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Effect of Age on Regulation of Human Osteoclast Differentiation

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Abstract

Human skeletal aging is characterized as a gradual loss of bone mass due to an excess of bone resorption not balanced by new bone formation. Using human marrow cells, we tested the hypothesis that there is an age-dependent increase in osteoclastogenesis due to intrinsic changes in regulatory factors [macrophage-colony stimulating factor (M-CSF), receptor activator of NF- κ B ligand (RANKL), and osteoprotegerin (OPG)] and their receptors [*c-fms* and RANK]. In bone marrow cells (BMCs), *c-fms* ($r=0.61$, $p=0.006$) and RANK expression ($r=0.59$, $p=0.008$) were increased with age (27–82 years, $n=19$). *In vitro* generation of osteoclasts was increased with age ($r=0.89$, $p=0.007$). In enriched marrow stromal cells (MSCs), constitutive expression of RANKL was increased with age ($r=0.41$, $p=0.049$) and expression of OPG was inversely correlated with age ($r=-0.43$, $p=0.039$). Accordingly, there was an age-related increase in RANKL/OPG ($r=0.56$, $p=0.005$). These data indicate an age-related increase in human osteoclastogenesis that is associated with an intrinsic increase in expression of *c-fms* and RANK in osteoclast progenitors, and, in the supporting MSCs, an increase in pro-osteoclastogenic RANKL expression and a decrease in anti-osteoclastogenic OPG. These findings support the hypothesis that human marrow cells and their products can contribute to skeletal aging by increasing the generation of bone-resorbing osteoclasts. These findings help to explain underlying molecular mechanisms of progressive bone loss with advancing age in humans.

Keywords

Aging; Osteoclast; Marrow Stromal Cell; RANKL; OPG

In humans, peak bone mass is reached during the third decade, with a subsequent decline in bone mass with age [Riggs *et al.*, 1981; Riggs *et al.*, 1982]. Skeletal aging is characterized as an excess of bone resorption that is not balanced by new bone formation. Although there is much information about sex hormone deficiency and development of osteoporosis, more information is needed about the mechanism(s) by which the aging process influences bone

loss. A better understanding of intrinsic age-related changes in human bone cells may offer new approaches to mitigate or avoid skeletal aging.

Osteoclasts are large, multinucleated cells derived from the non-adherent hematopoietic stem cells present in bone marrow [Walker, 1973]. Osteoclast differentiation is regulated locally through three major factors: macrophage-colony stimulating factor (M-CSF), receptor activator of NF- κ B ligand (RANKL), and osteoprotegerin (OPG). Binding of M-CSF to the *c-fms* cell surface receptor on osteoclast progenitors upregulates the expression of RANK, the receptor for RANKL [Arai *et al.*, 1991], which promotes osteoclastogenesis [Lacey *et al.*, 1998]. OPG is a soluble decoy receptor of RANKL and inhibits osteoclastogenesis [Yasuda *et al.*, 1998].

Adherent MSCs have potential to differentiate into multiple cell types, including osteoblasts. Many studies [Mueller and Glowacki, 2001; D'Ippolito *et al.*, 1991; Zhou *et al.* 2008; Zhou *et al.*, 2011; Geng *et al.*, 2011; Zhou *et al.*, 2012] but not all [Bellantuono *et al.*, 2009] show an age-related decline in osteoblastogenic potential of human MSCs. Studies in mice show an age-related increase in stromal/osteoblastic cell-mediated osteoclastogenesis [Perkins *et al.*, 1994; Kahn *et al.*, 1995; Cao *et al.*, 2005]. Expression of *RANKL* and *M-CSF* was increased in marrow stromal cells from older animals, *OPG* was decreased, and osteoclast formation was greater when stromal/osteoblastic cells from old rather than young donors were used to induce osteoclastogenesis [Cao *et al.*, 2003]. Previous studies with human cells showed an age-dependent increase in constitutive secretion of pro-osteoclastogenic IL-6 and IL-11 [Cheleuitte *et al.*, 1995]. The RANK/RANKL/OPG signaling pathway maintains the balance between the activity of osteoblasts and osteoclasts in order to prevent bone loss and ensure normal bone turnover [Trouvin *et al.*, 2010]. In a previous study, *OPG* expression was significantly lower in hMSCs obtained from subjects older than 65 years of age than from those younger than 55 years [Makhluf *et al.*, 2000]. These studies test the hypothesis that there are age-dependent changes in the expression of osteoclast differentiation factors and receptors in the RANKL/RANK/OPG and M-CSF/*c-fms* pathways in human marrow cells.

