Effects of Phytoestrogen α-ZAL and Mechanical Stimulation on Proliferation, Osteoblastic Differentiation, and OPG/RANKL Expression in MC3T3-E1 Pre-Osteoblasts

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Abstract—Estrogen and mechanical strain are two important regulators of bone remodeling. α-zearalanol (α-ZAL) is a new oomycete phytoestrogen that is similar to 17-beta estradiol, but has less adverse effects. The aim of this study was to investigate cell proliferation, differentiation, osteoprotegerin (OPG), and receptor activator of nuclear factor-κB ligand (RANKL) expression in the pre-osteoblast MC3T3-E1 cell line in response to α- ZAL, mechanical strain, or a combination of both. Cell proliferation was assessed by flow cytometry, and alkaline phosphatase (ALP) activity was measured using a spectrophotometric detection kit. mRNA expression of ALP, runt-related transcriptional factor 2 (Runx2), OPG, and RANKL genes was determined by real-time Reverse Transcription-Polymerase Chain Reaction (real-time RT-PCR). Protein expression of Runx2, OPG, and RANKL was determined by Western blotting. α-ZAL alone (10^{-6}–10^{-10} M) suppressed proliferation of MC3T3-E1 cells and promoted the activity and gene expression of ALP, an early marker of osteogenesis. It also enhanced Runx2 mRNA expression and the OPG/RANKL ratio. Mechanical strain alone promoted proliferation and differentiation of MC3T3-E1 cells. In addition, Runx2 protein, but not mRNA expression, was upregulated and the OPG/RANKL ratio increased. Although a combination of α-ZAL and mechanical strain inhibited cell proliferation, high concentrations of α-ZAL (10^{-6} M) combined with a high magnitude of strain (2500 $\mu\varepsilon$), significantly enhanced ALP activity. α-ZAL with a high magnitude of strain increased RUNX2 protein expression, but lowered the OPG/RANKL ratio. The OPG/RANKL ratio significantly increased in response to α-ZAL combined with a low magnitude of strain (1000 $\mu\varepsilon$). Thus, α-ZAL might be a promising candidate for the treatment of osteoporosis. Mechanical strain was effective in inducing osteogenic differentiation. Taken together, our data suggest that α-ZAL combined with a low, but not high magnitude of strain, may provide benefits for osteogenesis.

Keywords—α-Zearalanol, Mechanical strain, Osteoporosis, Bone formation.

INTRODUCTION

Age-related osteoblast (OB) dysfunction is one of the determinants for the development of osteoporosis (OP), which is a reduction in skeletal mass due to an imbalance between bone resorption and bone formation.[12] Clinically, estrogen replacement treatment (ERT) has long been considered a potent anti-resorptive agent, since estrogen regulates bone remodeling and balances interactions between OBs and osteoclasts (OCs).[19,21] Estrogen receptors are present in both OBs and OCs. On one hand, estrogen preferentially promotes the development of OBs over adipocytes from their common precursor cell[24]; it also increases OB proliferation, inhibits apoptosis,[10] and induces production of a number of proteins released by OBs, such as insulin-like growth factor-1, type I procollagen, transforming growth factor-β, and bone prophogenetic protein-6. Thus, estrogen tends to have an anabolic effect on isolated OBs.[11] On the other hand, estrogen suppresses osteoclast activity *via* increased osteoclast apoptosis, reduced OB/stromal cell production of receptor activator of nuclear factor-κB ligand (RANKL), and increased production of osteoprotegerin (OPG).[37] The widespread use of ERT could effectively eliminate postmenopausal osteoporosis.

However, it requires long-term, and even lifetime administration,[34] which may result in an increased incidence of breast cancer, thromboembolic events, and other adverse reactions.

Phytoestrogens are biologically active plant substances that are similar to estrogens and include genistein, daidzein, coumestrol, α-zearalanol, zearalenone, naringenin, taxifolin, and biochanin. Their similar structure to estrogen accounts for both their capability to bind the estrogen receptor (ER), and the fact that they possess most of the positive functions of estrogen. However, they have less negative effects than estrogen. Recently, phytoestrogens have been used for treatment of OP.[4,28] Shunling *et al.*[5] began their research on phytoestrogen replacement therapy in humans in 1996. Previous studies have shown that it has anti-atherosclerotic effects that are similar to the endogenous estrogen 17β-estradiol (E2), while its effects on uterus and mammary gland proliferation is significantly lower than the latter, suggesting its therapeutic implication in OP treatment. α-zearalanol (α-ZAL) is produced by mold and frequently infects pasture grasses, legumes, and corn. In veterinary medicine, α-ZAL is used in animal feed as a growth promoter, anabolic agent, and estrogenic agent. However, whether α-ZAL can be applied for treatment of osteoporosis has not been reported.

Mechanical forces are important regulators of bone remodeling.[29] Exercise programs can be developed that will effectively stimulate bone growth.[6] Many studies have shown that mechanical forces are crucial for the regulation of osteoblastic proliferation, differentiation, and apoptosis.[13,30] In our previous work, we found that physiological levels of mechanical strain [i.e., 1000 microstrain ($\mu\varepsilon$), 2500 $\mu\varepsilon$] could promote proliferation of mouse MC3T3-E1 pre-osteoblasts and directly upregulate the mRNA expression of osteogenic markers.[43] In this study, we combined α-ZAL with mechanical strain to examine their effects on OB proliferation and differentiation, as well as the expression of OPG and RANKL in pre-osteoblastic MC3T3-E1 cells.

MATERIALS AND METHODS

Cell Culture and Grouping

The mouse pre-osteoblast cell line, MC3T3-E1, was seeded at a density of 12,000 cells/cm^2 in a tissue culture flask with α-MEM (Invitrogen, San Diego, CA, USA) supplemented with 10% FBS and 1% penicillin–streptomycin (Hyclone, Logan, UT, USA). Cells were cultured at 37 °C under a humidified incubator of 95% air and 5% CO$_2$. The cells were divided into the

TABLE 1. Definition of combination group.

	L6	L8	L10	M6	M8	M10
α-ZAL (M)	10^{-6}	10^{-8}	10^{-10}	10^{-6}	10^{-8}	10^{-10}
Mechanical forces ($\mu\varepsilon$)	1000	1000	1000	2500	2500	2500

following three groups: (A) drug group, medium lacked drug or was supplemented with α-ZAL (10^{-6}, 10^{-8}, 10^{-10} M); (B) loading group, cells either lacked mechanical strain or were subjected to mechanical strain of 1000 and 2500 $\mu\varepsilon$ at 0.5 Hz for 1 h once a day.[45] In this study, we used a specially designed four-point bending device as the cell stretching apparatus to investigate the mechanical response in osteoblasts. The four-point bending device has been shown to produce homogenous, predominantly uniaxial strains of the cell culture substrate so that every cell is subjected to the same deformation[39]; (C) combination group, cells were simultaneously treated with α-ZAL (10^{-6}, 10^{-8}, 10^{-10} M) and mechanical strain as shown in Table 1.

