *Smad1,* and c) *Smad5* was assessed with qRT-PCR using *Gapdh* as a control (n=6). Protein was extracted following treatment with the phenolic fractions for 15 minutes, 1 hour or 4 hours. d) Representative western blots are shown at each time point. The relative abundance of e) phosphorylated p38, f) ERK, and g) Smad1/5 (n=3) are presented. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 4. DP fractions failed to rescue primary bone marrow-derived osteoblasts from the detrimental effects of TNF-α. Primary bone marrow stromal cells were treated with osteogenic media and allowed to differentiate for 7 days. Cells were then treated with TNF-α (1 ng/ml) and the DP fractions on day 7 of differentiation. a) Extracellular ALP was measured in the media following 3 or 7 days of treatment. Mineralized nodules were assessed using Von Kossa staining following 14 days of treatment with the DP fractions (10 µg/ml). b) Representative wells treated with DP-FrA and DP-FrB show increased

von Kossa staining and c) increased percentage of mineralized area (n=6). RNA was extracted following treatment with DP fractions for 1 hour. Relative mRNA expression of *Tak1* (d), *Smad1* (e), *Smad5* (f), *Smad6* (g), *Bmp2* (h), and *Runx2* (i) was assessed with qRT-PCR using *Gapdh* as a control. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other, $p < 0.05$.

