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J Dent Res 93(11):1108-1115, 2014

ABSTRACT

In a search for factors up-regulated by mechanical strain in osteocytes, we discovered that chemokine (C-C motif) ligand 7 (CCL7), a chemotactic myokine, was highly expressed in MLO-Y4 osteocyte-like cells. Although MLO-Y4 cells secrete potent chemotactic factors for osteoclast precursors, CCL7 was not responsible for this activity. CCL7 was increased in osteocytes in response to tooth movement *in vivo*. Since mechanical loading plays a crucial role in maintaining osteocyte viability, CCL7 was tested for protective activity and found to be protective against cell death induced by dexamethasone and etoposide. CCL7 specific antibody partially, but in combination with indomethacin, completely abrogated the protective effects of fluid flow shear stress against dexamethasone-induced cell death. CCL7 activated the β -catenin pathway through phosphorylation of glycogen synthase kinase 3 (GSK-3), suggesting that this pathway is responsible for the observed protective effects. A related cytokine, CCL2, also produced by MLO-Y4 cells but not regulated by mechanical loading, proved to be more potent and protected against cell death induced by not only dexamethasone, but also by Tumor Necrosis Factor α (TNF α). Whereas osteocytes may produce CCL2 in constitutively low levels, a major function of mechanically induced CCL7 may be to selectively protect osteocytes in an autocrine manner against glucocorticoid-induced cell death.

KEY WORDS: cell death, mechanical stress, chemokine, tooth movement, glucocorticoid, TNF α .

DOI: 10.1177/0022034514553008

Received June 10, 2014; Last revision September 3, 2014;
Accepted September 5, 2014

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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CCL7 Is a Protective Factor Secreted by Mechanically Loaded Osteocytes

INTRODUCTION

Osteocytes, which are terminally differentiated osteoblasts embedded in the mineralized matrix, compose 90-95% of all bone cells (Frost, 1960; Parfitt, 1977). Osteocytes regulate mineral homeostasis both locally and systematically (Feng *et al.*, 2006; Liu *et al.*, 2006) and orchestrate osteoblast and osteoclast activity in response to mechanical stimuli to regulate bone formation and resorption (Li *et al.*, 2008; Nakashima *et al.*, 2011). Osteocyte cell death is associated with age and pathological conditions and may be responsible for increased fragility due to loss of capacity to sense microdamage necessary for initiation of bone repair (Manolagas, 2000). Osteocyte viability therefore plays a significant role in the maintenance of bone homeostasis and integrity. Mechanical loading plays a role in maintaining osteocyte viability. Shear stress generated within the osteocyte lacuna-canalicular system by bone fluid is thought to be the form of mechanical loading to which the osteocyte is most likely exposed. Fluid flow shear stress (FFSS) inhibits osteocyte apoptosis induced by serum starvation (Bakker *et al.*, 2004) and by dexamethasone (dex) (Kitase *et al.*, 2010).

In a search for osteocyte factors that could respond to FFSS and recruit osteoclast precursors to target bone for resorption (see Appendix Tables 1 and 2), we identified one of the chemokines, CCL7, also known as monocyte chemotactic protein-3, MCP-3, produced by the osteocyte cell line murine long bone osteocyte Y4, MLO-Y4. Even though MLO-Y4 conditioned medium is chemotactic for osteoclast precursors, this activity could not be blocked by CCL7 specific antibody, suggesting the possibility of another function of CCL7 in osteocytes. Chemokines were first described based upon their capacity to stimulate recruitment of leukocytes to sites of inflammation and later to function as protective factors in many types of cells, including bone cells (Okamoto *et al.*, 2004; Wright *et al.*, 2005; Yano *et al.*, 2005). This raised the possibility that CCL7 could be a protective factor for osteocytes.

This study was performed to determine whether CCL7 could be a factor secreted by osteocytes in response to FFSS, responsible for mediating the protective effect of FFSS on glucocorticoid-induced osteocyte cell death. Previously, we had shown that prostaglandin E2 (PGE₂) produced by osteocytes in response to FFSS is a protective factor by activating the β -catenin pathway (Kitase *et al.*, 2010). Here we show that CCL7 is highly expressed in osteocyte-like MLO-Y4 cells and up-regulated by mechanical loading *in vitro* and *in vivo*. CCL7 selectively rescues osteocyte cell death induced by dex but not by the inflammatory cytokine, Tumor Necrosis Factor, TNF α . The

protective effect of FFSS is partially mediated by CCL7 but also through prostaglandin production and activation of the β -catenin signaling pathway.

