M-CSF Potently Augments RANKL-Induced Resorption Activation in Mature Human Osteoclasts

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Abstract

Macrophage-colony stimulating factor (M-CSF) is critical for osteoclast (OC) differentiation and is reported to enhance mature OC survival and motility. However, its role in the regulation of bone resorption, the main function of OCs, has not been well characterised. To address this we analysed short-term cultures of fully differentiated OCs derived from human colony forming unit-granulocyte macrophages (CFU-GM). When cultured on dentine, OC survival was enhanced by M-CSF but more effectively by receptor activator of NFκB ligand (RANKL). Resorption was entirely dependent on the presence of RANKL. Co-treatment with M-CSF augmented RANKL-induced resorption in a concentration-dependent manner with a (200–300%) stimulation at 25 ng/mL, an effect observed within 4–6 h. M-CSF co-treatment also increased number of resorption pits and F-actin sealing zones, but not the number of OCs or pit size, indicating stimulation of the proportion of OCs activated. M-CSF facilitated RANKL-induced activation of c-fos and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, but not NFκB nor nuclear factor of activated T-cells, cytoplasmic-1 (NFATc1). The mitogen-activated protein kinase kinase (MEK) 1 inhibitor PD98059 partially blocked augmentation of resorption by M-CSF. Our results reveal a previously unidentified role of M-CSF as a potent stimulator of mature OC resorbing activity, possibly mediated via ERK upstream of c-fos.

Introduction

The critical role of macrophage-colony stimulating factor (M-CSF) in osteoclast (OC) differentiation is well-studied, and its role in osteoclast survival and motility has been characterized. M-CSF is also known to enhance the number of OCs in cultures of CFU-GM precursors. However, the role of M-CSF in the regulation of bone resorption by mature OCs, which is the main function of these cells, is less clear. This knowledge gap is important, as M-CSF is involved in the regulation of bone health, and understanding its role in bone resorption could have implications for the treatment of bone diseases.

In short-term (6 h) semi-pure cultures of isolated neonatal rat OCs, human M-CSF enhanced survival, motility and cytoplasmic spreading, but inhibited resorbing activity, in a concentration-dependent manner; maximum effect at 0.5 ng/mL [8]. In contrast, in 6–18 h cultures of isolated human fetal OCs, M-CSF had no apparent effect on resorbing activity but did enhance survival; maximum effect at 50 ng/mL [9]. Experiments in a human bone marrow osteoclastogenesis assay suggested that resorption per OC was enhanced in the presence of M-CSF 50 ng/mL [10]. Likewise, in an osteoclastogenesis assay employing human colony forming unit-granulocyte macrophage (CFU-GM) precursors, we found that resorption per OC was increased in the presence of M-CSF 25 ng/mL [11]. However, investigation of resorbing activity in osteoclastogenesis assays is problematic because of the confounding effects of proliferation, differentiation and fusion. Furthermore, these findings were inconsistent with the widely held view [12–16] that, in fully differentiated OCs, the role of M-CSF is limited to regulation of survival and motility while receptor activator of NFκB ligand (RANKL) and perhaps interleukin-1 (IL-1) [17,18] are the key regulators of resorption activation.

To address this knowledge gap, we established a mature OC model based on that of Fuller and colleagues [17] that would allow the characterization of the role of M-CSF in the short-term regulation of resorption activation and survival in these cells. We generated OCs en masse by treating CFU-GM-derived cells with M-CSF and RANKL for 14d [19]. The OCs were then either detached from the plastic and cultured on dentine slices for 4–72 h for resorption and survival assays, or used in situ for transcription factor activation and signalling phosphorylation assays. We demonstrate that although RANKL is necessary for resorption, M-CSF 10–25 ng/mL potently augments RANKL-induced resorption, an effect that is not due to enhanced survival but, rather,
due to increased activation of resorption in OCs. We also demonstrate that M-CSF potentiates RANKL-induced c-fos
activation and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, and that the resorption-stimulating effect of M-
CSF is blocked by the mitogen-activated protein kinase (MEK)
inhibitor PD98059. This is the first study to definitively identify M-
CSF as a potent enhancer of resorption activation of mature OCs, a paradigm-shift for its role in physiology and pathophysiology.