Cells were seeded at a density of 10,000 cells/cm^2 in tissue culture dishes. After 24 h, the culture medium was replaced with culture medium containing drug. Then the cell cultures were subjected to a certain magnitude of mechanical strain until they reached 85% confluence at 72 h. Cells were analyzed by flow cytometry, an ALP assay, real-time RT-PCR, and Western blot analysis.

Measurement of Cell Proliferation by Flow Cytometry

The percentage of cells undergoing proliferation was determined by flow cytometry. Briefly, the cells were harvested, washed three times with PBS, and collected by centrifugation before being fixed in 75% cool ethanol and stored at 4 °C. Samples were washed two times with PBS to remove ethanol before flow cytometric analysis. Cells were stained with 10 pg/mL propidium iodide for 30 min for analysis. DNA content was measured and the cell cycle was analyzed using the EPICS flow cytometer (Coulter, Miami, FL, USA). FCM-DNA quantitative analysis divides diploid DNA content distribution into three parts, namely, the G_0/G_1, S, and G_2/M phases. Scattered cells above the ploidy-establishing peak and in the S and G2/M range represented the Proliferation Index (PI) and were counted as the percentage of the total number of OBs. The PI of each sample was calculated as follows to represent the proliferating cell population.[31]

$$PI(\%) = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$$

ALP Staining

Cells were harvested, rinsed three times with PBS, fixed in 95% ethanol, and then rinsed again with PBS. Cells were then stained with the ALP staining kit (Genmed, Shanghai, China) according to the manufacturer's instructions. Cell morphology was examined under a light microscope (Olympus, Japan).

ALP Activity Assay

Cells were lysed in 200 μL/dish of lysis buffer (10 mM Tris pH 8.0, 1 mM MgCl$_2$, 0.5% Triton X-100), sonicated, and centrifuged to remove cell debris. ALP activity in the cellular fraction was measured by a fluorometric detection kit (Nanjing Jiancheng Biotechnology Co. Ltd, Nanjing, China). The ALP activity of each sample was normalized to protein concentration.

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted with Trizol (Invitrogen), and RNA integrity was verified by denaturing agarose gel electrophoresis. The concentration of total RNA was determined by the Quant-iT RNA assay kit (Invitrogen). Reverse transcription was performed with 1 μg of RNA in a total volume of 20 μL per reaction using Revertra Plus (Toyobo, KITA-KU, OSAKA, Japan). Quantitative RT-PCR was performed to determine mRNA levels of ALP, RUNX2, OPG, and RANKL, using a pair of primers specific to those genes (Table 2) on a 7700 real-time PCR System (ABI, LA, CA, USA) with a Brilliant SYBR Green master mix. The amplification reaction included three steps: (1) 94 °C for 3 min; (2) 94 °C for 15 s; and (3) annealing and extension at each annealing temperature for 30 s. Steps 2 and 3 were repeated for 35 cycles. The fold change was calculated using the control sample Ct values at each specified time point as a calibrator using the $2^{-\Delta\Delta CT}$ method.[18] Three independent experiments were carried out to determine relative mRNA levels.

Western Blot Analysis

Cells were solubilized in modified RIPA buffer (1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethyl-sulfonyl fluoride, 1 mg/mL aprotinin, leupeptin, pepstatin, and 1 mM sodium ortho-vanadate in 50 mM Tris–HCl, pH 7.4). Approximately 30 μg of protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) to determine OPG and RANKL expression. Subsequently, the proteins were transferred to nitrocellulose membranes. The membranes were blocked in TBS-T with 5% milk for 1 h and probed overnight at 4 °C with rabbit anti-OPG and rabbit anti-RANKL antibodies (1:500, Boster, Wuhan, China). After washing, the membranes were incubated with HRP-conjugated secondary antibodies. The immunoreactive bands were visualized using the enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The optical density of the protein bands was determined with Gel Doc 2000 (Bio-Rad, WA, CA, USA). The expression of GAPDH was used as a loading control. Results were presented relative to control cells without treatment.

Statistical Analysis

All experiments were performed in triplicate and repeated at least three times. Data are presented as mean ± SD and were analyzed using one-way ANOVA followed by LSD multiple comparison tests to determine the significance between groups. Statistical analysis was performed using SPSS software version 13.0. The p values < 0.05 were considered statistically significant.

RESULTS

Effects of α-ZAL, Mechanical Strain, and a Combination of Both on the Proliferation of MC3T3-E1 Cells

We used flow cytometry to examine the cell cycle to determine the effects of drug, mechanical strain, and a

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TABLE 2. Oligonucleotides used in real-time RT-PCR.

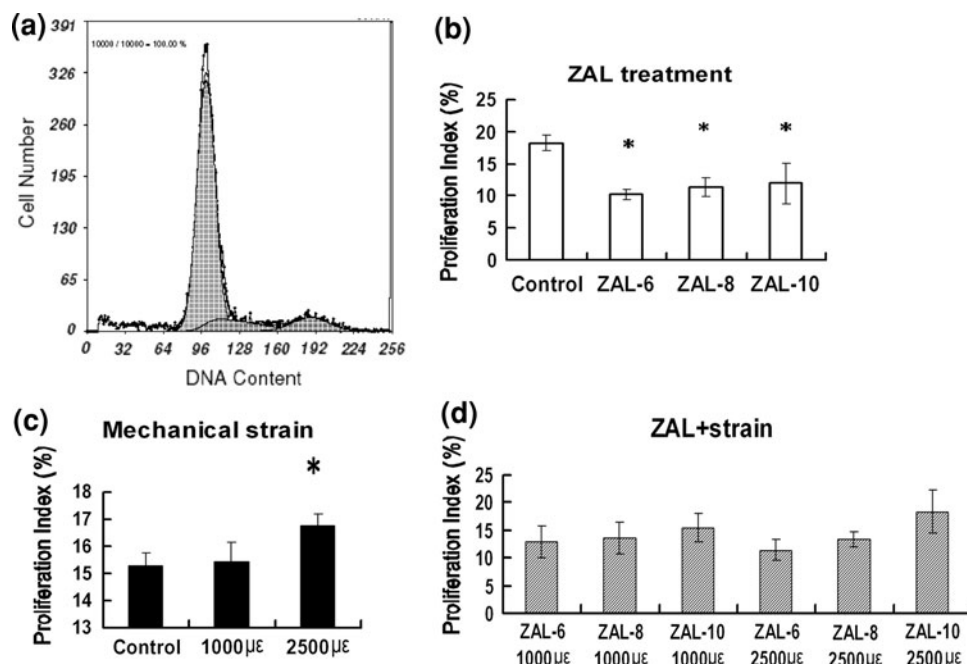| Gene	Primer sequence (5′–3′)	Products size (bp)	Annealing temperature (°C)
ALP (NM 007431.2)	F:CGGGACTGGTACTCGGATAA: R:ATTCCACGTCGGTTCTGTTC	157	58
RUNX2 (NM 001145920.1)	F: AGTAGCCAGGTTCAACGAT R:GGAGGATTTGTGAAGACTGTT	90	58
OPG (NM 008764.3)	F:AGTCTGAGGAAGACCATGAGR:AAACAGCCCAGTGACCATTC	205	56
RANKL (NM 011613.3)	F:CCAAGATGGCTTCTATTACCR:TCCCTCCTTTCATCAGGTTAT	152	53