Materials and Methods

Preparation of Low-Density Bone Marrow Cells

Bone marrow samples were obtained with IRB approval and annual review as femoral tissue discarded during total hip replacement for osteoarthritis [Zhou *et al.*, 2008]. Criteria for exclusion were rheumatoid arthritis, cancer, and other comorbid conditions that could affect skeletal metabolism including renal insufficiency, alcoholism, active liver disease, malabsorption, hyperthyroidism, ankylosing spondylitis, aseptic necrosis, hyperparathyroidism, morbid obesity, and diabetes. Also excluded were patients who were taking medications that may influence skeletal metabolism (*e.g.* thyroid hormone, glucocorticoids, NSAIDs, and bisphosphonates). There were equal numbers of men and women in these studies. Young was defined as < 50 years of age; older was defined as > 55 years of age [Zhou *et al.*, 2008]. Low-density mononuclear bone marrow cells (BMCs) from 9 young (mean 44.2 ± 7.2 years) and 10 older (mean 70.8 ± 9.3) subjects (age range, 27 to 82) were isolated by density centrifugation with Ficoll/Histopaque 1077 (GE Healthcare,

Piscataway, NJ) [Zhou *et al.*, 2008]. This procedure sediments dense differentiated cells, such as erythrocytes, granulocytes, and macrophages [Böyum, 1968] and enriches for undifferentiated mononuclear cells. Total RNA from 10×10^6 freshly isolated BMCs was extracted with Trizol reagent (Invitrogen) and 2 μ g was reverse-transcribed into cDNA with M-MLV (Promega). Constitutive expression of *c-fms* [Kirma *et al.*, 2007], *RANK* [Atkins *et al.*, 2000], and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was measured by semi-quantitative reverse-transcriptase PCR (RT-PCR). One-twentieth of the cDNA was used in each 50 μ l PCR reaction with conditions optimized to reflect the exponential phase of amplification (Table 1). PCR products were separated on 2% agarose gel electrophoresis, quantified by densitometry of captured gel images with KODAK Gel Logic 200 Imaging System and KODAK Molecular Imaging Software (New Haven, CT). Gene expression levels were normalized to the densitometric units of the internal control *GAPDH*.

Culture of Low-Density Bone Marrow Cells for Osteoclast Differentiation

Low-density mononuclear BMCs from 8 young (mean 44.5 ± 4.4 years) and 7 older (mean 67.6 ± 9.7 years) subjects (range, 36 to 84 years) were cultured for *in vitro* generation of osteoclasts. BMCs were cultured in 60-mm dishes (seeding density 1.43×10^6 cells/cm²) with phenol red-free α -MEM medium (Gibco), 10% Fetal Bovine Serum-heat inactivated (FBS-HI) (Invitrogen), 100 U/ml penicillin, 0.1 μ M streptomycin (Gibco), and 0.5 μ M fungizone. Half of the medium was changed twice each week with care to preserve the cell-cell interaction between adherent and non-adherent cells. With some samples, total RNA was isolated for expression of the osteoclast-specific marker, tartrate-resistant acid phosphatase (TRAP) [Atkins *et al.*, 2000; Eslami *et al.*, 2011]. Other cultures were cytochemically stained for TRAP [Eslami *et al.*, 2011]; TRAP-positive multinucleated cells with 3 or more nuclei were counted without knowledge of sample identity. In a validation study, *TRAP* gene expression was shown to be correlated with expression of cathepsin-K and with number of osteoclasts generated *in vitro* [Eslami *et al.*, 2011].

In other experiments, to test for responsiveness of *in vitro* osteoclastogenesis to exogenous regulators, recombinant human soluble RANKL (PreproTech, Rocky Hill, NJ) was added with each change of medium in BMC cultures from 3 younger subjects (mean age 46.7 ± 4.2 years). Recombinant human OPG (PreproTech) was added to cultures from 8 older subjects (mean 67.0 ± 7.0). Expression of *TRAP* is presented as mean $\pm$ S.D.

Preparation of Human Marrow Stromal Cells (MSCs)

Aliquots of 30×10^6 mononuclear cells isolated from 13 young (mean 44.6 ± 4.5 years) and 10 older (mean 68.6 ± 10.4 years) subjects were seeded in 100-mm tissue culture dishes. Non-adherent cells were removed at 18 hrs and adherent MSCs were expanded to passage 2. Upon near-confluence, RNA was isolated and assayed for constitutive expression of *RANKL* [Eslami *et al.*, 2011], *M-CSF* [Eslami *et al.*, 2011], *OPG* [Makhluf *et al.*, 2000] and *GAPDH* (Table 1).