Materials and Methods

Materials

Eagle’s MEM, penicillin/streptomycin, paraformaldehyde, Sigma
Cell Dissociation Solution (IX) non-enzymatic (Cat.
No. C5914), Fast Garnett GBC, MEK1/2 inhibitor PD98059
and naphthol AS-BI-phosphate were purchased from Sigma. Non-
essential amino acids (100X) and FBS were purchased from
Bovogen (Melbourne, Australia). Ficol-Paque was purchased from
Pharmacia Biotech. MethoCult GF H4534 (Isocove’s MDM
containing 1% methylcellulose, 30% FBS, 1% BSA, 10-3 M 2-
mercaptopoethanol, 2 mM L-glutamine, 10 ng/ml recombinant
human GM-CSF, 10 ng/ml IL-3, and 50 ng/ml stem cell factor)
was purchased from Stem-Cell Technologies. Human M-CSF and
neutralising polyclonal antibody against M-CSF was purchased from
Chemicon. Primary rabbit monoclonal antibodies raised against β-actin, c-fos and total or phosphorylated forms of IkBa
and ERK1/2 and HRP-conjugated anti-rabbit polyclonal antibody
were from Cell Signaling Technology (MA, USA); Soluble
RANKL, coupled to GST fusion protein (RANKL) was generously
provided by Drs Matthew Gillespie and Julian Quinn (Prince
Henry’s Institute, Monash Medical Centre, Melbourne, Australia).
Transcription factor activation kits incorporating detection
antibody for the p65 subunit of Nuclear Factor kappa B (NFkB)
and c-fos component of AP-1 were purchased from Pierce
Biotechnology (Illinois, USA). TransAM assay kits for Nuclear
factor of activated T-cells, cytoplasmic 1 (NFATc1) were from
Active Motif (California, USA).

Ethics statement

Human umbilical cord blood was obtained with written
informed consent from healthy donors under a protocol approved
by Barwon Health Human Research and Ethics Committee.

Mature human osteoclasts

Collection of human umbilical cord blood, isolation of a
mononuclear cell fraction, expansion of CFU-GM-derived OC
precursors and differentiation of mature human OCs have been
previously described [19]. Specifically, a mononuclear cell
fraction of cord blood (CBMC) was isolated by Ficol-Paque
density gradient centrifugation. CBMCs (3x10^6
cells/culture) were suspended in 3.0 ml Methocult GF H4534 in 6-well plates
and incubated at 37°C in humidified atmosphere of 5% CO2-air
for 10 days. Pooled colonies (CFU-GM-derived OC precursors)
were harvested into PBS. Precursors (5x10^6 cells/175 cm^2)
were then seeded into 175 cm^2 tissue culture flasks. The cells
were cultured in 20 mL of MEM containing 10% FBS,
nonessential amino acids, penicillin 50 U/ml, streptomycin
50 µg/ml, 2 mM L-glutamine, M-CSF (25 ng/ml), and RANKL
(125 ng/ml) for 14–21 days. The cultures were refreshed weekly
by replacing additives in one half volume of media. Mature OCs
were used in situ for signalling and transcription factor assays or
 dissociated and re-settled on dentine slices for survival and
resorption assays.

Mature OC survival and resorption assay

Mature OC cultures were treated with dissociation buffer
(5 mL/75 cm^2 flask) for 30 min at 37°C when most were detached
by agitation. Remaining adherent cells were removed with a cell
scraper. Detached OCs were washed, pelleted by centrifugation
(200 g; 2 min), resuspended in MEM/10% FBS and settled onto
round dentine slices (6 mm diameter) in 96-well plates (n = 500–
1000 OCs/well) and cultured for 4–72 h in 200 µL of media.
Cells were fixed in 1% formalin and reacted for TRAP as
previously described [19]. The formation of OCs was assessed by
transmission light microscopy; quantified using microcomputer
image analysis software (MCID - Imaging Research Inc. Ontario,
Canada). F-actin stained sealing zones were quantified by confocal
microscopy using rhodamine-conjugated phalloidin [20]. Cells
were removed from dentine slices by brief sonication in
chloroform:methanol 2:1. Xylene-free black ink was applied to
the surface of each slice and residual ink removed by wiping on
absorbent paper. Resorption was assessed by reflective light
microscopy and the percentage area resorbed was quantified using
MCID software. This method was validated by comparison with
scanning electron microscopy [21].