FIGURE 1. Effects of α-ZAL, strain, and a combination of both, on the proliferation of MC3T3-E1 cells: (a) profiles of control; (b–d) are PIs (%) of groups A, B, and C. α-ZAL inhibited PI at all detected concentrations (b). Mechanical strain at 2500 $\mu\varepsilon$ increased PI (c), while α-ZAL combined with strain had no difference on PI values (d). The results are expressed as mean ± SD from three individual experiments. *$p < 0.05$ vs. controls.

combination of both, on MC3T3-E1 cell proliferation after 72 h of culture. As shown in Fig. 1b, compared to cells without treatment, α-ZAL concentrations of 10^{-6} to 10^{-10} M inhibited the proliferation of MC3T3-E1 cells. In contrast, mechanical strain promoted the proliferation of MC3T3-E1 cells (Fig. 1c). Compared to control cells, 2500 $\mu\varepsilon$ strain significantly increased the PI ($p < 0.05$). The control data in groups A and B were a little inconsistent because the cells were from different batches, and thus had different physiological states. In the combination treatment group (Fig. 1d), there were no significant differences when drug and mechanical strain were coupled. Compared to the ZAL treatment group, the PI values increased at all concentrations and at different levels of mechanical strain, and the PI of M10 (ZAL-10, 2500 $\mu\varepsilon$) was close to that of the control, indicating that mechanical strain can relieve α-ZAL-induced inhibition of MC3T3-E1 cell proliferation.

ALP Staining and Effects of α-ZAL, Mechanical Strain, and a Combination of Both, on ALP Activity and ALP mRNA Expression

ALP is an early differentiation marker for OBs. Figure 2 shows ALP images of each group. Cells treated with 10^{-6} M of α-ZAL showed more positive staining than the control, indicating an increase in ALP activity. In addition, 2500 $\mu\varepsilon$ mechanical strain and a combination of 10^{-6} M α-ZAL and 2500 $\mu\varepsilon$ mechanical strain also increased ALP activity. Figure 3 shows the effects of drug, strain, and a combination of both on ALP activity. α-ZAL increased ALP activity in a dose-dependent manner with a significant increase at a concentration of 10^{-6} M (Fig. 3a, $p < 0.05$). Mechanical strain of 2500 $\mu\varepsilon$ promoted ALP activity (Fig. 3c, $p < 0.05$). When the two factors were combined (Fig. 3e), mechanical strain of 2500 $\mu\varepsilon$ led to higher ALP activity than that of 1000 $\mu\varepsilon$ at all drug concentrations ($p < 0.05$). Maximum ALP activity was achieved with α-ZAL-6 plus 2500 $\mu\varepsilon$, which was fourfold more than the control. This indicates that a high magnitude of strain can better promote ALP activity in combination with α-ZAL. We also determined whether such upregulation of ALP activity resulted from an increase in ALP mRNA levels. As shown in Figs. 3b, d, and f, ALP mRNA expression after treatment with α-ZAL showed the same trend as ALP activity. Mechanical strain of 2500 $\mu\varepsilon$ led to higher ALP mRNA levels than that of 1000 $\mu\varepsilon$ or control (Fig. 3d, $p < 0.05$). In the combination group, ALP mRNA expression after treatment with ZAL-6 and strain of 2500 $\mu\varepsilon$ was enhanced fourfold compared to the control.
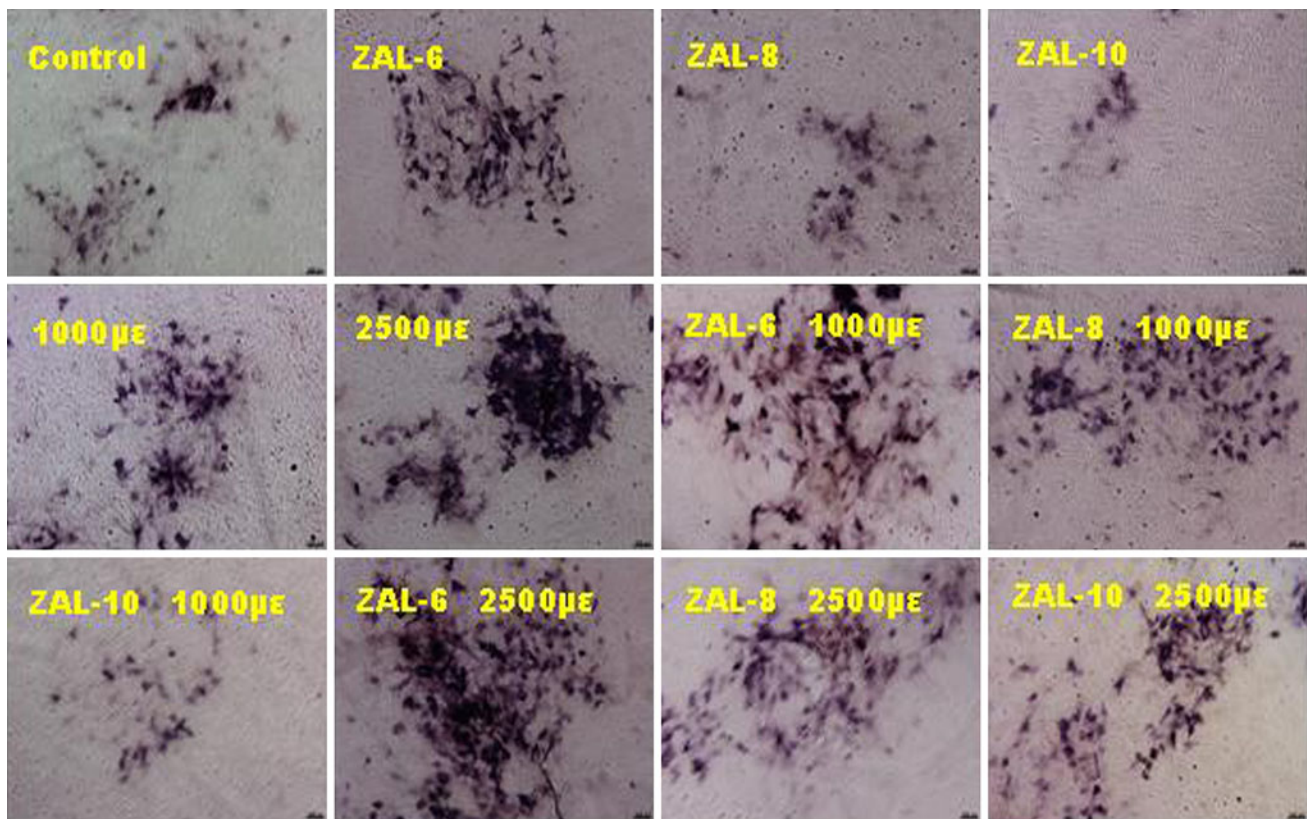
FIGURE 2. ALP staining of cells subjected to α-ZAL and mechanical strain. ZAL-6, 2500 $\mu\varepsilon$ mechanical strain, ZAL-6 plus 1000 $\mu\varepsilon$ strain, and ZAL-6 plus 2500 $\mu\varepsilon$ strain have relatively more blue areas. Scale bar—100 μm.