MATERIALS & METHODS

Cell Culture

MLO-Y4 osteocyte-like cells were cultured on collagen type I-coated plates (rat tail collagen type I, 0.15 mg/mL) in alpha minimum essential medium (α -MEM) supplemented with 2.5% fetal bovine serum, 2.5% bovine calf serum in a 5% CO₂ incubator at 37°C. Both 2T3 and MC3T3-E1 osteoblast-like cells were cultured in α -MEM supplemented with 10% FBS.

Quantitative Real-time Polymerase Chain-reaction (PCR)

Taqman real-time quantitative PCR analysis was performed as previously described (Kitase *et al.*, 2010). Relative expression levels were obtained by the $\Delta\Delta C_t$ method. *GAPDH* expression levels were used for normalization.

Enzyme-linked Immunosorbent Assay (ELISA)

MLO-Y4 cells were incubated in 48-well plates at 1×10^4 cells/0.3 mL medium/well in the presence or absence of 1 μ M dex for 24 and 48 hr. After treatment, soluble CCL7 was quantitated with a commercially available kit according to manufacturer's protocols (Bender Medsystems, Vienna, Austria). The values are expressed as fold increase after normalization with cell number.

Northern Blot Analysis

MLO-Y4 cells were exposed to 2 hr of FFSS at 16 dynes/cm². After FFSS, the cells were incubated for 30 min and 24 hr. Northern blot analysis was performed as described previously (Chen *et al.*, 1998) with a mouse 1 kb CCL7 complementary (c)DNA probe.

Tooth Movement and *in situ* Hybridization

All animal procedures were performed in compliance with the regulations and guidelines of the Animal Care Committee of The University of Texas Health Science Center at San Antonio. Three-month-old C57BL6 mice were anesthetized, and an opening coil spring was bonded directly to the right incisor and maxillary first molar as described previously (Pavlin *et al.*, 2000). A continuous force (10–12 g) was applied for 24 hr. The left side served as a control for each mouse. The maxillary complex was dissected, fixed, decalcified, dehydrated through methanol, embedded in paraffin, and sectioned at 7- μ m thickness. The sections were processed for *in situ* hybridization with mouse ribonucleic acid (RNA) probes labeled with ³²P-rUTP as previously described (Pavlin *et al.*, 2000).

Quantification of Cell Death

MLO-Y4 cells stably transfected with nuclear green fluorescent protein (GFP) were plated at 1×10^4 /cm² on a collagen-coated 48-well plate with 3–4 wells for each experimental condition. MC3T3-E1 cells were plated at 4×10^4 /cm². Cells were pre-

treated with recombinant murine CCL7, CCL2 (R&D Systems, Minneapolis, MN, USA) and/or CD40L (Amgen, Thousand Oaks, CA, USA) for 30 min, followed by treatment with 1 μ M dex, 50 μ M etoposide (Sigma-Aldrich, St. Louis, MO, USA), or 1 ng/mL TNF α (R&D Systems) for 6 hr. CD40L was used as positive control, because it was previously shown to inhibit apoptosis of MLO-Y4 cells induced by the above three apoptotic agents (Ahuja *et al.*, 2003). In this assay, phenol red free α -MEM (Invitrogen, Carlsbad, CA, USA) was used for both MLO-Y4 and MC-3T3-E1 cells.

The Nuclear Fragmentation Assay

MLO-Y4 cells exhibiting chromatin condensation and nuclear fragmentation were detected by fluorescence microscopy. Five hundred cells were examined for each experimental condition by systematic random sampling. Digital images were acquired with an optronics camera and analysis imager software.

The Trypan Blue Exclusion Assay

After treatment, adherent cells were released by trypsin-ethylenediaminetetraacetic acid (EDTA), combined with floating cells, and pelleted by centrifugation. Then, 0.04% trypan blue (Sigma-Aldrich) was added, and cells exhibiting both nuclear and cytoplasmic staining were determined by means of a hemocytometer under a light microscope. One hundred cells *per* experimental condition were counted. The percentage of MLO-Y4 cells stained with trypan blue has previously been shown to correlate with that of apoptotic cells (Plotkin *et al.*, 1999).