Figure 5. The relative abundance of neochlorogenic acid and crytopchlorogenic acid in DP-FrA and DP-FrB is represented. Bars represent the percentage of detected compounds in each fraction that is neochlorogenic acid or cryptochlorogenic acid.
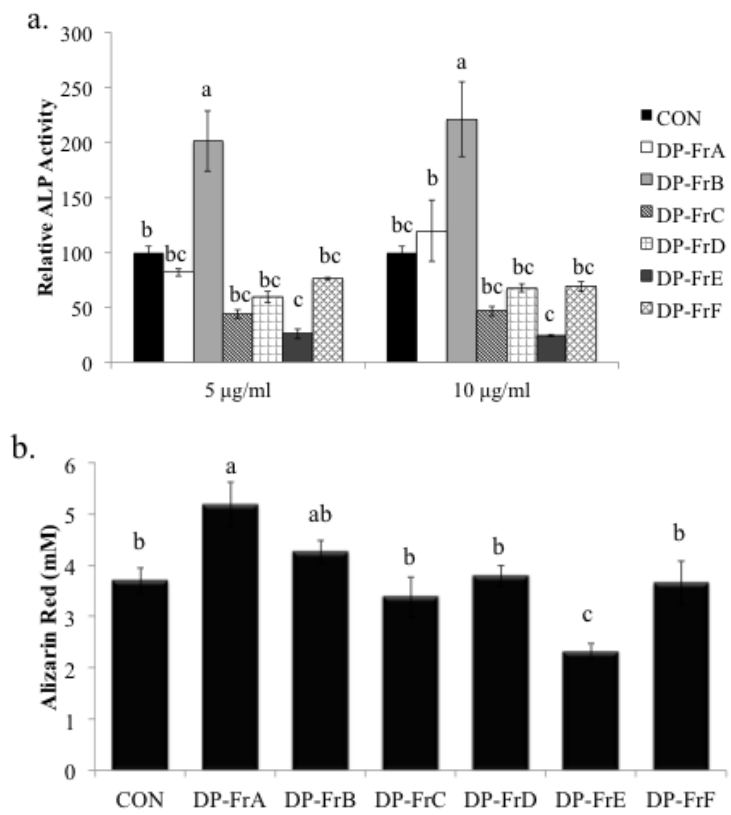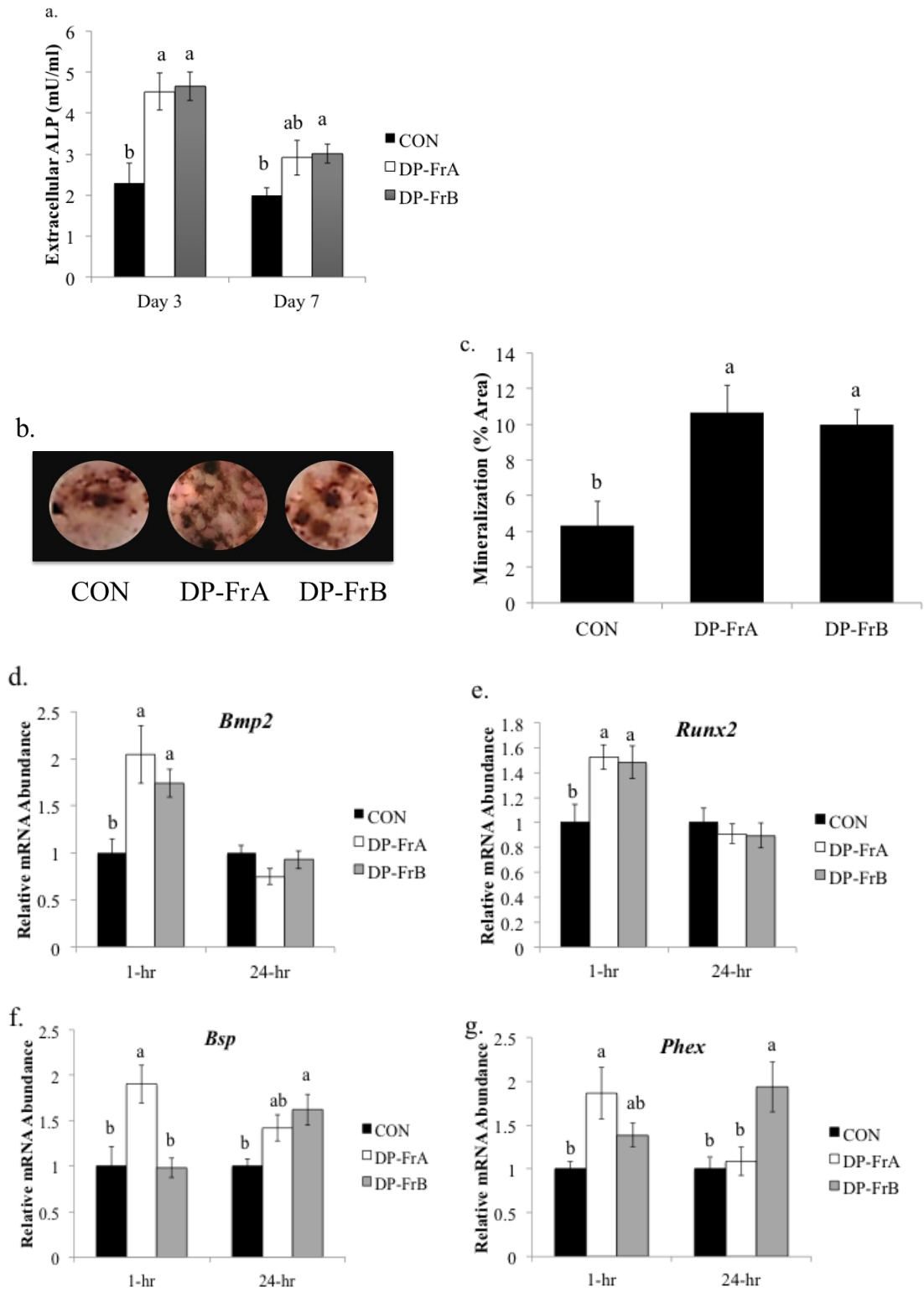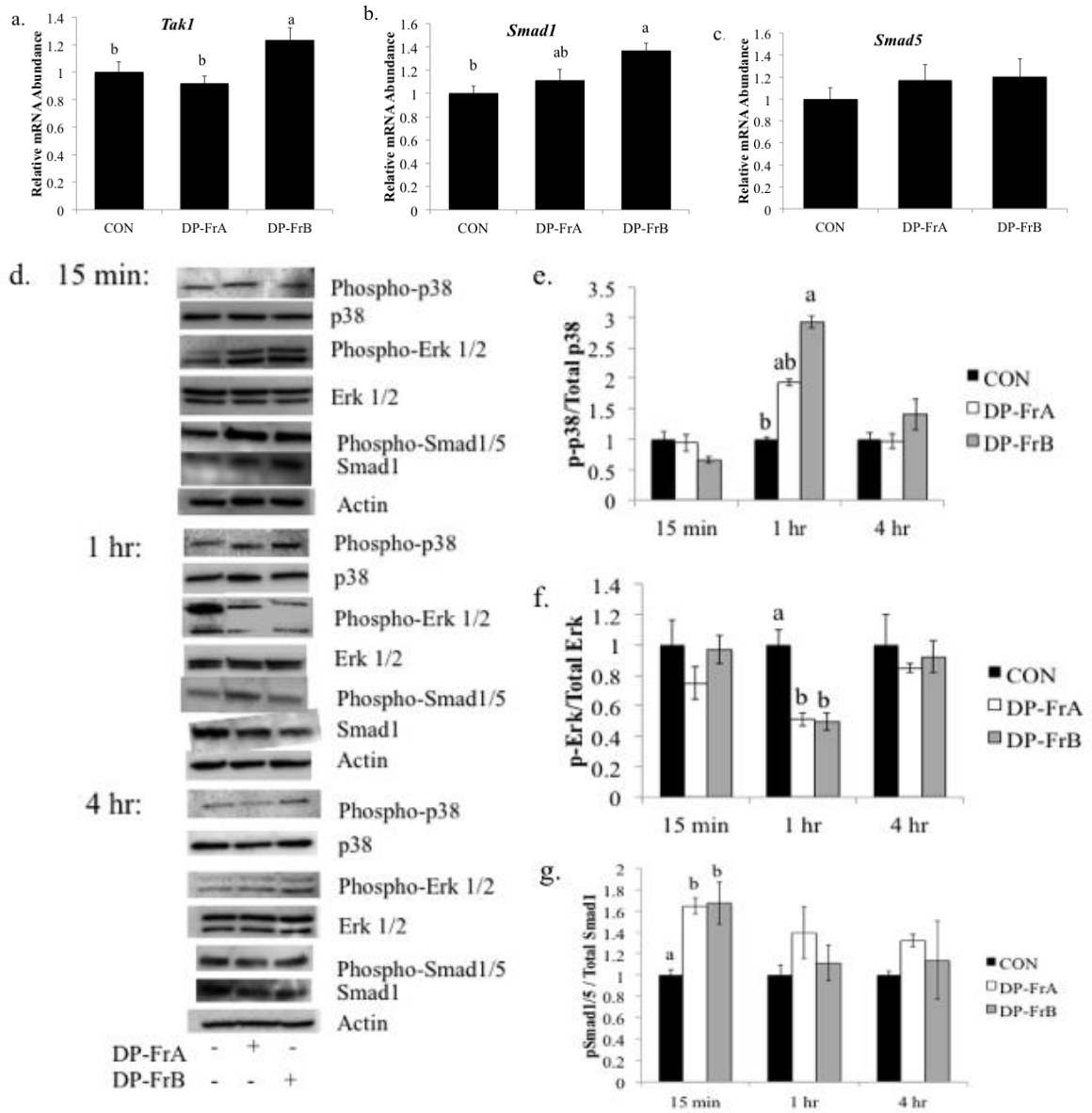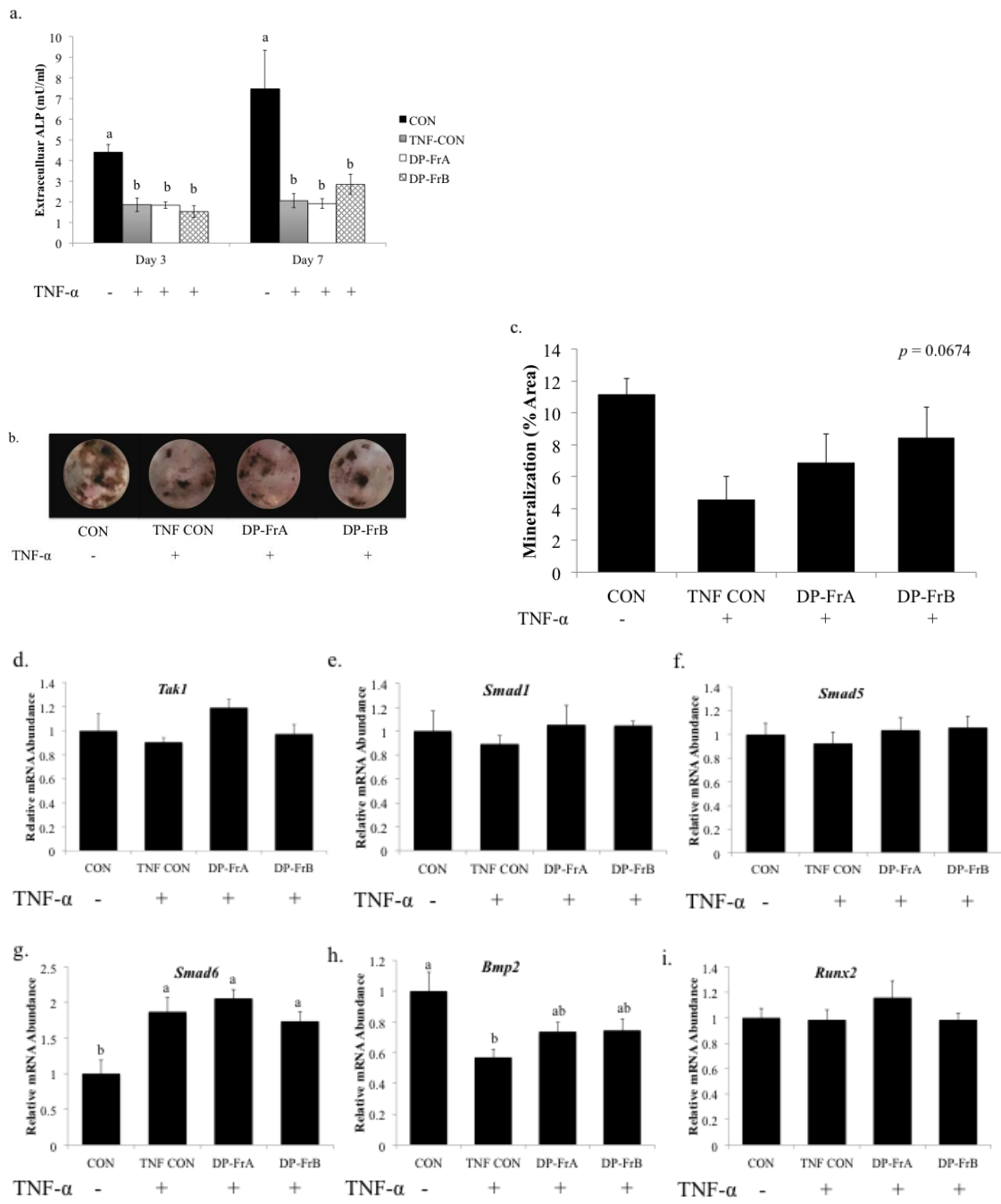
Figure 1.