Effects of α-ZAL, Mechanical Strain, and a Combination of Both on RUNX2 mRNA and Protein Expression

RUNX2 is an essential mediator of the OB phenotype and plays a pivotal role in the process of OB differentiation. Figures 4a, c, and e show the effects of α-ZAL, mechanical strain, and a combination of both on Runx2 expression. α-ZAL upregulated Runx2 mRNA expression in a dose-dependent manner (Fig. 4a, $p < 0.05$) with no effect on RUNX2 protein expression (Fig. 4b, $p > 0.05$), indicating that α-ZAL upregulates Runx2 expression at the transcriptional level to regulate osteogenesis differentiation. Mechanical strain downregulated Runx2 mRNA expression, but increased its protein expression (Figs. 4c, d); very little drug effects were seen in cells subjected to combination treatment. RUNX2 mRNA expression in cells subjected to a low magnitude of strain (1000 $\mu\varepsilon$) was significantly higher than control cells and cells subjected to a high magnitude of strain (2500 $\mu\varepsilon$). On the contrary, protein levels in cells subjected to a low magnitude of strain were significantly lower than in cells subjected to a high magnitude of strain.

Effect of α-ZAL, Mechanical Strain, and a Combination of Both on OPG and RANKL mRNA Expression

Although α-ZAL had no significant effect on OPG mRNA levels (Fig. 5a), RANKL mRNA levels significantly decreased (Fig. 5b); thus, the OPG/RANKL mRNA ratio increased compared to the control in the ZAL treatment group (Fig. 6a), indicating that α-ZAL favors osteogenesis and inhibits bone resorption. Mechanical strain (2500 $\mu\varepsilon$) increased OPG and RANKL mRNA expression (Figs. 6c, d). Furthermore, there was a higher increase in OPG over RANKL expression. Therefore, although the OPG/RANKL ratio slightly increased, this increase was not statistically significant.

The results from the combination treatment group were much more complicated. Treatment of cells with α-ZAL (10^{-6} M) led to increased OPG production in response to a low magnitude of strain (1000 $\mu\varepsilon$), but had no effect in response to a high magnitude strain (2500 $\mu\varepsilon$), as shown in Fig. 5e. Furthermore, drug combined with a low magnitude of strain (1000 $\mu\varepsilon$) led to high levels of OPG production, but low levels of RANKL production compared to drug combined with a high magnitude of strain (2500 $\mu\varepsilon$) (Figs. 5e, f). As a
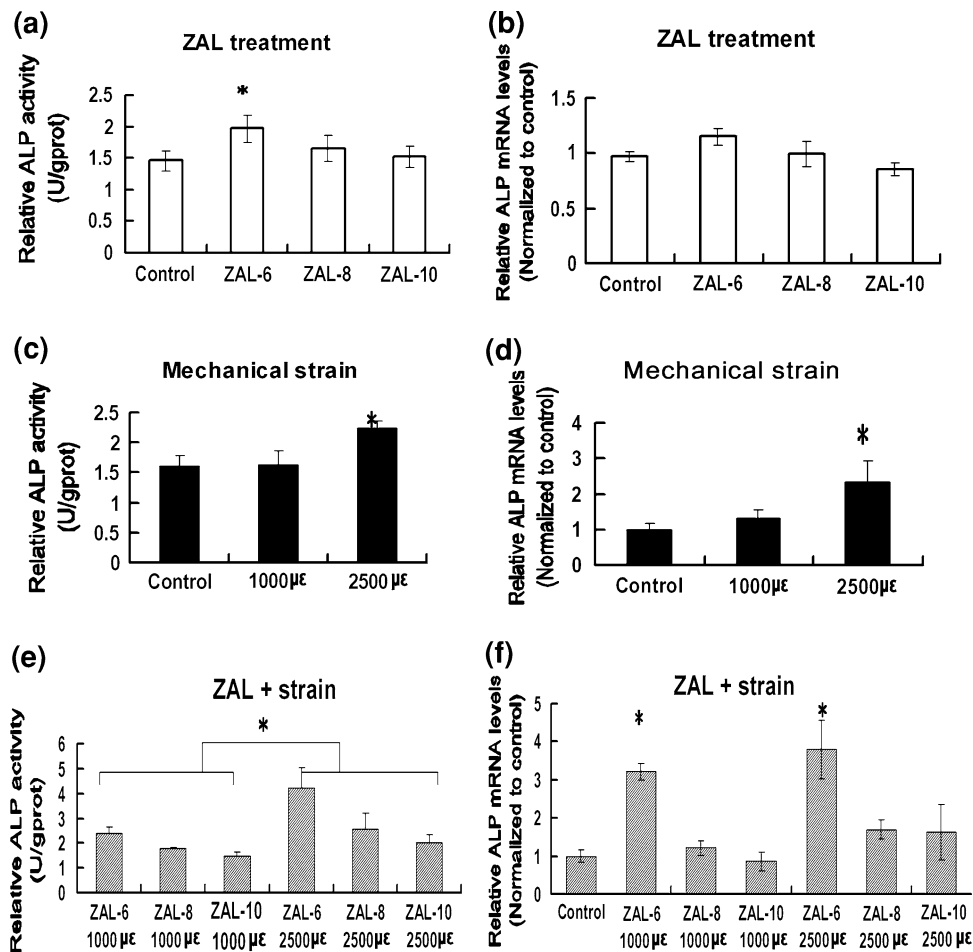
FIGURE 3. Effects of α-ZAL, mechanical strain, and a combination of both on ALP activity and ALP mRNA expression in MC3T3-E1 cells treated with α-ZAL or mechanical strain for 72 h. 10^{-6} M of α-ZAL (a) and 2500 $\mu\varepsilon$ mechanical strain (c) significantly increased ALP activity. α-ZAL alone had no effect on ALP mRNA expression (b). 2500 $\mu\varepsilon$ of mechanical strain (d) significantly increased ALP mRNA expression. Cells treated with both 10^{-6} M α-ZAL and 2500 $\mu\varepsilon$ mechanical strain showed high ALP activity (e) and high ALP mRNA expression (f). Results are shown as mean ± SD from three independent experiments. *$p < 0.05$ vs. control or between groups.

result, a low magnitude of strain (1000 $\mu\varepsilon$) combined with α-ZAL significantly increased the OPG/RANKL ratio in a dose-dependent manner due to high expression of OPG in response to α-ZAL treatment; a high magnitude of strain (2500 $\mu\varepsilon$) combined with α-ZAL decreased the OPG/RANKL ratio compared to the control due to high RANKL expression in response to mechanical strain (Fig. 6c).