Western Blot Analysis

MLO-Y4 cells were incubated with 100 ng/mL CCL7 with or without pre-incubation with wortmannin for 30 min. After treatment, the cells were processed for Western blot analysis according to previously described methods (Kitase *et al.*, 2010) with the primary antibodies anti-phospho-GSK-3 α / β (1:1,000, R&D Systems) and anti-GSK-3 α and β (1:1,000, Cell Signaling Technology, Danvers, MA, USA).

Immunofluorescence Staining for Detection of β -catenin

MLO-Y4 cells were treated with 100 ng/mL CCL7 for 1 hr. After treatment, immunofluorescence was performed as previously described (Kitase *et al.*, 2010). The primary antibody against β -catenin (E-17) (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used.

Microarray Analysis and Osteoclast Precursor Chemotaxis Assay

Methods for these experiments are listed in the Appendix.

Statistical Analysis

Data are expressed as means $\pm$ SD. The statistical significance of difference between mean values was determined by one-way ANOVA followed by Tukey-Kramer *post hoc* analysis except in Fig. 3C, which was done by simple-effect one-way ANOVA. $P < .05$ was considered significant.

MLO-Y4 Osteocyte-like Cells Express Large Amounts of CCL7, Which Is Regulated by Fluid Flow Shear Stress, FFSS

CCR2, a receptor for both CCL7 and CCL2, was increased two-fold at the same time-point and reduced by 24 hr. Northern blot analysis confirmed that FFSS increased CCL7 mRNA 3.5-fold at 2 hr and 16-fold at 24 hr after cessation of FFSS (Fig. 1C). This suggests the potential for autocrine effects of CCL7, especially in response to FFSS.

The tooth movement model is ideal for examining gene expression in cells, since both resorption and formation occur simultaneously. The application of force in the coronal region causes a tipping tooth movement, resulting in compressive stress (Pr) in the coronal portion of the mesial periodontium and in the apical portion of the distal periodontium in the distal root. Fig. 1D shows a dark-field view of the maxillary first molar. Strong hybridization signals were observed in both the alveolar bone and periodontal ligament on the pressure side. Regions under pressure showed a strong differential signal, while the tension region showed a uniform, non-specific hybridization signal (Figs. 1Da, 1Db). Fig. 1Dc shows late osteoblasts-early

osteocytes (Ocy) on the pressure side with strong positive signals for CCL7. This is consistent with *in vivo* microarray data (Paic *et al.*, 2009), which shows that both mature osteoblasts (*Col2.3 cyan*⁺) and pre-osteocytes/osteocytes (*DMP1topaz*⁺) express CCL7 with the average intensity of 2139 and 797, respectively.

CCL7 Significantly Inhibits Cell Death of MLO-Y4 and MC3T3-E1 Cells Induced by Dexamethasone, Dex

Even though CCL7 is highly expressed in osteocytes and regulated by mechanical stimuli both *in vitro* and *in vivo*, antibody to CCL7 could not block the chemotactic activity of MLO-Y4 cells on MOPC-5 cells, nor did addition of CCL7 protein have any effect (see Appendix Table 2). Since FFSS has a protective effect on osteocyte viability (Kitase *et al.*, 2010), we hypothesized that CCL7 may function as a protective factor. CCL7 prevented dex-induced cell death in a dose-dependent manner, as determined by both nuclear fragmentation and trypan blue exclusion (Fig. 2A). CCL7 also inhibits dex-induced cell death in osteoblast MC3T3-E1 cells (Appendix Table 3).

CCL7 Partially Mediates the Protective Effects of Fluid Flow Shear Stress on Dex-induced Cell Death

Antibody to CCL7 (1 $\mu\text{g/mL}$) significantly but not completely reduced the protective effects of FFSS (16 dynes/cm²) on dex-induced cell death (Fig. 2B, left). Control IgG had no effect. However, the combination of anti-CCL7 antibody (1 $\mu\text{g/mL}$) and indomethacin (100 nM) completely abrogated the protective effects of FFSS (Fig. 2B, right). The combination of indomethacin and control IgG had a partial effect.