Figure 2.

Figure 3.

Figure 4.

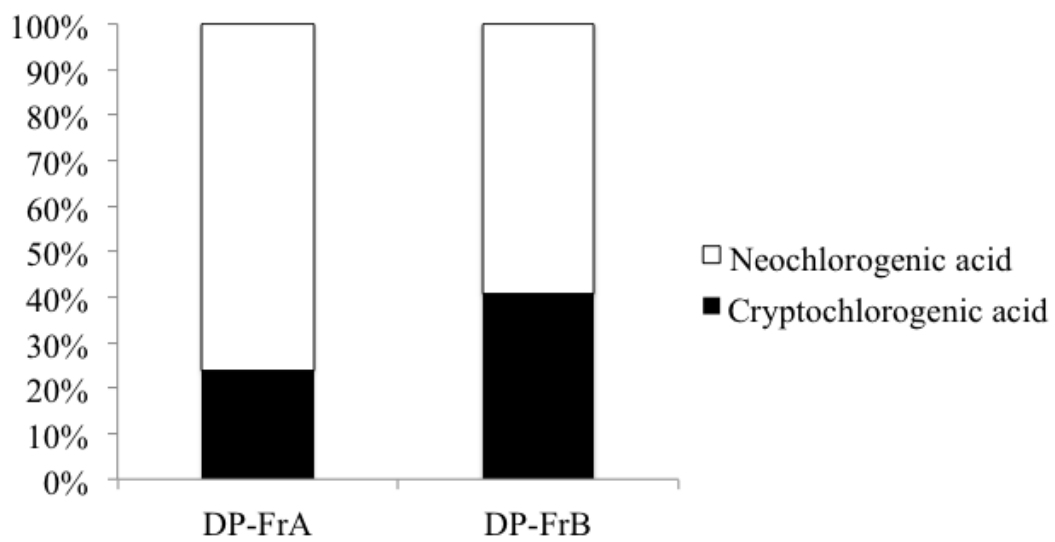Figure 5.

Legend:
□ Neochlorogenic acid
■ Cryptochlorogenic acid

CHAPTER V

SUMMARIES, CONCLUSIONS, AND RECOMMENDATIONS

Previous studies have demonstrated that a crude ethanol extract of the polyphenols in

dried plum restores bone in osteogenic animals and can simultaneously increase

osteoblast activity and decrease osteoclast activity. However, the type of polyphenolic

compounds within the extract and the mechanisms by which they influence bone cell

activity have remained in question. The current studies were conducted to determine

which fraction(s) of the polyphenolic extract were able to positively affect osteoblast and

osteoclast differentiation and activity in primary cell cultures, and the mechanism through

which these effects are mediated. Screening assays revealed that DP-FrE and DP-FrF

downregulated osteoclast differentiation under normal and inflammatory conditions.

These effects on osteoclast differentiation were confirmed in primary bone marrow-

derived osteoclasts in conjunction with a decrease in osteoclast resorption pit formation

resulting from the downregulation of *Nfatc1*, the master regulator of osteoclastogenesis.