NFkB, c-fos and NFATc1 transcription factor activity

Mature OC cultures in 6-well plates were rinsed, pre-treated
with or without M-CSF (37.5 ng/mL) for 18 h in MEM/10%
FBS and then treated with RANKL (125 ng/mL) or vehicle
for 30 min. Nuclear extracts (Ne-PER; Pierce, IL, USA) were
assessed for activation of NFkB, c-fos and NFATc1 using
ELISA-based transcription factor assay kits. Briefly, an oligonu-
cleotide containing the consensus binding sequence for the
relevant transcription factor is immobilised to a 96-well plate.
Transcription factor contained in nuclear extracts binds specifi-
cally to this oligonucleotide and is detected through use of an
antibody directed against either p65, in the case of NFkB, c-fos or
NFATc1. Addition of a secondary antibody conjugated to horseradish
peroxidase provides sensitive chemiluminescent or
colorimetric readout that is quantified by spectrophotometry.

ERK1/2, IkBα signalling and c-fos Western Blot

Mature OCs in 25 cm^2 flasks were serum-starved for 18 h when
media was replaced with MEM/10%FBS with vehicle, RANKL
(125 ng/mL), M-CSF (25 ng/mL) or both. Cytoplasmic extracts
(M- 
PER, Pierce, IL, USA) were collected at 0, 5 min and 10 min.
For c-fos expression, mature OCs were treated with or without
M-CSF for 18 h before cell lysis. Total protein was subjected to SDS-
PAGE on 4–20% gradient gels (NuSep, Georgia, USA). Protein
gels were transferred to nylon membranes (Amersham Biosciences,
Buckinghamshire, UK) overnight at 4°C (50 V; 25 mA) and
blotted in PBS containing 5%BSA; 0.2% Tween 20 for 1 h.
Immunodetection used primary rabbit monoclonal antibodies that
recognised either total or phosphorylated forms of IkBα, ERK1/2,
c-fos, or β-actin (Cell Signaling Technology, MA, USA).
Secondary antibody was HRP-conjugated anti-rabbit polyclonal
antibody (Cell Signaling Technology, MA, USA) A chemilumi-
nescent substrate followed by autoradiography was used for
detection (GE Healthcare Bio-Sciences, NJ, USA). MCID
software was used for densitometric analysis.

Statistical analyses

Data are expressed as the mean ± SEM where applicable.
Differences between groups where determined using one-way
ANOVA followed by Fisher’s multiple comparison test, two-way
ANOVA – general linear model (GLM) followed by Tukey’s
post-hoc test or unpaired T-test. Statistical significance was set at $P<0.05$. Treatment groups with annotations containing the same letter are not significantly different. (For example: 3 groups annotated with “a”, “b” and “c”, are all different to each other. In the case of 3 groups annotated with “a”, “ab” and “b”, “a” is different to “b”, whereas “a” and “ab” are not different, likewise “ab” and “b”.)

**Results**

M-CSF acutely augments RANKL-induced resorption in mature human OCs

We assessed the survival and resorbing activity of mature human OCs by seeding freshly harvested cells onto dentine slices and culturing them for 72 h. Survival of OCs cultured in media/10% FBS alone was enhanced (62% increase) by the addition of M-CSF (25 ng/mL) but more effectively so (165% increase) by RANKL (125 ng/mL). Neither M-CSF nor RANKL had any substantial effect on OC plan area (size). In the absence of RANKL, resorption was negligible. Activation of RANKL-induced resorption per OC was markedly enhanced (300% increase) by the addition of M-CSF (25 ng/mL) (Fig. 1A). In six independent experiments, the addition of M-CSF (25 ng/mL) resulted in a 220±12% (mean±SEM) increase in resorption compared to RANKL (125 ng/mL) alone (p = 0.004, not shown). This effect was concentration-dependent (Fig. 1B). At M-CSF concentrations between 50 and 200 ng/mL, a concentration-dependent, although non-significant, trend to decreased resorption was apparent. In cultures treated with RANKL only, co-treatment with a neutralising antibody to M-CSF had no significant effect on survival or resorption, indicating that endogenous M-CSF [11] is not necessary for RANKL-induced survival or resorption activation (Fig. 1B). A 130% stimulation of resorption in the presence of M-CSF (37.5 ng/mL) was accompanied by an

![Figure 1. M-CSF augments RANKL-induced resorption and F-actin sealing zone formation by harvested OCs.](image-url)
equivalent increase in sealing zone formation when compared to treatment with RANKL alone (Figs. 1C and 1D).