CCL7 Selectively Rescues MLO-Y4 Cell Death Induced by Dexamethasone and Etoposide, but Not by TNF α , Whereas CCL2 Potently Blocks Cell Death Due to Dex and TNF α

CCL7, 100 ng/mL ($\cong 1 \times 10^{-8}$ M), protects against etoposide similar to dex (Fig. 3A); however, it failed to prevent TNF α -

Figure 2. CCL7 blocks dex-induced cell death in MLO-Y4 cells and is partially responsible for the protective effects of fluid flow shear stress. **(A)** Nuclear fragmentation (upper) and trypan blue exclusion (lower) data showing significant blocking effects of CCL7 at 100 and 250 ng/mL on dex-induced cell death. Representative images of nuclear fragmentation of MLO-Y4 cells with each treatment are shown on the right. * $p < .05$ vs. control. # $p < .05$ vs. pro-apoptotic agent alone. **(B)** Effects of FFSS, α -CCL7Ab, and IgG on dex-induced cell death (left). Effects of FFSS, α -CCL7Ab, IgG, and indomethacin on dex-induced cell death (right). * $p < .05$ vs. control. # $p < .05$ vs. dex alone. + $p < .05$ vs. dex+FFSS. ^ $p < .05$ vs. dex+FFSS+ α -CCL7Ab (left) or dex+FFSS+ α -CCL7Ab+indomethacin (right).

induced cell death (Fig. 3B). Even when the CCL7 concentration was increased to 1 $\mu\text{g/mL}$ ($\cong 1 \times 10^{-7}$ M), 20 times greater than the concentration sufficient to significantly inhibit dex-induced cell death, no significant effect on TNF α -induced cell death was observed. Like CCL7, CCL2 can inhibit dex-induced cell death,

Figure 3. CCL7 blocks cell death due to etoposide as well as dex, but not to TNF α , whereas CCL2 and CD40L block the effects of TNF α . (A) Effects of 100 and 250 ng/mL of CCL7 on cell death induced by 50 μ M etoposide as compared with dex. (B) No effect of CCL7 up to 1,000 ng/mL was observed against 1 ng/mL TNF α -induced cell death. (C) CCL2 is more potent than CCL7 in blocking both dex- and TNF α -induced cell death. * p < .05 vs. control. # p < .05 vs. pro-apoptotic agent alone. (D) CD40L protects against dex-induced cell death similar to CCL7, but unlike CCL7, and like CCL2 protects against TNF α -induced cell death. * p < .05 vs. control. # p < .05 vs. pro-apoptotic agent alone.

but at a lower dose (10 ng/mL $\cong$ 1×10^{-9} M). Unlike CCL7, CCL2 inhibited TNF α -induced cell death at a concentration of 10 ng/mL ($\cong$ 1×10^{-9} M) (Fig. 3C). Next, the potency of CCL7 was compared with that of CD40L, which was previously shown to prevent apoptosis of MLO-Y4 cells and osteoblast-like MC3T3-E1 cells (Ahuja *et al.*, 2003). Fig. 3D shows that CCL7 can prevent MLO-Y4 cell death induced by dex at a six-fold lower amount (100 ng/mL $\cong$ 1×10^{-8} M) than CD40L (1 μ g/mL $\cong$ 6×10^{-8} M). However, like CCL2, CD40L will block TNF α -induced cell death, although at a much higher amount (Fig. 4D). Therefore, these three agents show similar activities, but distinct differences in potencies and target specificity.

CCL7 Activates the β -catenin Pathway

We have previously shown that the β -catenin pathway is responsible for the protective effects of FFSS, prostaglandin, and muscle-secreted factors against osteocyte/osteoblast cell death (Kitase *et al.*, 2010; Jähn *et al.*, 2012). To determine if CCL7 also utilizes this pathway, we performed Western blot analysis for GSK3 phosphorylation and nuclear translocation of β -catenin. CCL7 was able to increase the phosphorylation of both GSK-3 α and GSK-3 β isoforms approximately two- to three-fold compared with the control (Fig. 4A). To determine if the PI3K/Akt pathway is responsible for the phosphorylation of GSK-3 induced by CCL7,

we used a PI3K inhibitor, wortmannin. Wortmannin inhibited the phosphorylation of GSK-3 induced by CCL7 (Fig. 4A). In addition, immunofluorescent staining with a specific antibody to β -catenin showed that CCL7 promoted nuclear translocation of β -catenin, as indicated by the bright nuclear staining (Fig. 4B).