Effect of α-ZAL, Mechanical Strain, and a Combination of Both on OPG and RANKL Protein Expression

The protein expression of OPG and RANKL had a different pattern than mRNA expression. Only 10^{-6} M of α-ZAL increased OPG expression (Fig. 7a), with no significant effects on RANKL protein levels (Fig. 7b). Hence, α-ZAL increased the OPG/RANKL ratio at a concentration of 10^{-6} M (Fig. 8a). Mechanical strain

increased OPG expression in a magnitude-dependent manner (Fig. 7c), with no significant effects on RANKL expression (Fig. 7d), thereby increasing the OPG/RANKL ratio (Fig. 8b). α-ZAL treatment combined with a low magnitude of strain (1000 $\mu\varepsilon$) dose-dependently increased OPG expression (Fig. 7e) without affecting RANKL levels. α-ZAL treatment combined with a high magnitude of strain dose-dependently downregulated RANKL protein levels (Fig. 7f). As a result, a low magnitude of strain (1000 $\mu\varepsilon$) combined with α-ZAL elevated the OPG/RANKL ratio in a dose-dependent manner (Fig. 8c).

DISCUSSION

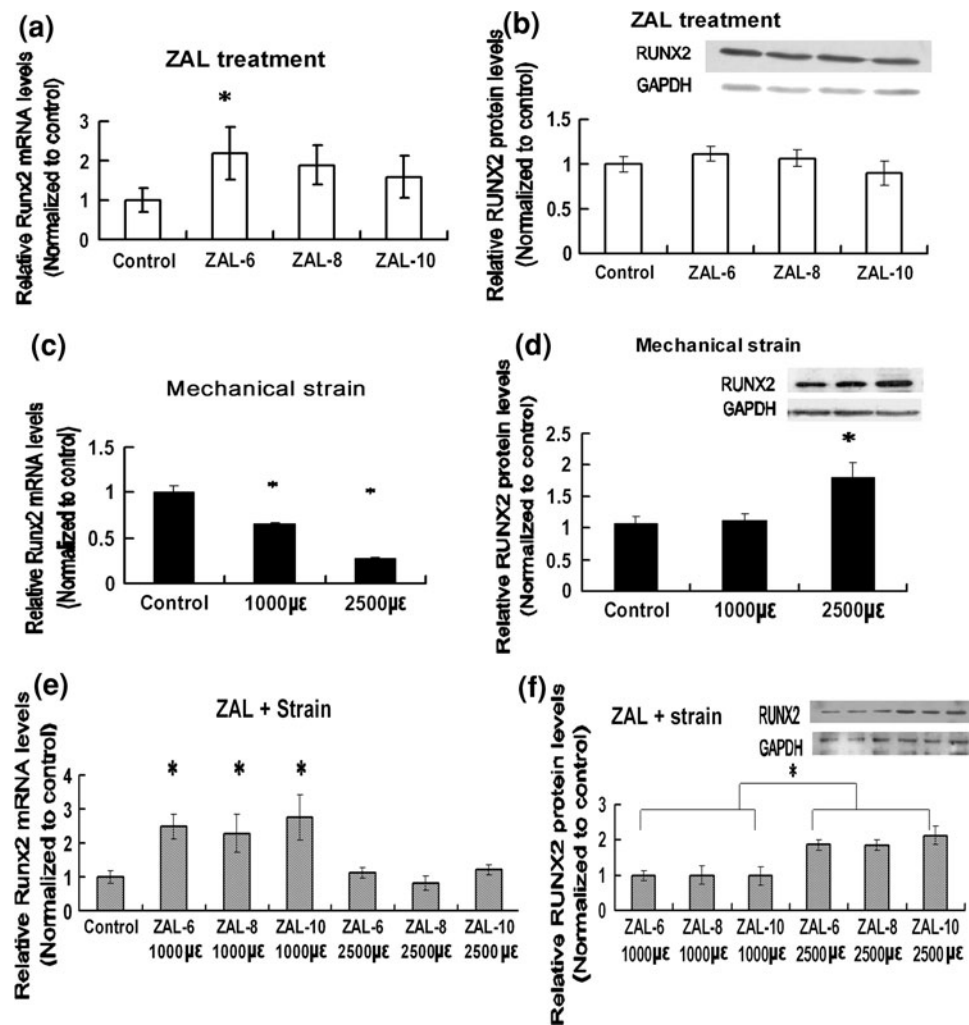Estrogen and mechanical strain are two important regulators in bone remodeling. Low concentrations of

FIGURE 4. Effects of α-ZAL, mechanical strain, and a combination of both on RUNX2 mRNA and protein expression (a, c, and e): mRNA expression; (b, d, and f): protein expression. MC3T3-E1 cells were treated with α-ZAL, mechanical strain, or a combination of both for 72 h. 10^{-6} M of α-ZAL (a), drug with low magnitude of strain (1000 $\mu\varepsilon$) (e) significantly increased Runx2 mRNA expression after treatment. Mechanical strain (c) significantly downregulated Runx2 mRNA expression. α-ZAL alone (b) had no effect on RUNX2 protein expression. Protein expression was opposite that of mRNA expression in cells treated with drug and mechanical strain combined with drug (d, f). Results are shown as mean ± SD of data from three independent experiments. *$p < 0.05$ vs. controls or between groups.

estrogen and the appropriate mechanical strain can promote the activation of OBs and bone formation.[38] However, their mechanisms of action are not the same. Estrogen acts on bone cells both directly and indirectly.[27] The ER, including ERα and ERβ, are present in OBs. E2 directly binds to the ER, which results in estrogen response element (ERE)-linked transcriptional activation. ER mediates most functions of estrogen, including regulation of OB proliferation and differentiation, synthesis of the extracellular matrix (ECM), inhibition of bone resorption, and promotion of osteoclast apoptosis.[16,41] Indirectly, it increases production of a number of cytokines, such as IGF-1, TGF-β, and OPG. These cytokines are produced by osteoblasts in autocrine and paracrine manners, and

transmit chemical signals and molecular signals, respectively, to complete their functions.[15,26,36]