In our 72 h cultures, we could not exclude the possibility that the observed resorption-stimulating effect was due to ongoing OC differentiation in the presence of M-CSF. In CFU-GM osteoclastogenesis cultures we previously found a linear increase in OC plan area over three weeks due to progressive cell fusion [19] yet in the current study, no substantive increase in OC plan area was observed with M-CSF, suggesting an absence of significant cell fusion. Nevertheless, to exclude this possibility, we conducted experiments over a shorter time of 4–24 h. Since the small amount of dentine resorption present in these short-term assays could not be accurately quantified using whole-slice image analysis, we counted resorption pits at higher magnification and individually measured their plan area and diameter or, in the case of non-circular “trail or trench” pits, trail pit linear length (trail length). In these experiments, OCs were treated with RANKL (125 ng/mL) alone or together with M-CSF (25 ng/mL) (Figs. 2A and 2B). No significant effect on OC number was seen over this period. In the presence of RANKL only, the resorption pit number, size and trail length increased with time in a linear manner. Co-treatment with M-CSF increased the pit number and pits per OC at each time point (200% at 24 h) but had no substantive effect on the pit size or trail length (Fig. 2A). The effect of M-CSF at 6 h in an independent experiment is shown in Fig. 2B. The addition of M-CSF to RANKL had no effect on OC characteristics but increased the number of pits and pits per OC by 66% and 62%, respectively.

**M-CSF pre-treatment facilitates RANKL-induced activation of c-fos but not of NFkB**

In mature human OCs, NFATc1 is not activated by either RANKL or M-CSF. Acute treatment with RANKL of OCs that had been serum-starved for 18 h produced a 320% activation of NFκB at 30 min (Fig. 3A). Pre-treatment with M-CSF (37.5 ng/mL) for 18 h had no effect on basal or RANKL-induced activation of NFκB (Fig. 3A). In contrast, in control OCs, RANKL did not activate c-fos, whereas pre-treatment with M-CSF increased basal activation by 70% and RANKL-induced activation by 190% (Fig. 3A). We also found that M-CSF pre-treatment increased basal c-fos protein expression by 41.5% (Fig. 3B). No effect on NFATc1 activation was seen with M-CSF pre-treatment or acute RANKL treatment (Fig. 3A).

**Phosphorylation of ERK1/2 by M-CSF is augmented by co-treatment with RANKL in mature human OCs**

Since MEK/ERK pathways are known to be involved in both RANK and c-fms signalling [22–24], we hypothesised that they may be involved in the observed effect of M-CSF on RANKL-
induced resorbing activity. Furthermore, c-fos induction by ERK is well characterised [25,26] and in the current study we have shown that c-fos is activated by M-CSF. In serum-starved OCs we found no detectable levels of constitutively phosphorylated ERK1/2. Treatment with M-CSF activated phosphorylation, with a peak increase of 477% at 5 min, dissipating to 70% at 10 min (Fig. 4A). Treatment with RANKL produced only a weak phosphorylation of ERK1/2 at 5 min, in comparison to M-CSF, with negligible levels detected at 10 min. Co-treatment with RANKL and M-CSF increased ERK1/2 phosphorylation at 5 min (44% increase) and 10 min (+19%), compared to M-CSF alone. Levels of total ERK1/2 were unchanged over the time-course investigated.

M-CSF alone decreased phosphorylated IκBα by 42% at 10 min, whereas RANKL exposure stimulated phosphorylation by 278%. There was no additional effect in combination with M-CSF alone. Total IκBα was unchanged over the time-course (Fig. 4B).