Dex Inhibits the Expression of CCL7 and CCL2 in MLO-Y4 Cells

By gene array analysis (Fig. 4C), dex suppressed CCL7 mRNA levels approximately three- to four-fold. Dex also inhibited other chemokines such as CCL2, but had no effect on CCR1 and CCR2. Real-time PCR analysis also validated that the expression of CCL7 mRNA in MLO-Y4 cells was 3–4 times lower compared with that in each untreated control (Fig. 4D, left). ELISA was performed to determine the effects of dex on CCL7 protein expression in MLO-Y4 cells (Fig. 4D, right). Dex significantly inhibited the expression level of CCL7 approximately two- to three-fold compared with each control.

DISCUSSION

In this study, a novel function for the chemokine CCL7 in osteocytes is described: that of an autocrine, mechanically regulated agent that provides protection against glucocorticoid-induced cell death. The chemokine CCL2 is also produced by osteocytes, but at lower levels, is not mechanically regulated but is more potent and is effective against TNF α -induced cell death. Therefore, the osteocyte may have several mechanisms for maintaining viability against numerous apoptotic agents under different conditions.

The expression and function of chemokines and their receptors in bone have been reported mainly in osteoblast and osteoclast lineage cells (Ota *et al.*, 2013; Shahnazari *et al.*, 2013; Wintges *et al.*, 2013; Govey *et al.*, 2014). Analysis of recent emerging data shows that chemokines produced by osteocytes may regulate bone homeostasis (Chen *et al.*, 2010; Hoshino *et al.*, 2013; Leucht *et al.*, 2013). In the present study, mechanical stimulation was shown to increase expression of CCL7 in MLO-Y4 cells within hours after the application of FFSS. By *in situ* hybridization, CCL7 increased in osteocytes as well as in late osteoblasts near the pressure side of alveolar bone in the tooth movement model. The CCL7 receptors, CCR1 and CCR2, were also elevated in MLO-Y4 cells and increased by FFSS. These findings suggest that CCL7 in response to mechanical loading may act in an autocrine and paracrine fashion to maintain osteocyte function and viability.

Figure 4. The effects of CCL7 are mediated through the β -catenin pathway. Dex inhibits the expression of the protective factors CCL7 and CCL2 in MLO-Y4 cells. **(A)** The effects of CCL7 appear to be mediated through the β -catenin pathway. Western blot analysis showing phosphorylation of GSK-3 α / β . The intensity of phosphorylated GSK-3 α / β was normalized by each GSK-3 α and β total protein. **(B)** Immunofluorescence of β -catenin showing nuclear translocation with CCL7 treatment. **(C)** Results taken from a gene array analysis (Kitase *et al.*, 2010; the dataset will be submitted to the GEO Data Base Repository, <http://www.ncbi.nlm.nih.gov/geo/>) showing the expression levels of chemokines, their receptors, and BMP7 at 4 and 24 hr of dex exposure. Expression levels are described as fold change based on expression levels in non-treated MLO-Y4 cells at 4 and 24 hr, respectively. Genes positively regulated by dex, Gilz, and Fkbp51 are also shown. NC, not changed. **(D)** Quantitative real-time PCR (left) and ELISA (right) showing CCL7 expression in MLO-Y4 cells at 24 and 48 hr of dex exposure. **p* < .05 vs. each control.

Glucocorticoid treatment is the primary therapy for many inflammatory and autoimmune diseases, but it is well-known to cause the adverse effect of secondary osteoporosis. Increased osteocyte apoptosis is thought to be responsible for increased skeletal fragility and fracture risk due to reduced capacity to sense and repair microdamage (Tomkinson *et al.*, 1997; Weinstein *et al.*, 1998, 2000; O'Brien *et al.*, 2004). Long-term use of corticosteroids has been reported to have adverse effects on oral and craniofacial bones such as the mandible (Beeraka *et al.*, 2013). Osteocyte viability is therefore thought to be essential in maintaining bone homeostasis and integrity (Manolagas, 2000). Mechanical loading at physiological levels is well-known to maintain osteocyte viability, while pathological loading results in osteocyte cell death (Verborgt *et al.*, 2000; Noble *et al.*, 2003; Bakker *et al.*, 2004; Aguirre *et al.*, 2006; Mann *et al.*, 2006). Previous studies showed that chemokines can elicit protective effects against apoptosis in many cell types, including bone cells (Okamatsu *et al.*, 2004; Wright *et al.*, 2005; Yano *et al.*, 2005). We therefore examined whether CCL7 can protect osteocytes against glucocorticoid-induced cell death.