Statistical Analyses

Group data are presented as mean values $\pm$ standard deviation. Quantitative data were analyzed with Mann-Whitney test, Spearman correlation test, or paired t-test when appropriate. Because subanalyses did not detect gender differences in any of the studies, data for cells from men and women were pooled. A value of $p < 0.05$ was considered significant. If the p value was higher than 0.05 but less than 0.10, it was described as a trend.

Results

Effects of Subject Age on Receptors *c-fms* and Rank in Bone Marrow Cells

Constitutive expression of *c-fms* in freshly isolated BMCs was correlated with age (Fig. 1A, $r=0.61$, $p=0.006$); the amount of *c-fms* mRNA was 1.7-fold greater in BMCs from the older subjects (0.67 ± 0.23) than for the younger subjects (0.40 ± 0.16 , $p=0.017$). Constitutive *RANK* expression was also correlated with age ($r=0.59$, $p=0.008$); the amount of *RANK* mRNA was 1.9-fold greater in BMCs from the older subjects (0.61 ± 0.32) than for the younger subjects (0.32 ± 0.36 , $p=0.028$). Further, there was a strong positive correlation between *RANK* and *c-fms* expression ($r=0.85$, $p<0.0001$; Fig. 1B).

Effects of Subject Age on *In Vitro* Differentiation of Osteoclasts

In vitro osteoclastogenesis was assessed in a series of BMC cultures by enumeration of TRAP-positive multinucleated cells (Fig. 2A). Osteoclastogenesis was correlated with age (Spearman $r=0.89$, $p=0.007$, Fig. 2B); the number of osteoclasts generated in cultures of cells from the older subjects (939 ± 124) was 1.9-fold higher than for the younger group (489 ± 145 , $p=0.029$, Fig. 2B, inset). Osteoclastogenesis was also monitored by expression of *TRAP* and was correlated with age ($r=0.75$, $p=0.037$) (Fig. 2C); expression of *TRAP* was 1.3-fold higher in BMC cultures from older subjects (1.08 ± 0.06) than for younger subjects (0.84 ± 0.15 , $p=0.029$, Fig. 2C, inset). The number of *in vitro*-generated osteoclasts was correlated with expression of *TRAP* ($r=0.74$, $p=0.046$).

Expression of *TRAP* in a second series of 15 samples was also correlated with age ($r=0.60$, $p=0.019$, Fig. 2D); expression of *TRAP* was 1.4-fold higher in BMC cultures from older subjects (0.97 ± 0.27) than for younger subjects (0.69 ± 0.21 , $p=0.014$, Fig. 2D, inset).

In Vitro Effects of Recombinant OPG and RANKL

To assess the responsiveness of *in vitro* osteoclastogenesis to exogenous regulatory factors, we tested for the expected effects of a range of doses of RANKL on osteoclastogenesis with BMCs from young subjects and the effects of a range of doses of OPG on cells from older subjects. With BMCs from 3 subjects between 42 to 50 years of age, addition of 10 ng/mL RANKL increased *TRAP* expression by $20.2 \pm 12.7\%$ (Fig. 3A, $p=0.044$), and 25 ng/mL RANKL increased *TRAP* expression by $29.4 \pm 12.3\%$ ($p=0.041$). With cells from 8 subjects between 58 and 79 years of age, addition of 1 ng/mL OPG inhibited *TRAP* expression to an average 93.2% (Fig. 3B, $p=0.035$) and addition of 10 ng/mL OPG inhibited *TRAP* expression to an average 87.4% relative to each control ($p=0.004$).

Effects of Subject Age on RANKL and OPG in Human Marrow Stromal Cells

We tested whether subject age influences constitutive expression of osteoclastogenic regulatory factors in stromal cells. Constitutive expression of pro-osteoclastogenic *RANKL* in hMSCs was correlated with age (Fig. 4A, $r=0.41$, $p=0.049$); there was a trend of 2.3-fold higher expression of *RANKL* in MSCs from older subjects (0.35 ± 0.27) than for younger subjects (0.15 ± 0.11 , $p=0.072$). Constitutive expression of anti-osteoclastogenic *OPG* in hMSCs was inversely correlated with age ($r=-0.43$, $p=0.039$); there was a trend for 1.4-fold higher expression of *OPG* in younger subjects (0.73 ± 0.28) than that in older group (0.55 ± 0.19 , $p=0.088$). There was an age-related increase in the ratio of *RANKL/OPG* ($r=0.56$, $p=0.005$) (Fig. 4B). *M-CSF* expression did not appear to vary with age in this series.