Mechanical strain also plays an important role in bone homeostasis. Application of mechanical strain increases the cell proliferation of OBs, increases cell proliferation, increases cell mitosis, and increases the synthesis of ECM (collagen, non-collagen protein and proteoglycan).[22] Many studies have demonstrated that mechanical strain can influence the expression of bone morphogenetic proteins (BMPs), suggesting the potent regulatory role of the BMP/Smad signaling pathway in the mechanical signal transduction in osteoblasts.[25] Mechanical loading influences cell function depending upon mode of action, duration, and loading size. In our previous work, we found that OBs were more
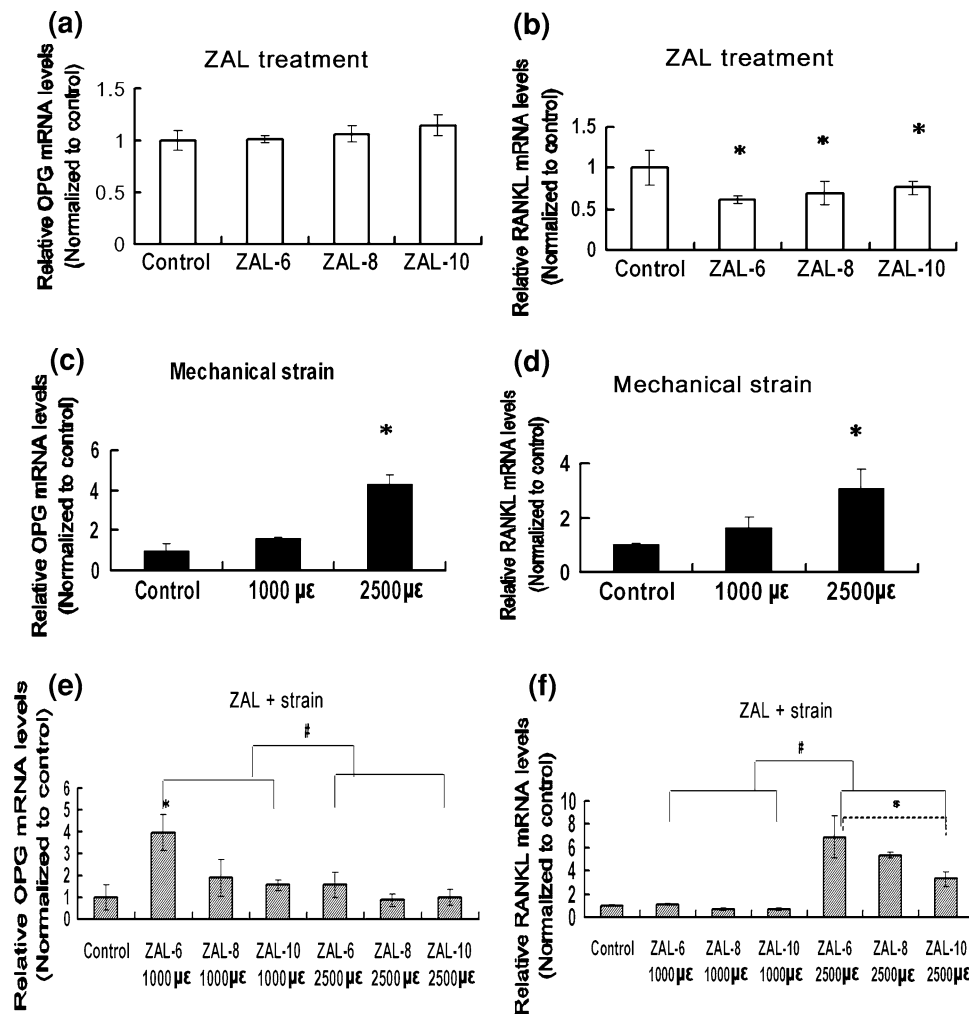
FIGURE 5. The effect of α-ZAL, mechanical strain, and a combination of both on OPG and RANKL mRNA expression. (a, c, and e): OPG expression; (b, d, and f): RANKL expression. MC3T3-E1 cells were treated with α-ZAL, mechanical strain, or a combination of both for 72 h. α-ZAL had no effect on OPG expression (a), but decreased RANKL expression in a dose-dependent manner (b); mechanical strain of 2500 $\mu\varepsilon$ significantly enhanced both OPG expression (c) and RANKL expression (d); OPG mRNA expression in cells treated with drug and a low magnitude of strain (1000 $\mu\varepsilon$) (e) was higher than that of drug with a high magnitude of strain (2500 $\mu\varepsilon$). RANKL expression in cells treated with drug and a low magnitude of strain (1000 $\mu\varepsilon$) (f) was lower than that in cells treated with drug and a high magnitude of strain (2500 $\mu\varepsilon$). Results are shown as mean ± SD of data from three independent experiments. *$p < 0.05$ vs. controls or between groups (e, f).

sensitive to intermittent or cyclical stress. Intermittent mechanical stimulation (1 h per day) promoted DNA synthesis and alkaline phosphatase activity.[46] Therefore, we selected intermittent mechanical strain in this study.

Studies have shown that estrogen and mechanical strain may share common signaling transduction pathways; however, knowledge about this is very limited. Zaman *et al.*[48] found that mechanical strain activates estrogen response elements in ROS.SMER#14 cells (ROS 17/2.8 cells stably transfected with ERα). Lambertini *et al.*[9] demonstrated that the human ERα gene is a target of the Runx2 transcription factor in OBs, which is the most critical transcription factor that

regulates OB differentiation and bone formation *in vitro* and *in vivo*.[23] In addition, the ER was shown to cross-talk with Wnt signaling to regulate early gene expression in response to mechanical strain in osteoblastic cells.[1] In our study, the results of OB proliferation and differentiation by α-ZAL treatment combined with mechanical strain also demonstrated that they share some common actions.

Reduction of OB proliferation is one of the important cellular mechanisms of OP. ALP activity is an osteoblast differentiation marker and bone turnover indicator. Although α-ZAL alone inhibited cell proliferation at a concentration of 10^{-6} to 10^{-10} M, mechanical strain promoted cell proliferation. As a
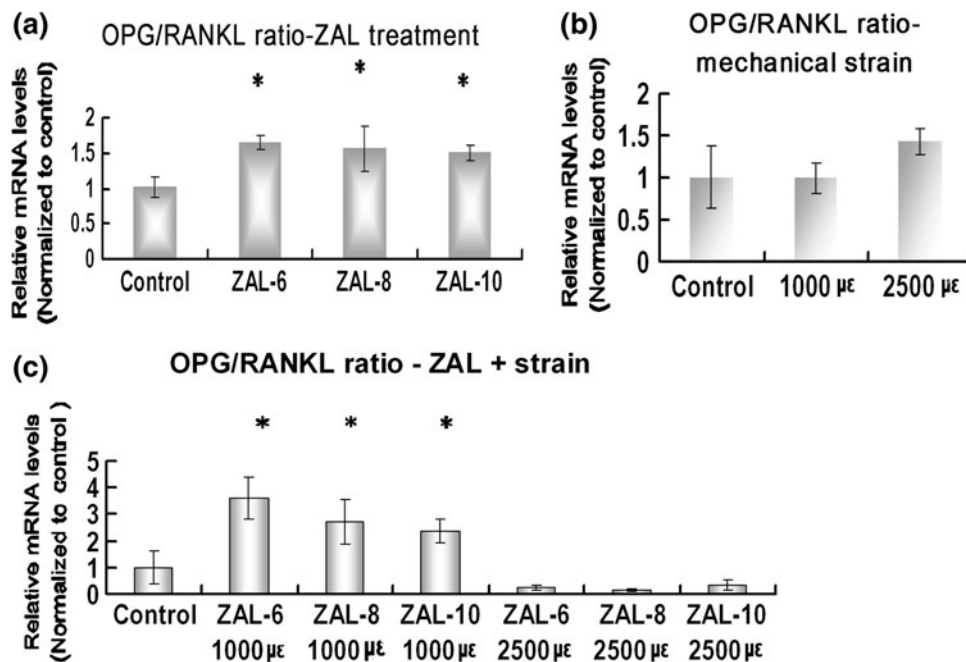
FIGURE 6. Effect of α-ZAL, mechanical strain, and combination of both on the OPG/RANKL mRNA ratio. MC3T3-E1 cells were treated with α-ZAL, mechanical strain, or a combination of both for 72 h. α-ZAL (a) enhanced the ratio at the mRNA level. Mechanical strain (b) had no effect on the ratio. The ratio increased in response to a low of magnitude strain (1000 $\mu\varepsilon$) coupled with α-ZAL (c). Results are shown as mean ± SD of data from three independent experiments. *$p < 0.05$ vs. controls.