The present study clearly shows that CCL7 is very potent at protecting both osteocytes and osteoblasts from dex-induced

cell death, comparable with previously reported protective agents (see Appendix Table 4), but not from cell death induced by TNF α , which is involved in postmenopausal primary osteoporosis (Tomkinson *et al.*, 1997; Teitelbaum, 2004), in contrast to CCL2 and other protective agents. Parathyroid hormone (PTH) has been shown to have selective ability as a protective factor similar to CCL7 (Jilka *et al.*, 1999), indicating that both PTH and CCL7 may inhibit cell death through similar mechanisms. This has implications for the treatment of primary and secondary osteoporosis.

How this selectivity is achieved is not clear, but could be due to differences between glucocorticoid- and cytokine-specific receptors and their death signaling pathways. Clearly, glucocorticoid functions through a classic nuclear receptor and membrane signaling to regulate target genes responsible for apoptosis induction (Schmidt *et al.*, 2004; Gu *et al.*, 2005) and involves disruption of mitochondria permeability, followed by release of cytochrome C and subsequent activation of caspase 9, which initiates the apoptosis cascade (Yoshino *et al.*, 2001). TNF α induces apoptosis through direct ligation of the death receptor, TNFR1, followed by activation of caspase 8, resulting in initiation of the caspase cascade and amplification through

mitochondria (Thorburn, 2004). This suggests that the magnitude of TNF α -induced cell death might be much greater than that of glucocorticoid-induced cell death. The protective effects of chemokines are reported to be associated with regulation of the BCL-2 protein family, which functions within mitochondrial membranes (Boehme *et al.*, 2000; Li *et al.*, 2003; Tarzami *et al.*, 2005). Therefore, the survival signals induced by CCL7 and CCL2 might be more potent against glucocorticoid-induced cell death, which is initiated by mitochondrial disruption.

CCL2 shares the same CCR2 receptor with CCL7 and in the present study significantly rescued cell death mediated by TNF α . CCL2 was also more potent with respect to inhibiting dex-induced cell death compared with CCL7 (10 ng/mL $\approx$ 1×10^{-9} M vs. 50 ng/mL $\approx$ 5×10^{-9} M). The different potencies of CCL7 and CCL2 may be due to their different binding affinities for CCR2 (Kurihara and Bravo, 1996), the promiscuous nature of chemokines in binding to other receptors, or heterogeneity due to glycosylation and/or amino terminal processing. This may explain the functional disparity between CCL7 and CCL2. Further investigation will be required to understand the mechanism responsible for the selective protective effect of CCL7.

Osteocyte viability is crucial for bone homeostasis. To maintain osteocyte viability, protective factors are produced systematically and locally. Recently, we identified prostaglandin as an osteocyte autocrine, early-protective factor induced by mechanical loading within minutes (Kitase *et al.*, 2010). Analysis of the data presented here suggests that CCL7 is a late-protective factor elevated within a few hours of load similar to BMP7, recently described as a protective factor (Wang and Guo, 2013). The PI3K/Akt/GSK-3/ β -catenin pathway has been shown to play a critical role in maintaining osteocyte viability in response to mechanical stimuli (Kitase *et al.*, 2010; Wang and Guo, 2013). As with prostaglandin and BMP7, CCL7 appears to be protective through activation of the β -catenin pathway. Interestingly, gene array analysis showed that dex inhibits the expression of osteocyte protective factors CCL7, CCL2, Cox-1 and -2 (enzymes for prostaglandin synthesis), but not BMP7. Analysis of the present data therefore suggests that mechanical loading may have the capacity to increase osteocyte viability factors to prevent osteocyte cell death, thereby maintaining the integrity of bone.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (NIH)/National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), grant PO1 AR046798 to S.E.H. and L.F.B. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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