Discussion

These studies show an age-related increase in *in vitro* osteoclastogenesis with human marrow cells. Further, they show that constitutive expression of multiple osteoclast regulatory factors and receptors are influenced by age (age range 27 to 87 years). The addition of exogenous osteoclastogenic factors was not required for osteoclast formation in this study because high seeding density and presence of supporting stromal cells and their products facilitate *in vitro* osteoclast differentiation, compared with other human or murine cell methods [Koshihar *et al.*, 2002; MacDonald *et al.*, 1987; Thavarajah *et al.*, 1991]. Koshihara *et al.* studied marrow cells from elderly women with hip fracture (age range 64-96), using 20% selected horse serum and adding 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$ [Koshihar *et al.*, 2002]; those stimuli may have been needed because of the lower seeding density in their experiments ($0.5 \times 10^6/\text{cm}^2$) than in ours ($1.43 \times 10^6/\text{cm}^2$). Many studies with murine marrow require exogenous growth factors, such as RANKL or M-CSF, to stimulate osteoclast formation [Kahn *et al.*, 1995; Udagawa *et al.*, 1999]. Using supplements and selected sera may confound assessment of constitutive osteoclastogenesis, a goal of this investigation. Phenol red-free growth medium was used to avoid the indicator's estrogenic effects [Berthois *et al.*, 1986]. Osteoclast differentiation in BMC cultures was the result of interactions between osteoclast progenitors in the non-adherent fractions and regulatory factors produced by the adherent stromal cells. As evidence of clinical relevance of studies with human marrow cultures, Koshihara *et al.* found an inverse correlation between bone mineral density of the spine and *in vitro* osteoclastogenesis [Koshihara *et al.*, 2002].

To validate the responsiveness of the culture system to exogenous factors, we added recombinant OPG and soluble RANKL to BMC cultures from older and younger subjects, respectively. The inhibition of osteoclastogenesis by exogenous recombinant OPG was expected [Dunn *et al.*, 2007] and suggests *in vitro* rejuvenation of old marrow cells. Stimulation of osteoclast differentiation by RANKL with cells from younger subjects is consistent with other studies [Matsuzaki *et al.*, 1998].

Availability of a wide range of age of subjects undergoing orthopedic surgery with discarded marrow made possible these analyses of the effects of age on osteoclastogenesis regulatory factors. Age had multiple effects on constitutive expression of receptors and endogenous regulatory factors. Age-related increases in the expression of both *c-fms* and *RANK* may indicate an age-dependent increase in the number osteoclast progenitors and/or the number

of receptors per cell, which would increase their responsiveness to M-CSF and RANKL, as suggested in mice [Cao *et al.*, 2005]. A correlation between *c-fms* and *RANK* expression is consistent with mouse data that binding of M-CSF to *c-fms* up-regulates the expression RANK receptors on the cell surface and in turn increases the response of osteoclast progenitors to RANKL [Arai *et al.*, 1991].

In addition to the intrinsic differences in expression of the receptors *c-fms* and *RANK*, the age-related increase in *in vitro* osteoclastogenesis may be explained by changes in MSCs and their regulation of osteoclast differentiation. Human MSCs showed an age-related increase in pro-osteoclastogenic *RANKL* and a decrease in anti-osteoclastogenic *OPG*. These findings are consistent with those in mice [Cao *et al.*, 2005], but, unlike in mouse cells, age-related effects on *M-CSF* expression were not observed in these human MSCs. Jiang *et al.* detected elevated *RANKL* expression in fresh whole marrow aspirates from older subjects, but did not provide data on *OPG* or on the receptors [Jiang *et al.*, 2008]. Our data indicate a correlation between age and the ratio of *RANKL* to *OPG*. The ratio of *RANKL* to *OPG* may be a key determinant for the levels of osteoclastogenesis and bone resorption. Abdallah *et al.* showed that the ratio of *RANKL*/*OPG* was significantly higher in iliac bone biopsies from women with hip fractures than for controls and was associated with increased fracture susceptibility [Abdallah *et al.*, 2005]. In a comparison of marrow cells from postmenopausal women, we reported that the *RANKL*/*OPG* ratio in cells from women taking the anti-osteoporosis drug alendronate at the time of marrow procurement was 52% of that for age-matched control women [Eslami *et al.*, 2011]. That difference in the *RANKL*/*OPG* ratios indicates that the marrow cells retain effects of *in vivo* alendronate therapy and that alendronate acts to suppress osteoclast differentiation. Gori *et al.* reported that support of human osteoclastogenesis by stromal-osteoblast lineage cells is developmentally regulated; undifferentiated human MSCs expressed a high *RANKL*/*OPG* ratio to initiate and support osteoclast formation, but as *in vitro* differentiation to mature osteoblasts occurred, the *RANKL*/*OPG* ratio decreased and osteoclast formation was not supported [Gori *et al.*, 2000]. Giner *et al.* found the same decline in *RANKL*/*OPG* as osteoblast cultures matured [Giner *et al.*, 2008].