**Blockade of MEK/ERK signalling inhibits M-CSF-induced stimulation of resorption**

The results above indicated that ERK1/2 phosphorylation was enhanced by co-treatment with M-CSF and RANKL, suggesting that MEK/ERK signalling may be involved in M-CSF enhancement of RANKL-induced resorption activity. To test this possibility, we examined the effects of PD98059, a specific inhibitor of the ERK1/2 activator MEK-1, on this effect. Mature OCs cultured on dentine slices were pre-treated for 1 h with PD98059 (5 μM), or vehicle, prior to treatment with RANKL alone or together with M-CSF for 72 h. Pre-treatment with PD98059 had no effect on OC survival, size or RANKL-induced resorption (Fig. 5). In cultures pre-treated with vehicle, treatment with RANKL and M-CSF produced a 330% increase in resorption compared to RANKL alone. This effect was attenuated in the presence of PD98059. In the experiment shown in Fig. 5, resorption in M-CSF group is not significantly different (ANOVA) to that of the RANKL-only control in the presence of PD98059. However, in 3 independent experiments PD98059 reduced the resorption-stimulating effect of M-CSF by 52.3 ± 6.2% but resorption in the M-CSF group remained significantly greater than the control (p = 0.023, paired T-test).

**Discussion**

Utilising a human, mature OC activation assay, we have demonstrated that M-CSF effectively activates OC resorption, in
addition to its roles in survival and motility. It is established that RANKL rapidly activates resorption in isolated mouse OCs [27], increasing actin-ring formation within 30 minutes. Furthermore, treatment of mice with intravenous RANKL increases ionized calcium at 1 h, consistent with acute activation of mature OCs [27]. In our system, exposure to RANKL alone initiates, within 4 h, the formation of resorption pits that progressively enlarge with time, while the addition of M-CSF results in a rapid increase in the relative number of pits, which are not larger or longer, indicating a fundamental difference in the mechanism. The rapidity of this effect suggests that it is not due to an effect on OC differentiation but, rather, activation of mature cells. Pits progressively accumulate so the increased number could be due to an increase in the proportion of OCs activated to form pits, or an increase in the activation frequency among the same proportion of the OCs. However, since the presence of an actin ring indicates current OC activation [28], the observed increase in sealing zone number confirms that the increased pit number is due to a greater proportion of activated OCs.

Functional RANK is expressed on mature OCs and RANKL stimulates their activation [27,29,30]. Since resorption is RANKL-dependent, we surmised that augmentation of RANK signalling by M-CSF was a possible mechanism of its stimulation of OC activation. In OC precursors, a number of signalling cascades are known to be activated downstream of RANK leading to activation of NFκB, AP-1, p38, ERK, Src and NFAT (reviewed by Boyle et al. [12]). However, most of this knowledge is based on investigations done in rodent osteoclast generation models and the signalling pathways involved in the activation of mature OCs have not been well characterised. Likewise, signalling downstream of c-fms, the tyrosine kinase receptor of M-CSF is not well characterised in mature OCs although the important roles of Src kinase in resorption [31,32], PI-3-kinase in motility [33] and PI-3-kinase as well as MEK 1 in survival [34] are well established. Therefore, we investigated the effects of acute treatment of OCs with RANKL on activation of NFκB, c-fos and NFATc1, and the modulation of this by M-CSF. In fully differentiated OCs under these experimental conditions, RANKL activation of NFκB is independent of M-CSF. However, RANKL activation of c-fos is dependent on the presence of M-CSF. NFATc1 appears to have no role in RANKL-induced activation of differentiated OCs. These results are consistent with the results of our resorption assays where RANKL initiates sub-maximal resorption activity independent of M-CSF while M-CSF augments RANKL-induced resorption, the former mediated by NFκB and the latter by c-fos.

Yao et al. [35] have previously shown that M-CSF treatment of mature mouse OCs results in increased c-fos expression and transcriptional activation [35]. The key role of c-fos in OC differentiation is well established [36,37]. Our results indicate that in mature OCs c-fos activation is not essential for the initiation of resorption but that its activation by M-CSF greatly augments RANKL-induced activation of resorption. Moreover, although NFATc1 is critical in osteoclast differentiation it appears to play no part in resorption activation of fully differentiated OCs. Lee and Kim [26] have previously highlighted that existing data show that signalling pathways stimulated by RANKL are different in OC precursors and mature OCs [26].

Miyazaki et al. [24] used adenoviral gene transfer methods to activate and inhibit ERK and NFκB in semi-pure mouse OC cultures. Activation of ERK increased OC survival and inhibition of ERK decreased survival but neither had any effect on their bone-resorbing activity. In contrast, activation and inhibition of NFκB increased and decreased OC resorbing activity, respectively, but had no effect on survival [24]. We have shown that in fully differentiated human OCs, functional MEK/ERK pathways are not essential for the pro-survival and pro-resorption effects of RANKL but these pathways are involved in M-CSF augmentation of RANKL-induced resorption activation. The involvement of ERK is consistent with the involvement of c-fos activation that we have demonstrated. The results do not exclude the possibility that other MAPK parallel cascades, such as JNK and p38 are also involved [25].