result, we found that OB proliferation under a combination of both drug treatment and mechanical strain (10^{-10} M, 2500 $\mu\varepsilon$) was similar to that of normal cells. Furthermore, the highest concentration of α-ZAL (10^{-6} M) combined with highest physical mechanical strain (2500$\mu\varepsilon$) induced a fourfold increase in ALP activity, suggesting that a combination of both favors bone formation.

RUNX2 is the most critical transcription factor in the regulation of osteogenic differentiation. It binds to the OB-specific cis-acting element 2 (OSE2), which is found in the promoter region of all major OB-specific genes (i.e., osteocalcin, type I collagen, bone sialoprotein, osteopontin, ALP, and collagenase-3), and controls their expression.[8] Runx2 is regulated by a variety of cytokines, hormones, and endogenous substances. In our study, α-ZAL increased RUNX2 mRNA expression, which is consistent with an increase in‘ ALP activity, indicating that the phytoestrogen α-ZAL can promote the differentiation of OBs. This might be related to estrogen-stimulated Runx2-II promoter activity.[44] However, RUNX2 protein expression did not increase. This discrepancy may be due to the complicated transcriptional and post-transcriptional regulation of RUNX2, including epigenetic mechanisms and ubiquitin–proteasome-mediated hydrolysis.[35,47] A high magnitude of mechanical strain alone increased RUNX2 protein expression, which is consistent with an increase in ALP activity. The possible

mechanism underlying this affect is that mechanical strain stimulated an increase in bone morphogenetic protein (BMP) expression, indirectly mediated by Dxl, resulting in an increase in RUNX2 expression.[17] However, mechanical decreased Runx2 expression at the mRNA level. It is possible that the level of gene product is controlled, via negative feedback, by its own abundance (negative auto-regulation mechanism). Among gene 5′ expression regulation sequence there are at least three binding sites for themselves. As long as there is one complete self-binding site exists, over-expression of Runx2 can autoregulatory inhibit its gene expression.[33] There was also a discrepancy between OPG and RANKL mRNA and protein expression levels. A possible reason is that both OPG and RANKL are secreted cytokines, which when secreted into the culture medium, cannot be fully detected in cells.[7,14] ALP activity, however, was consistent with ALP mRNA levels. The precise mechanisms underlying the effects of drug and mechanical strain still need to be determined.

Osteoblasts express key factors that modulate osteoclastogenesis, including receptor activator of nuclear factor-κB ligand (RANKL), a member of the tumor necrosis factor (TNF)-ligand family, and osteoprotegerin (OPG). RANKL binds to its receptor RANK on osteoclast progenitors and stimulates their differentiation and activity. OPG acts as a non-signaling decoy receptor, binds RANKL, and prevents
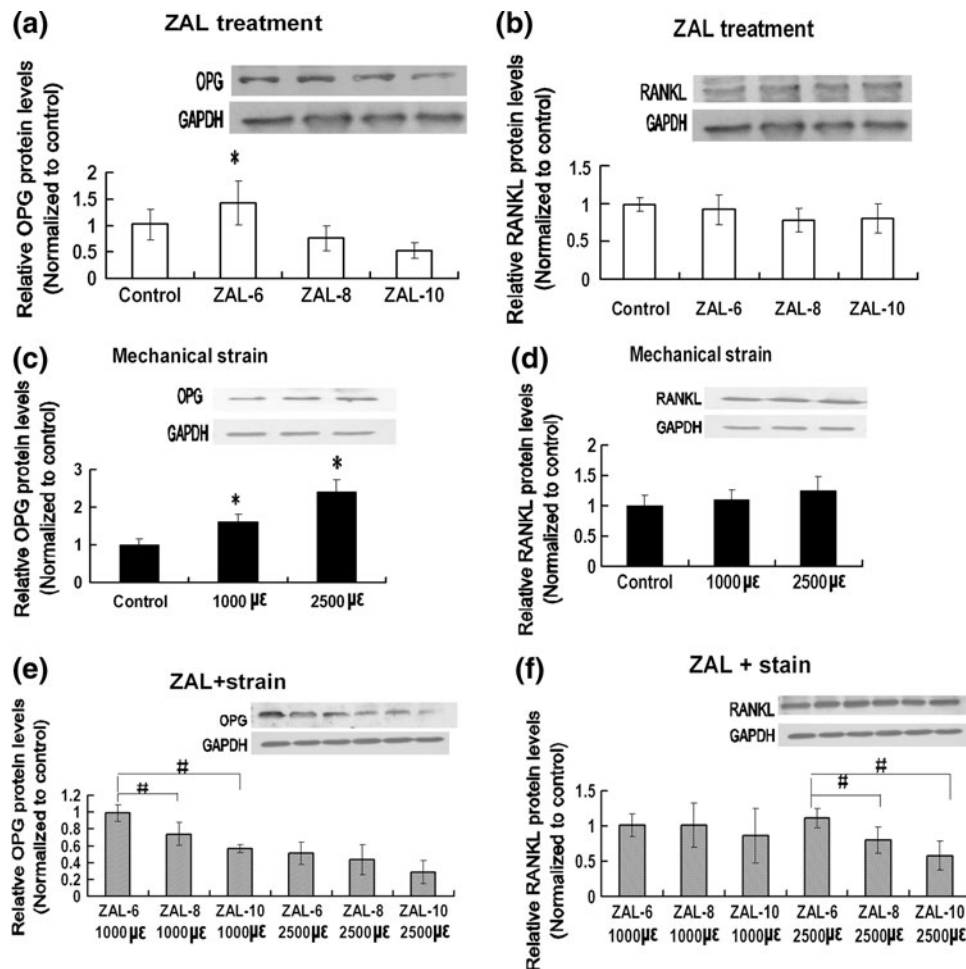
FIGURE 7. Effect of α-ZAL, mechanical strain, and a combination of both on OPG and RANKL protein expression. (a, c, and e): OPG expression; (b, d, and f): RANKL expression. MC3T3-E1 cells were treated with α-ZAL, mechanical strain, or a combination of both for 72 h. 10^{-6} M of α-ZAL (a) and mechanical strain (c) significantly increased OPG expression, while the two factors had no effect on RANKL expression (b, d). Drug combined with a low magnitude of strain increased OPG expression in a dose-dependent manner (e), but had no effect on RANKL expression (f); drug coupled with a high magnitude of strain decreased RANKL expression in a dose-dependent manner, but had no effect on OPG expression. Results are shown as mean ± SD of data from three independent experiments. *$p < 0.05$ vs. controls, #$p < 0.05$ between groups.

