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Continuously Generated H_2O_2 Stimulates the Proliferation and Osteoblastic Differentiation of Human Periodontal Ligament Fibroblasts

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ABSTRACT

Numerous studies have shown that hydrogen peroxide (H_2O_2) inhibits proliferation and osteoblastic differentiation in bone-like cells. Human periodontal ligament fibroblasts (PLF) are capable of differentiating into osteoblasts and are exposed to oxidative stress during periodontal inflammation. However, the cellular responses of PLF to H_2O_2 have not been identified. In this study, we examined how H_2O_2 affects the viability and proliferation of PLF by exposing the cells to glucose oxidase (GO) or direct addition of H_2O_2. We also explored the effects of GO on the osteoblastic differentiation of PLF and the mechanisms involved. The viability and proliferation in PLF were increased with the addition of 10 mU/ml GO but not by volumes greater than 15 mU/ml or by H_2O_2 itself. GO-stimulated DNA synthesis was correlated with the increase in cyclin E protein levels in the cells. Osteoblastic differentiation of PLF was also augmented by combined treatment with GO, as evidenced by the increases in alkaline phosphatase activity, mineralization, collagen synthesis, and osteocalcin content in the cells. The inductions of runt-related transcription factor 2 and osterix mRNA and proteins were further increased in PLF incubated in combination with GO compared to those in untreated cells. These results demonstrate that the continuous presence of H_2O_2 stimulates the proliferation of PLF and augments their potential to differentiate into osteoblasts through the up-regulation of bone-specific transcription factors. Collectively, we suggest that H_2O_2 may elicit the functions of PLF in maintaining the dimensions of the periodontal ligament and in mediating a balanced metabolism in alveolar bone. J. Cell. Biochem. 113: 1426–1436, 2012.

KEY WORDS: PERIODONTAL LIGAMENT FIBROBLASTS; GLUCOSE OXIDASE; HYDROGEN PEROXIDE; PROLIFERATION; OSTEOBLASTIC DIFFERENTIATION

The human periodontal ligament (PDL) is a band of fibrous connective tissue located between the tooth-root cementum and the alveolar bone. The PDL consists of a heterogeneous cell population, where fibroblasts are the predominant cell type [Bordin et al., 1984; McCulloch and Bordin, 1991]. PDL plays important roles in maintaining the stability and function of teeth. It has been suggested that PDL fibroblasts (PLF) are a source of osteoblasts required for alveolar bone remodeling by physiological or mechanical stimuli [Roberts et al., 1982]. PLFs are naturally osteogenic and are able to differentiate into osteoblasts [Heo et al., 2010]. Numerous studies also demonstrate the potential of PLFs in regulating both osteoblastic and osteoclastic differentiation within periodontal tissue [Kook et al., 2009, 2011; Wattanaroonwong et al., 2011].

Cellular oxidative stress occurs if reactive oxygen species (ROS) are overproduced or if their removal is reduced. Persistent and prolonged oxidative stress causes various pathological disorders such as stroke, heart attack, aging, and several degenerative diseases [Buttke and Sandstrom, 1994; Chandra et al., 2000]. ROS can be also produced continuously by periodontal-pathogenic bacteria or their

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by-products. ROS causes oxidative damage of periodontal tissue and cells and eventually mediates periodontitis [Ara et al., 2009]. There are also reports supporting the relationship between the antioxidant status in plasma and the risk of periodontitis [Chapple et al., 2007; Ekuni et al., 2009]. Chaves Neto et al. [2011] demonstrated that oxidative stress inhibited the proliferation of bone cells, and this result was facilitated when the cells were incubated with osteoblast-inducing media. These reports indicate that the processes required for alveolar bone remodeling are affected by intracellular redox states. Furthermore, the modes of action of PLF in response to ROS are quite important because of their potentials to regulate osteoblastic and osteoclastic activations.

Hydrogen peroxide (H_2O_2) is generated by almost all sources of oxidative stress and can penetrate cellular membranes [Forman and Torres, 2001; Nordberg and Arner, 2001]. H_2O_2 is also widely used in dental clinics to bleach teeth, enhance gingival healing, and diminish bacterial populations in dental plaque [Tipton et al., 1995]. Many studies have shown the susceptibility of bone cells to H_2O_2, where both the proliferation and differentiation of osteoblasts were sensitively suppressed by exposure to this agent [Choi et al., 2009; Chaves Neto et al., 2011; Kim et al., 2010]. However, it is important to note that cellular responses to H_2O_2 can differ depending on the type of cells and the concentration of H_2O_2. In lymphoma cells, H_2O_2 treatment induced growth inhibition and apoptosis [Son et al., 2009], whereas this same treatment stimulated proliferation in several other cell types [Timblin et al., 1995; Herbert et al., 1996]. Exposure of BHK-21 fibroblasts to a low H_2O_2 level (1 μM) stimulated proliferation, but treatment with 100 μM H_2O_2 induced growth inhibition and apoptotic cell death in the cells [Burdon et al., 1996]. We previously reported on the dual modes of action of H_2O_2 according to the methods of exposure, that is, temporal versus continuous presence of this agent in the cultures [Son et al., 2009]. Furthermore, glucose oxidase (GO) that continuously generates H_2O_2 at relatively low concentrations significantly increased the proliferation rate in bovine aortic endothelial cells, but a direct addition of H_2O_2 inhibited the proliferation of the cells [Ruiz-Ginés et al., 2000].