Our novel finding that M-CSF regulates the rate of osteoclastic bone resorption in vitro, independent of OC formation, is supported by data from two murine in vivo studies. The first investigated the capacity of cell-surface M-CSF (csCSF-1) to restore in vivo M-CSF function in the CSF-1-deficient osteopetrotic (Csf1r<sup>−/−</sup> /Csf1r<sup>−/−</sup>) background [6]. Transgenic expression of csCSF-1 corrected the gross defects of these mice, including growth retardation and failure of tooth eruption. However, residual osteopetrosis and significantly delayed trabecular bone resorption in the subepiphyseal region of the long bones, along with incomplete correction of hematologic abnormalities were found. Complete restoration was achieved by transgenic expression of the full length CSF-1 gene, which encodes both circulating CSF-1 and some csCSF-1 [38]. Only partial correction of osteopetrosis in csCSF-1 transgenic mice was observed, even though there was an equivalent number and size of OCs to wild-type controls. This finding prompted the authors to conclude that while local csCSF-1 expression was sufficient to support osteoclastogenesis, the rate of bone resorption in these animals lacking circulating M-CSF was slower [6]. These investigators subsequently demonstrated [7] that the osteopetrosis phenotype of CSF-1- and CSF1R-deficient mice could be replicated by post-natal administration of a neutralising anti-CSF-1 antibody. The number of OCs was dramatically reduced at 15.5 but not at 36.5 or 64.5 days post-natal, and increased trabecular bone density remained. Very low to undetectable levels of circulating antibody were present in the older mice, suggesting that some M-CSF was present (although not measured in this study) but at sub-optimal levels for OC resorption to proceed [7].

Figure 4. Activation of ERK1/2 and IκBα by RANKL and M-CSF in mature OCs. Mature OCs were serum starved for 18 h then acutely treated with RANKL (125 ng/mL) or M-CSF (25 ng/mL) alone, or in combination. Cytoplasmic protein extracts were harvested and subjected to SDS-PAGE followed by western analysis using antibodies against total and phosphorylated ERK1/2 and IκBα. A. Augmentation of phospho-ERK1/2 in the presence of RANKL and M-CSF. B. M-CSF has no effect on phospho-IκBα: representative data from 2 independent experiments.

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In healthy individuals, serum M-CSF increases from approximately 12 ng/mL in the early twenties to 20 ng/mL in the eighties, corresponding to the concentration range where we observed in vitro a three- to four-fold increase in resorption [39]. In women, the serum bone resorption marker, C-telopeptide, increases in a near linear manner from age 21 to 71 years and bone mineral density declines substantially with ageing [40]. Thus, the possibility exists that increasing circulating M-CSF is involved in “normal” age-related bone loss. Furthermore, as we have previously reviewed, systemic and/or local M-CSF concentrations are increased in a variety of inflammatory and neoplastic conditions known to be associated with bone loss [11].

Karsdal et al. [41] pointed out that osteopetrotic mutations resulting in reduced or absent OCs are associated with decreased bone formation, whereas osteopetrotic mutations that result in increased numbers of non-resorbing OCs are associated with increased bone formation and suggested that nonresorbing osteoclasts provide anabolic signals for osteoblasts [41]. It has subsequently been shown that secreted products of OCs, spingosine-1-phosphate and bone morphogenic protein 6 stimulate migration and osteoblast differentiation of human mesenchymal stem cells [42].

We hypothesize that incomplete blockade of c-fms signalling, or downstream signalling pathways, may provide a potential means to attenuate bone loss. In this situation, OC differentiation will proceed but the proportion activated to resorb will be lower, resulting in reduced bone resorption yet maintenance of OC-derived anabolic signals to osteoblasts.

Author Contributions
Conceived and designed the experiments: JMH FMC MAK GCN. Performed the experiments: JMH FMC NJP. Analyzed the data: JMH FMC. Contributed reagents/materials/analysis tools: JMH FMC NJP. Wrote the paper: JMH FMC NJP GCN.

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