Two recent studies demonstrated that for mice, osteocytes, rather than osteoblasts or MSCs, are the major source of *RANKL* for osteoclast formation [Nakashima *et al.*, 2011; Xiong *et al.*, 2011]. There is as yet no information about relative effects of human osteocytes in regulating osteoclast differentiation. An important question related to skeletal aging is whether and how osteocytes could contribute to age-related osteoclastogenesis [Matsuo, 2012] in view of the fact that aging is associated with fewer osteocytes in bone for both humans [Busse *et al.*, 2010] and mice [Manolagas and Parfitt, 2010]. There is some uncertainty regarding humans and mice and age-related changes in bone remodeling. Studies in mice indicate that cancellous bone remodeling either declines [Almeida *et al.*, 2007; Shahnazari *et al.*, 2012] or increases [Cao *et al.*, 2005]. Whereas the effects of age on bone remodeling have been relatively well studied in women, there have been few studies in men. In women, advancing age is associated with a decline in periosteal bone formation, a decline in the volume of bone formed by each basic multicellular unit (BMU), continued resorption by each BMU, and high remodeling after menopause [Seeman, 2008]. Available data

indicate that loss of trabecular bone is predominantly due to decreased formation at the level of individual bone remodeling units and that an increase in remodeling rate does not play a major role in men [Compston, 2011].

Age-related intrinsic changes in human MSCs described here for expression of *RANKL* and *OPG* add to previously reported age-related increases in other pro-osteoclastogenic cytokines interleukin 6 [Cheleuitte *et al.*, 1995; Koshihara *et al.*, 2002], interleukin 11 [Cheleuitte *et al.*, 1995], and prostaglandin E₂ [Koshihara *et al.*, 2002]. Human osteoblasts also show effects of age; osteoblast from elders expressed more IL-6 and less OPG than cells from young subjects [Eriksen *et al.*, 2010]. Taken together with the finding of an age-related decrease in proliferation and osteoblast differentiation in hMSCs [Zhou *et al.*, 2008], these findings support the hypothesis that human marrow cells and their products can contribute to skeletal aging by increasing the generation of bone-resorbing osteoclasts and by decreasing the renewal of bone-forming osteoblasts. These findings help to explain underlying molecular mechanisms of progressive bone loss with advancing age in humans.

In conclusion, these studies show an age-related increase in human osteoclast differentiation, an increase in expression of *c-fms* and *RANK* in osteoclast progenitor cells, as well as an increase in constitutive expression of pro-osteoclastogenic *RANKL* and decrease in anti-osteoclastogenic *OPG* by human MSCs. Age-related changes in these multiple ligands and receptors provide compounding mechanisms for increased bone resorption. The *RANKL*/*OPG* ratio in hMSCs may be an important age-related regulator of osteoclast differentiation. Taken together with the finding of an age-related decrease in proliferation and osteoblast differentiation in hMSCs, these findings support the hypothesis that human marrow cells and their products can contribute to skeletal aging by increasing the generation of bone-resorbing osteoclasts and by decreasing the renewal of bone-forming osteoblasts. These findings help to explain underlying molecular mechanisms of progressive bone loss with advancing age in humans.

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Fig. 1.