Despite the critical roles of PLF in the process of alveolar bone remodeling and in the maintenance of tooth function and stability, little information on the mode of action of PLF in response to H_2O_2 is available. Therefore, we examined the viability and proliferation of PLF after exposure to GO or to H_2O_2 itself. We also explored the effects of H_2O_2 on osteoblastic differentiation of the cells. In addition, we aimed to determine the mechanisms by which H_2O_2 affects the proliferation and differentiation of PLF. In the present study, we demonstrated for the first time that continuously generated H_2O_2 stimulates both the proliferation rates and osteoblastic differentiation in PLFs depending on the concentrations exposed.

MATERIALS AND METHODS

CHEMICALS AND LABORATORY WARES

Fetal bovine serum (FBS) was purchased from Gibco-BRL (Gaithersburg, MD). The primary antibodies specific for actin, runt-related transcription factor-2 (Runx2), osterix, osteopontin, cyclins, and cyclin-dependent kinase-2 (CDK2), and the secondary goat-anti rabbit antibody were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise specified, the other chemicals and laboratory items were purchased from Sigma Chemical Co. (St. Louis, MO) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ), respectively.

CELL CULTURES AND H_2O_2 EXPOSURE

PLFs were obtained from healthy male volunteers aged 20–30 years and cultured according to methods described elsewhere with slight modifications [Kook et al., 2009]. All the donors gave written informed consent for use of their tissues. This study was approved by the Ethical Committee of Chonbuk National University Hospital. Here, single cell suspensions of PLFs were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 IU/ml penicillin G and 100 μg/ml streptomycin) in 100-mm culture dishes. The cultures were switched to a fresh batch of medium every 3 days. When PLFs reached >90% confluence, the cells were suspended in the culture medium at a density of 5×10^5/ml and then seeded in 6-well and 96-well flat-bottomed plates with 2 ml and 200 μl per well, respectively. When the cells reached 70–80% confluence, the medium was changed to a fresh culture medium to proliferate or to differentiate into osteoblasts and exposed to various concentrations of GO or H_2O_2. In this study, 10 nM *d*examethasone, 50 μM *a*scorbic acid, and 20 mM β-*g*lycerophosphate, named DAG, were used to induce osteoblastic differentiation of PLFs. All the experiments were performed using fibromodulin-positive PLFs (>95%) at passages 4 through 8. In the present study, PLFs were exposed to GO ranging from 0 to 50 mU/ml or H_2O_2 at various doses (10 μM to 2 mM) through its direct addition into the cultures.

H_2O_2 DETERMINATION

The levels of H_2O_2 in the culture medium were determined using Amplex® Red Hydrogen Peroxide Assay Kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instruction. In brief, PLFs suspended in DMEM without phenol red were divided to culture plates in the presence and absence of DAG. The culture supernatants (50 μl/sample) were collected at various times after GO exposure and mixed with the same volume of the Red reagent in 96-multiwell plates. After incubation for 30 min at room temperature, horseradish peroxidase (0.1 U/ml) was added into the mixtures. Finally, the absorbance was measured at 560 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

MEASUREMENT OF CELL VIABILITY AND DNA SYNTHESIS

Cell viability was determined using water-soluble tetrazolium salt (WST)-8 reagent. Briefly, GO- or H_2O_2-exposed cultures were treated with WST-8 reagent at various times after exposure. Following incubation for an additional 4 h, absorbance was measured at 450 nm using a microplate reader (Molecular Devices). The level of DNA synthesis in the cells was measured by adding 0.5 μCi of ^{3}H-thymidine deoxyribose (TdR; Amersham Pharmacia Biotech, Inc., Piscataway, NJ) to each well of the 96-multiwell plates during the 12 h prior to cell harvesting. After collection of the cells using a harvester (Inotech, Inc., Dietikon, Switzerland), beta emission from

the ^{3}H-TdR-incorporated cells was measured for 1 min using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

ASSAY OF CELLULAR ANTIOXIDANT ACTIVITIES

PLFs were exposed to increasing GO concentrations for 24 h and then adjusted to the analyses of intracellular antioxidant activities such as superoxide dismutase (SOD), catalase, and reduced glutathione (GSH). In this study, the assay kits specific for SOD (CAT No. 706002, Cayman Chemical Company, Ann Arbor, MI), catalase (CAT No. A22180, Invitrogen, Carlsbad, CA), and GSH (CAT No. ADI-900-160, Enzo Life Sciences, Plymouth Meeting, PA) were used to determine the respective activities. All the experiments were carried out according to the manufacturer's instructions.