Mechanisms by which DP-FrE and DP-FrF downregulated *Nfatc1* expression included

the suppression of Erk1/2 and p38 MAPK signaling, as well as a reduction in intracellular

calcium levels in the differentiating osteoclast. The decreased calcium levels can be

attributed, at least in part, to a downregulation of costimulatory receptors involved in calcium signaling, including OSCAR, TREM2, and SIRPβ1. Importantly, the effects of DP-FrE and DP-FrF on down-regulating Nfatc1 were confirmed in an osteoblast and osteoclast co-culture system. In terms of bone formation, DP-FrA and DP-FrB improved osteoblast activity under normal conditions. Mechanisms by which osteoblast activity were improved included an upregulation of *Runx2*, which is essential to osteoblast differentiation. The upregulation of *Bmp2* mRNA expression indicates that an increase in *Runx2* is at least in part due BMP signaling. Providing further support of enhanced BMP signaling was the increased activation of the MAPK p38, the signaling cascade of which is initiated upon BMP receptor stimulation, with treatment with the fractions. Coinciding with these alterations in BMP signaling, mineralized nodule formation was increased by both DP-FrA and DP-FrB. This is due, in part, to the upregulation of *Bsp* and *Phex* expression*,* both of which are involved in matrix mineralization, by DP-FrA and DP-FrB. However, when the osteoblast precursors were challenged with TNF-α, creating an inflammatory environment, neither DP-FrA or DP-FrB were able to significantly restore osteoblast function, although there was a trend for improved mineralization. These studies demonstrate that fractions of a polyphenolic extract from dried plum improve bone health by suppressing osteoclast differentiation and activity, as well as by increasing osteoblast activity.

Conclusions

Purpose: The purpose of this project was to determine the bioactive component(s) in dried plum responsible for the beneficial effects on bone metabolism and the mechanisms through which these components enhance osteoblast activity and suppress osteoclast activity under normal and inflammatory conditions.

Central Hypothesis: Polyphenolic compounds in dried plum favorably affect bone metabolism by suppressing osteoclast activity and increasing osteoblast activity. These effects are mediated by alterations in calcium and MAPK signaling in osteoclasts and BMP signaling in osteoblasts.

Specific Aim 1: To investigate the effects of different fraction(s) of a dried plum polyphenol extract on osteoclasts *in vitro* under normal and inflammatory conditions and how these effects are mediated.

*Working Hypothesis 1***:** The polyphenolic compounds in dried plum will attenuate the increase in osteoclast differentiation and activity in normal and inflammatory cell culture systems by suppressing calcium and MAPK signaling.

Sub-Aim 1.1: To determine the fraction(s) and dose of the dried plum polyphenol extract that most effectively reduces osteoclastogenesis in murine immortal cell lines.

Preliminary experiments were performed to determine the ability of the polyphenolic fractions to dose-dependently downregulate osteoclast differentiation using the murine immortalized macrophage/monocyte cell line, RAW 264.7 cells. DP-FrA, DP-FrE and DP-FrF most effectively downregulated the number of TRAP$^+$ osteoclasts per well at doses of 1 μg/ml and 10 μg/ml under normal as well as inflammatory conditions. These fractions and doses were then further examined in murine primary bone marrow-derived osteoclast cultures to confirm these findings and to begin to investigate their mechanism of action.

Sub-Aim 1.2: To examine how the active polyphenolic fraction(s) alter osteoclast differentiation (i.e., quantification of TRAP$^+$ cells) and activity (i.e., resorption pit formation) using primary bone marrow-derived osteoclast cultures.

Under normal conditions, only DP-FrE and DP-FrF suppressed osteoclastogenesis. The ability of DP-FrE and DP-FrF to alter osteoclast activity was examined via a resorption pit assay. Both fractions reduced the area of dentin disc resorbed by osteoclasts at doses of 1 and 10 μg/ml indicating that the reduction in osteoclast numbers translated to a decrease in osteoclast activity.

Next, due to the role of TNF-α in upregulating osteoclast differentiation and activity, and therefore bone resorption, the capacity of DP-FrE and DP-FrF to reduce osteoclast number and activity in the presence of TNF-α was investigated. Both DP-FrE and DP-FrF reduced osteoclast number and activity compared to the TNF-α treated control to a level similar to that of the control not treated with TNF-α. These findings

indicate that DP-FrE and DP-FrF can attenuate the TNF-α-induced increase in osteoclast differentiation and activity, making the fractions a promising potential component of prevention or treatment strategies for inflammation-induced bone loss.

Sub-Aim 1.3: To examine the extent to which the active polyphenolic fraction(s) alter key regulators of osteoclast differentiation by way of calcium and MAPK signaling pathways.