Effects of subject age on constitutive expression of *c-fms* and *RANK* in low-density mononuclear bone marrow cells (BMCs) isolated from 19 subjects (age range, 27 to 82 years). (A) There were age-dependent increases in expression of *c-fms* (Spearman $r=0.61$, $p=0.006$) and *RANK* ($r=0.59$, $p=0.008$) in BMCs. Expression of *c-fms* and *RANK* was 1.7-fold ($*p=0.017$) and 1.9-fold ($*p=0.028$) higher, respectively, in BMCs from the older subjects (inset, shaded bars, >55 years, $n=10$) than for the younger subjects (open bars, <50 years, $n=9$). (B) There was a correlation between *RANK* and *c-fms* gene expression ($r=0.85$, $p<0.0001$).

Fig. 2.

Effect of subject age on *in vitro* osteoclastogenesis in cultures of BMCs after 14 days. (A) Photomicrographs show TRAP-positive multinucleated cells in BMC cultures from a 74-year-old female subject (*right*), compared with a 42-year-old female subject (*left*). (B) The number of generated osteoclasts was positively correlated with age of subject ($r=0.89$, $p=0.007$, $n=8$). The number of osteoclasts was 1.9-fold ($p=0.029$) higher in BMC cultures from the older subjects (inset, shaded bar, $n=4$) than for the younger subjects (open bar, $n=4$). (C) Expression of *TRAP* in a series of samples was correlated with age ($r=0.75$, $p=0.037$, $n=8$). *TRAP* gene expression was 1.3-fold ($p=0.029$) higher in BMC cultures from the older subjects (inset, shaded bar, $n=4$) than for the younger subjects (open bar, $n=4$). (D) For a series of 15 different samples, *TRAP* mRNA was correlated with the age of subjects ($r=0.60$, $p=0.019$). *TRAP* gene expression was 1.4-fold ($p=0.014$) higher in older subjects (inset, shaded bar, $n=8$) than for the younger subjects (inset, open bar, $n=7$).

Fig. 3.

Effects of exogenous factors on osteoclast differentiation in BMC cultures from young or old subjects. (A) Recombinant human RANKL stimulated osteoclast differentiation in BMC cultures from 3 subjects younger than 50 years. Bars represent the mean values $\pm$ S.D.

* $p=0.044$; ** $p=0.041$ (B) Recombinant human OPG inhibited osteoclast differentiation in BMC cultures from 4 to 8 subjects older than 55 years. Bars represent the mean values of 4-8 specimens. * $p=0.035$; ** $p=0.004$

Fig. 4.

Effects of subject age on constitutive expression of osteoclast differentiation factors in hMSCs. (A) With the age of subjects ($n=23$), there was a positive correlation for *RANKL* expression ($r=0.41$, $p=0.049$) and an inverse correlation for *OPG* ($r=-0.43$, $p=0.039$). There was a trend of 2.3-fold ($p=0.072$) higher expression of *RANKL* in hMSCs from the older subjects (shaded bar, $n=10$) than for the younger subjects (open bar, $n=13$) and a trend of 1.4-fold ($p=0.088$) higher expression of *OPG* in hMSCs from the younger subjects (open bar, $n=13$) than that for the older subjects (shaded bar, $n=10$). M-CSF expression did not appear to vary with age. (B) The ratio of RANKL/OPG was significantly correlated with the age of subject ($r=0.56$, $p=0.005$).

Table 1
Primer pairs and amplification conditions

Target mRNA	Primer sequences (5' → 3')	Annealing temperature (°C)	Product size (bp)	Reference
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	60	452	Clontech, BC004109.1
OPG	F: GAACCCAGAGCGAAATACA R: CGCTGTTTTACAGAGGTCA	60	441	Makhluf <i>et al.</i> , 2000
c-fms	F: CAGATTGGTATAGTCCGCTCTCT R: TCCAACACTATTGTCAAGGGCAAT	60	360	Kirma <i>et al.</i> , 2007
RANK	F: CCTACGCACAAGGCGAAGATGC R: CGTAGACCACGATGATGTCGCC	62	702	Atkins <i>et al.</i> , 2000
TRAP	F: CTGGCTGATGGTGCCACCCCTG R: CTCTCAGGCTGCAGGCTGAGG	61	469	Eslami <i>et al.</i> , 2011
RANKL	F: ATCCCATCTGGTTCCCATAA R: CCCTGACCAATACTTGGTGC	55	276	Eslami <i>et al.</i> , 2011
M-CSF	F: ATGACAGACAGGTGGAAGTGCAGTGTAAGG R: TCACACAACCTTCAGTAGGTTCAGGTGATGGGC	60	495	Clontech, XM_002150.2