activation of RANK, resulting in decreased osteoclast recruitment. The RANK/RANKL/OPG axes couple OB and osteoclast activity, thereby controlling the balance between bone formation and resorption.[2] The ratio should be considered a quantitative osteogenic activity standard, which represents drug, strain, or a combination of both on bone tissue metabolism *in vitro*. OPG and RANKL are regulated by a variety of hormones and endogenous substances. Osteotropic factors, such as parathyroid hormone, $1,25(OH)_2D_3$, and prostaglandin, increase the ratio of RANKL and OPG in favor of RANKL, and can support osteo-clastogenesis, whereas estrogens can inhibit osteoclast recruitment by changing the RANKL/OPG ratio in favor of OPG.[42] Our study confirmed that α-ZAL,

similar to E2, can increase OPG expression, which supports inhibition of osteoclast activity. Various studies have examined whether and how mechanical strain is involved in the regulation of OPG and RANKL expression with mixed results. For example, Sanchez *et al.* found that continuous mechanical loading decreased OPG expression in OBs.[32] Ludwika *et al.*[20] gave intermittent mechanical strain to human OBs and found increased mRNA expression of RANKL. They also found that continuous loading had no significant effect on RANKL expression. The expression of OPG was not significantly influenced. Another study by Kim *et al.* focused on oscillatory fluid flow[3]; the authors used ST-2 murine bone marrow stromal cells and found a significant increase in OPG
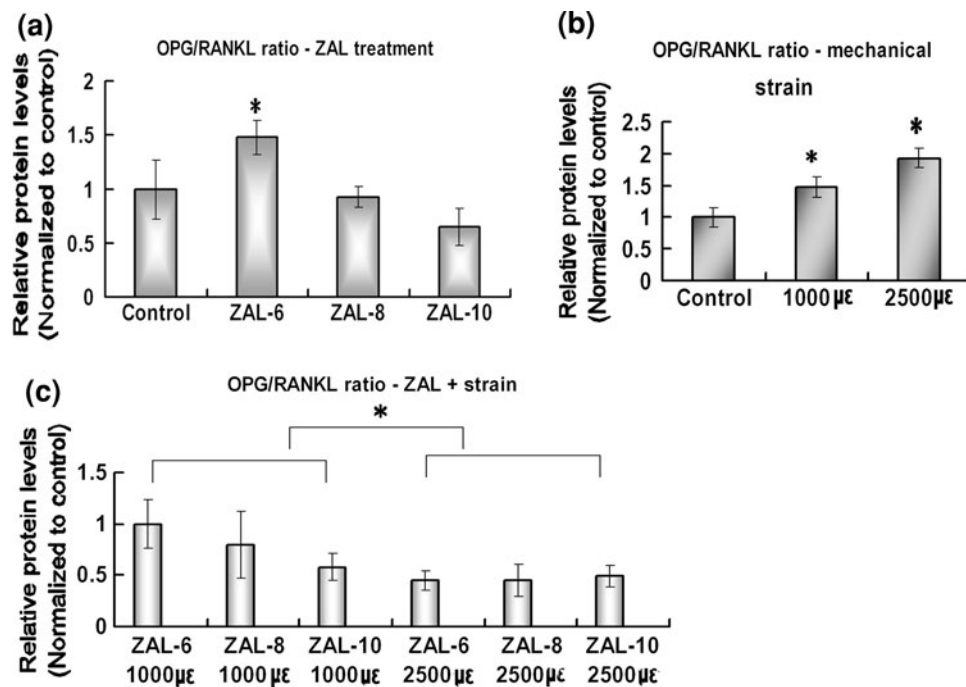
FIGURE 8. Effect of α-ZAL, mechanical strain, and a combination of both on the OPG/RANKL protein ratio. MC3T3-E1 cells were treated with α-ZAL, mechanical strain, or a combination of both for 72 h. α-ZAL with 10^{-6} M (a) and mechanical strain (b) increased the OPG/RANKL ratio. α-ZAL coupled with a low magnitude of strain (1000 $\mu\varepsilon$) dose-dependently increased the ratio (c). Results are shown as mean ± SD of data from three independent experiments. *$p < 0.05$ vs. controls or between groups.

expression and decrease in RANKL expression with increasing load duration up to 2 h. This suggests that changes in OPG/RANKL ratio in response to mechanical strain depend upon the context, cell type, mode of stress load, and magnitude of strain. We found that the responses of OPG and RANKL to joint stimulation of estrogen and strain were more complicated. In general, a low magnitude of strain coupled with a high drug concentration will help improve the OPG/RANKL ratio. A high magnitude of strain is not conducive to an increase in the OPG/RANKL ratio following estrogen stimulation. More interestingly, a low magnitude of strain combined with α-ZAL treatment dose-dependently increased OPG expression, which was inhibited under a high magnitude of strain coupled with α-ZAL treatment. On the contrary, a high magnitude of strain coupled with α-ZAL dose-dependently increased RANKL expression, but had no effect under a low magnitude of strain coupled with α-ZAL, indicating that the expression of OPG and RANKL is regulated by different signal transduction pathways. Lin *et al.*[40] also demonstrated that the ERK-MAPK pathway was involved in RANKL mRNA expression in response to mechanical strain, while OPG mRNA expression was mediated by cyclooxygenase or prostaglandin synthesis, and this effect partly involved an intracellular tyrosine kinase cascade. However, little is known about the upstream signaling of OPG and RANKL. The precise mechanisms underlying drug and strain effects still need to be determined.

In conclusion, we studied the effect of two kinds of stimuli on osteoblastic proliferation, differentiation, and the capability of inhibiting osteoclast activity. ALP (ALP activity and ALP mRNA levels) and RUNX2 (mRNA and protein expression) were used as markers to evaluate osteogenic differentiation, and the OPG/RANKL ratio (mRNA and protein expression) represented the capability of inhibiting OC activity. Our results showed that mechanical strain could increase osteogenic differentiation in the presence of α-ZAL in a magnitude-dependent manner, but had little effect on cell proliferation. However, a low magnitude of strain, but not high magnitude, was conducive to inhibiting OC activity in the presence of α-ZAL. Our results suggest that α-ZAL combined with a low of magnitude strain may provide benefits for treatment of OP.

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