To identify potential mechanisms by which DP-FrE and DP-FrF downregulate osteoclast differentiation and activity, calcium and MAPK signaling pathways essential to osteoclast differentiation were examined. Our data demonstrate that the DP-FrE and DP-FrF suppress osteoclastogenesis under normal and inflammatory conditions by downregulating *Nfatc1* in murine primary bone marrow-derived osteoclasts. Both calcium and MAPK signaling play a role in the regulation of *Nfatc1* expression. DP-FrF, and DP-FrE to a lesser extent, suppressed intracellular calcium levels in differentiating osteoclasts under normal conditions. Coinciding with this finding was the downregulation of costimulatory receptors known to play a role in calcium signaling, including OSCAR, SIRPβ1, and TREM2. These findings suggest that Dp-FrE and DP-FrF downregulate osteoclast differentiation, in part, by suppressing the calcium oscillations required for the function of calcium-dependent enzymes that regulate Nfatc1 auto-amplification. In addition to suppressing calcium signaling, the phosphorylation and activation of Erk1/2, was also suppressed by treatment with the fractions. A downregulation of activated Erk1/2 provides an additional mechanism by which the polyphenolic fractions reduce

osteoclast number, as Erk1/2 is known play a role in the proliferation and survival of osteoclasts. These findings demonstrate that DP-FrE and DP-FrF reduce osteoclast numbers, at least in part, by suppressing MAPK and calcium signaling pathways essential to osteoclast differentiation and survival.

Sub-Aim 1.4: To examine how the fraction(s) of the dried plum polyphenolic extract alter osteoclast differentiation using murine primary co-cultures, a system that allows osteoblast and osteoclast interaction, in normal and inflammatory conditions.

While a mono-culture of bone marrow-derived osteoclast cells provides important insights for examining signaling mechanisms involved in osteoclastogenesis, the culture system does not account for the coupled activity of osteoblasts and osteoclasts that occurs *in vivo*. Therefore, to begin to address whether these polyphenolic fractions can reduce osteoclast differentiation in an environment that more closely reflects the bone microenvironment *in vivo*, a co-culture system was used. Under normal conditions, DP-FrE and DP-FrF tended ($p = 0.0530$) to suppress osteoclast differentiation. Under inflammatory conditions, the fractions attenuated the TNF-α-induced increase in osteoclast number, although the magnitude of response was much greater in DP-FrF than in DP-FrE. The reduction in osteoclast number under both normal and inflammatory conditions can be attributed, in part, to a downregulation of *Nfatc1* expression. Furthermore, under inflammatory conditions, suppression of *Rankl* expression suggests there is a decrease in RANKL binding to RANK on osteoclast precursors, which is required to initiate the signaling cascades that upregulate *Nfatc1* expression. Increased

osteoclast differentiation in the presence of TNF-α can be attributed both to the upregulation of RANKL production by osteoblasts and some immune cell populations, as well as enhanced sensitivity of osteoclast precursors to RANKL stimulation. These findings suggest that the fractions may reduce osteoclast differentiation indirectly by influencing the production of RANKL by osteoblasts, as well as by acting directly on osteoclast precursors by downregulating *Nfatc1* expression, similar to the observations in the bone marrow-derived osteoclast mono-cultures.

In summary, fractions of a crude polyphenolic extract of dried plum that suppressed osteoclast differentiation and activity and enhanced osteoblast activity and function were identified. The fractions most effective in reducing osteoclast differentiation and activity exerted these effects, at least in part, by suppressing calcium and MAPK signaling in differentiating osteoclasts. These fractions were able to suppress osteoclast differentiation and activity under both normal and inflammatory conditions, demonstrating their potential as a component of a treatment strategy for inflammatory conditions of bone loss, such as that observed with estrogen deficiency and rheumatoid arthritis.

Specific Aim 2: To investigate the effects of different fraction(s) of a dried plum polyphenol extract on osteoblasts *in vitro* under normal and inflammatory conditions and how these effects are mediated.

Working Hypothesis 2: The polyphenolic compounds in dried plum will enhance osteoblast differentiation and activity by enhancing BMP signaling and altering MAPK signaling in normal and inflammatory cell culture systems.

Sub-Aim 2.1: To determine the fraction(s) and dose of a dried plum polyphenol extract that results in the greatest increase in osteoblast activity (i.e., ALP) and function (i.e., formation of mineralized nodules) in murine immortal cells lines.

The ability of the polyphenolic fractions to increase osteoblast activity and function was screened in the murine immortalized calvarial pre-osteoblast cell line, MC3T3-E1 cells. Six initial polyphenolic fractions were assessed for their effects on osteoblast ALP production and mineralized nodule formation. Two of the fractions, DP-FrA and DP-FrB, increased osteoblast activity and mineralized nodule formation compared to control. Therefore, the bioactivity of these two fractions in enhancing osteoblast activity and function was further assessed in murine primary bone marrow-derived osteoblasts.