PROPIDIUM IODIDE (PI) STAINING AND CELL CYCLE ANALYSIS

PLFs were exposed to various GO concentrations. At various times, the cells (2×10^6 cells) were fixed with 70% ethanol at 4°C for 24 h and then incubated at room temperature for 1 h in a staining mixture (500 μl) containing 125 μl of phosphate-buffered saline (PBS), 125 μl of 1 mg/ml RNase, and 250 μl of 50 μg/ml PI. Ten thousand cells per experiment were counted to measure the PI intensity using a FACS Vantage® system (Becton-Dickinson), and cell cycle progression was analyzed using the WinMDI 2.9 program.

WESTERN BLOT ANALYSIS

Cell lysates were prepared in NP-40 lysis buffer (30 mM Tris–Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF, and protease inhibitor mixture containing 1 μg/ml each of aprotinin and leupeptin). Protein contents were quantified using the Bradford method [1976]. Samples of extracts containing equal amounts of protein (30 μg/sample) were analyzed by SDS–PAGE (10–15% gels) and blotted onto polyvinyl difluoride membranes. The blots were probed with primary antibodies and incubated with horseradish peroxidase-conjugated anti-IgG in blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence (Santa Cruz Biotechnology) and exposed to X-ray film (Eastman-Kodak, Rochester, NY).

ALKALINE PHOSPHATASE (ALP) ACTIVITY ASSAY

PLFs were incubated in osteoblast-inducing medium with and without various GO concentrations (0–10 mU/ml), 500 U/ml SOD, or 500 U/ml catalase. At various times, the cells were washed several times with PBS (pH 7.4), sonicated for 20 s at 4°C, and then centrifuged at 12,000*g* for 15 min. Protein levels were determined by the Bradford method [1976], and the normalized protein lysates were adjusted for the determination of ALP activity. ALP activity was determined at 37°C in a buffer (10 mM $MgCl_2$ and 0.1 M alkaline buffer, pH 10.3) supplemented with 10 mM *p*-nitrophenylphosphate as the substrate. The reaction was stopped by adding 0.5 N NaOH, and absorbance was measured at 405 nm. ALP activity was expressed as nmol/min/mg of protein.

ALIZARIN RED STAINING

The degree of mineralization in PLFs was determined in 6-well plates using Alizarin red staining. In brief, PLFs were exposed to GO in osteoblast-inducing medium with and without antioxidant enzymes. At various times, they were fixed with ice-cold 70% (vol/vol) ethanol for 1 h. The cells were stained with 0.2% Alizarin red S in distilled water for 30 min at room temperature. After destaining and air-drying, the cell culture plates were evaluated under light microscopy. In order to quantify the red dye, the stains were solubilized with 10% acetylpyridinum chloride by shaking for 20 min, and the absorbance was measured using a microplate reader.

MEASUREMENT OF COLLAGEN AND OSTEOCALCIN

Collagen contents in PLF were determined by the Sirius Red-based colorimetric assay. In brief, PLFs were treated with various GO concentrations (0–10 mU/ml) and/or antioxidant enzymes in DMEM containing dexamethasone, ascorbic acid, and glycerophosphate. The medium was changed every 2 days. Ten days after treatment, the cells were fixed with Bouin's fluid for 1 h and washed several times with distilled water. The culture plates were stored at room temperature for drying before being stained with Sirius Red dye reagent for 1 h. After two washes with 10 mM HCl, the cells were treated with 100 mM NaOH, and the absorbance at 550 nm was measured. In addition, the osteocalcin contents in PLF were measured at 10 days after GO treatment using a sandwich ELISA assay kit (Biomedical Technologies, Inc., Stoughton, MA). All the experiments were performed according to the manufacturer's instructions. The contents of collagen and osteocalcin were expressed as μg or ng per 10^6 cells.