Sub-Aim 2.2: To examine how the active polyphenolic fraction(s) alter osteoblast activity (i.e., ALP) and function (mineralized nodule formation) using primary osteoblast cultured under normal and inflammatory conditions.

Based on the results of the screening assay, the ability of DP-FrA and DP-FrB to enhance osteoblast activity and function was assessed in murine primary bone marrow-derived osteoblasts. Under normal conditions, both DP-FrA and DP-FrB increased ALP production and mineralized nodule formation. However, under inflammatory conditions, neither DP-FrA or DP-FrB were able to attenuate the suppressive effect of TNF-α on ALP production and mineralized nodule formation.

Sub-Aim 2.3: To examine the mechanisms (i.e., BMP and MAPK signaling pathways) by which the most bioactive fraction(s) of the dried plum polyphenolic extract increase osteoblast differentiation and activity.

Alterations in BMP and MAPK signaling pathways by DP-FrA and DP-FrB were examined using gene and protein expression analyses to determine the mechanisms by which osteoblast activity is increased. Within one hour of treatment, both fractions significantly upregulated the gene expression of *Bmp2* and *Runx2*. To further examine signaling pathways that could provide explanation for the upregulation of *Runx2* and osteoblast activity, genes and proteins within the BMP signaling pathway were assessed. Within 15 minutes of treatment, gene expression of *Tak1*, which is activated upon BMP receptor stimulation and initiates MAPK signaling within the BMP signaling cascade,

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was upregulated by DP-FrB only. Additionally, gene expression of *Smad1*, which also is activated upon BMP receptor stimulation, was also upregulated by the fractions, although the magnitude of response was greatest with DP-FrB. Providing further evidence of upregulated BMP signaling was the increased phosphorylation of the MAPK p38, the activation of which relies on TAK1 activation. Interestingly, phosphorylation of Erk1/2 was suppressed by treatment of the fractions. However, while Erk1/2 plays an essential role in the proliferation and early differentiation of osteoblast precursors, the activation of Erk1/2 is inhibitory to the maturation and mineralization activity of osteoblasts. In addition to the upregulation of signaling involved in osteoblast differentiation, genes involved in bone mineralization (i.e., *Bsp* and *Phex*) were significantly upregulated by DP-FrA within one hour of treatment and by DP-FrB following 24 hours of treatment.

Under inflammatory conditions, while neither of the fractions was able to attenuate the TNF-α-induced suppression of ALP production, there was a trend ($p =$ 0.0674) for increased mineralized nodule formation with DP-FrA and DP-FrB compared to the TNF-α treated control in primary bone marrow-derived osteoblasts. While TNF-α did not significantly affect *Runx2* gene expression following one hour of treatment, *Bmp2* was significantly downregulated. Neither fraction was able to fully rescue the cells from this suppression of *Bmp2*. In addition, TNF-α significantly upregulated the expression of *Smad6*, which is inhibitory to osteogenic Smad1/5/8 signaling, and neither fraction was able to attenuate this response.

Previously, it was demonstrated that a crude ethanol extract of the polyphenols from dried plum upregulated *Runx2* and mineralization capacity in MC3T3-E1 cells. This study is the first to show that the most hydrophilic fractions of the crude polyphenolic

extract from dried plum had similar effects on osteoblast activity and function in murine

primary bone marrow-derived osteoblasts. Furthermore, the enhanced BMP signaling

observed with treatment with these polyphenolic fractions advances our understanding of

how dried plums up-regulate osteoblast's ability to form calcified nodules. However,

while the crude ethanol extract of polyphenols from dried plum was able to attenuate the

detrimental effects of TNF-α on osteoblast activity and function in MC3T3-E1 cells, the

magnitude of response to the polyphenolic fractions in an inflammatory environment was

not the same in primary bone marrow-derived osteoblasts.

In summary, the fractions most bioactive in enhancing osteoblast activity were

identified. These fractions increased osteoblast activity, at least in part, by upregulating

BMP signaling in osteoblasts. However, while osteoblast activity was enhanced by

treatment with the fractions under normal conditions, the fractions were unable to protect

the osteoblasts from the suppressive effects of TNF-α. These are the first studies

identifying the most bioactive components of the crude polyphenolic extract of dried

plum. In addition, these studies are the first to show alterations in calcium and MAPK

signaling in osteoclasts and BMP signaling in osteoblasts with treatment of components

of dried plum.

Recommendations for Future Research

These studies advance the understanding of the bioactive components of dried

plum that are responsible for improving bone metabolism, as well as the potential

mechanisms by which these components affect osteoclast and osteoblast function. Further

investigation is necessary to fully elucidate the bioactive components in these fractions and the mechanisms by which they affect bone metabolism *in vivo*.

While previous findings that a crude polyphenol extract could protect osteoblasts from the suppressive effects of TNF-α, the magnitude of response to DP-FrA and DP-FrB in primary bone marrow-derived osteoblasts treated with TNF-α was not as prominent in this study. It may be that higher doses or the possibility of a combination of fractions is required to restore bone formation under inflammatory conditions. Alternatively, the identification of certain components of the polyphenolic extract from dried plum that are most bioactive in improving osteoblast activity and in reducing osteoclast activity in this study provides insight into certain types of dried plum or other fruits (i.e., those high in these particular polyphenols or bioactive components) that could be consumed as a preventative or treatment measure for osteoporosis. Further investigation is needed to characterize the types of polyphenols that should be targeted.

In addition to characterizing the types of compounds within these fractions that are responsible for the enhance osteoblast activity and suppressed osteoclast activity, further understanding of the mechanisms by which they may do so *in vivo* is necessary. The *in vitro* monoculture systems used in these studies are valuable systems to examine signaling mechanisms. However, they do not allow for the coupling of osteoblast and osteoclast activity that occurs *in vivo*. The co-culture system allowed for a more physiologically relevant environment in that osteoblasts and osteoclasts are able to communicate with each other. In this system, it was determined that the fractions downregulated osteoclast differentiation under inflammatory conditions. There was a trend for reduced osteoclast numbers under normal conditions, but unlike in the mono-

culture system in which osteoclast precursors are bombarded with RANKL treatment to induce differentiation, osteoclast differentiation is solely induced by signaling from the osteoblasts in the co-culture system. Therefore, in an environment that more closely mimics the *in vivo* environment, significant downregulation of osteoclast differentiation may not be desirable, as some osteoclast activity is required for maintenance of bone integrity. The mechanisms by which the fractions downregulate osteoclast differentiation in the co-culture system are not yet clear, and further investigation is warranted. In addition, the immune system plays a major role in bone metabolism *in vivo*, and osteoclasts are derived from the same lineage as monocyte/macrophages. Therefore, investigation of how the fractions affect activation of these immune cells much like they do osteoclasts may provide another mechanism by which the fractions are beneficial to bone health. Finally, it must be determined whether alterations in bone metabolism due to treatment with the fractions observed in murine models of osteoporosis translate to humans. This could be accomplished by examining whether consumption of certain cultivars of dried plum which contain a higher amount of the components within these fractions provides a greater improvement in bone health than is currently observed with the cultivar of dried plum most commonly consumed. The future investigations suggested above will assist in the progression of potential treatment development.

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APPENDICES

Oklahoma State University
Institutional Animal Care and Use Committee (IACUC)

Protocol Expires: 10/19/2014

Date : Thursday, October 20, 2011   Animal Care and Use Protocol (ACUP) No :   HE116

Proposal Title: Osteoprotective Activity of a Dried Plum Extract

Principal
Investigator:

Brenda J. Smith
Nutritional Sciences
301 HES
Campus

Reviewed and   Full Committee
Processed as:

Approval Status Recommended by Reviewer(s) : Approved

The revised protocol is approved. You are approved to use a maximum of 101 C57BL/6 mice for the next three years:

45 female mice - 4 weeks old
8 pregnant female mice - 12-16 weeks old
48 neonatal mice - 2-3 days old

Signatures :

Charlotte L. Ownby

Charlotte Ownby, IACUC Chair

Thursday, October 20, 2011
Date

cc: Department Head, Nutritional Sciences
Director, Animal Resources

VITA

Jennifer L. Graef

Candidate for the Degree of

Doctor of Philosophy

Thesis: BIOACTIVE COMPOUNDS IN DRIED PLUM ENHANCE OSTEOBLAST ACTIVITY VIA BMP PATHWAYS AND DECREASE OSTEOCLAST ACTIVITY BY SUPPRESSING INTRACELLULAR CALCIUM AND ACTIVATION OF MAPKS

Major Field: Nutritional Sciences

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in July, 2016.

Completed the requirements for the Master of Arts in Dietetics at University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma in 2010.

Completed the requirements for the Master of Science in Exercise Physiology at University of Oklahoma, Norman, Oklahoma in 2009.

Completed the requirements for the Bachelor of Science in Biology at University of Wisconsin-Oshkosh, Oshkosh, Wisconsin in 2007.

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Academy of Nutrition and Dietetics

Oklahoma Dietetic Association