RNA ISOLATION AND REAL-TIME RT-PCR

The total RNA was extracted from PLFs using STAT-60 (Tel-Test, Inc., Friendwood, TX) and the real-time quantification of RNA targets was performed in the Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, NSW, Australia) using a QuantiTect SYBR Green RT-PCR kit (QIAGEN, CA), as described elsewhere [Heo and Lee, 2011]. The primers used were 5′-GAGGGACTATGGCGTCAAACA-3′ (sense), 5′-GGATCCCAAAAGA-AGCTTTGC-3′ (antisense) for Runx2, and 5′-TCAGCCGCCCCGA-TCTTCCA-3′ (sense), 5′-AATGGGTCCACCGCGCCAAG-3′ (antisense) for osterix. The temperature of the PCR products was increased from 65°C to 99°C at a rate of 1°C/5 s, and the resulting data was analyzed using the software provided by the manufacturer.

STATISTICAL ANALYSIS

Unless specified otherwise, all the data are expressed as the mean ± standard deviation (SD) from three or more independent experiments. A one-way analysis of variance (ANOVA) followed by a Scheffe's test was used for multiple comparisons using the SPSS program (version 18.0). A value of $P < 0.05$ was considered statistically significant.

RESULTS

CONTINUOUS GENERATION OF H_2O_2 IN GO-EXPOSED PLFS

The concentrations of H_2O_2 in the culture supernatants were augmented proportionally to the amounts of GO added (Fig. 1A). The H_2O_2 levels produced were increased continuously after the addition of GO, peaked at around 4 h, and gradually decreased after

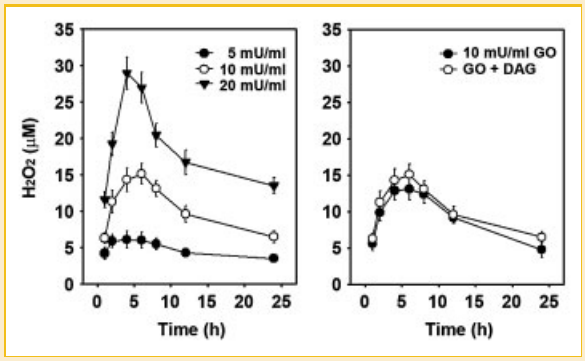

Fig. 1. Continuous and dynamic production of H_2O_2 in the cultures of PLFs exposed to GO. PLFs were exposed to 5, 10, and 20 mU/ml GO (left panel) or 10 mU/ml GO with DAG (10 nM *d*examethasone, 50 μM *a*scorbic acid, and 20 mM β-*g*lycerophosphate) (right panel) and then the culture supernatants were collected at various times intervals after GO exposure during 24 h incubation. The results indicate the mean ± SD from triplicate experiments.

6 h. This dynamic pattern was more apparent in the culture supernatants exposed to higher doses of GO, such that 20 mU/ml GO treatment produced 28.9 μM at 4 h as a maximum concentration and this was reduced up to 13.5 μM at 24 h. These results were quite similar to the previous reports showing that GO treatment generates H_2O_2 continuously at low concentrations with a complex dynamic [Lee et al., 2006; Son et al., 2009; Kaczara et al., 2010]. The patterns of GO-mediated production were not affected by DAG at a significant level (Fig. 1B). In parallel with the previous report [Kaczara et al., 2010], the amounts of H_2O_2 generated by GO were also not influenced by the presence of 10% FBS (data not shown).

GO TREATMENT AFFECTS THE VIABILITY AND PROLIFERATION OF PLF BASED ON CONCENTRATION

The PLFs showed multiple responses to GO depending on the concentration. The viability of PLF was increased when exposed to 10 mU/ml GO for 24 h, whereas the same viability was markedly reduced after exposure to 25 mU/ml GO for the same time period (Fig. 2A). GO treatment (10 mU/ml) also resulted in the increase in TdR uptake in PLFs (Fig. 2B). This was accompanied by a significant increase in cells in the G_2/M phase of cell cycle progression (Fig. 2C). In contrast, the direct addition of H_2O_2 suppressed the viability of PLFs in a dose-dependent manner (Fig. 2D). The viability of the cells was reduced to approximately 30% of that of the untreated control cells when they were exposed to 1 mM H_2O_2. Pretreatment of PLFs with 500 U/ml catalase almost prevented a complete GO-mediated reduction in viability (Fig. 2E).

GO EXPOSURE AT LOW CONCENTRATIONS ENHANCES SOD ACTIVITY IN PLFs

SOD activity in PLFs was affected based on the concentration of GO added. Treatment with GO ranging from 1 to 10 mU/ml augmented SOD activity in the cells, but the addition of 20 mU/ml GO significantly reduced the activity (Fig. 3A). GO exposure did not increase catalase activity in the cells but reduced it in a dose-dependent manner (Fig. 3B). However, the reduction in GSH in PLFs was not changed with the addition of GO at the concentrations examined.

GO TREATMENT INCREASES THE PROLIFERATION RATE IN PLFS INCUBATED IN OSTEOGENIC MEDIUM

We subsequently explored whether GO treatment affects the proliferation rate of PLFs incubated in osteogenic medium

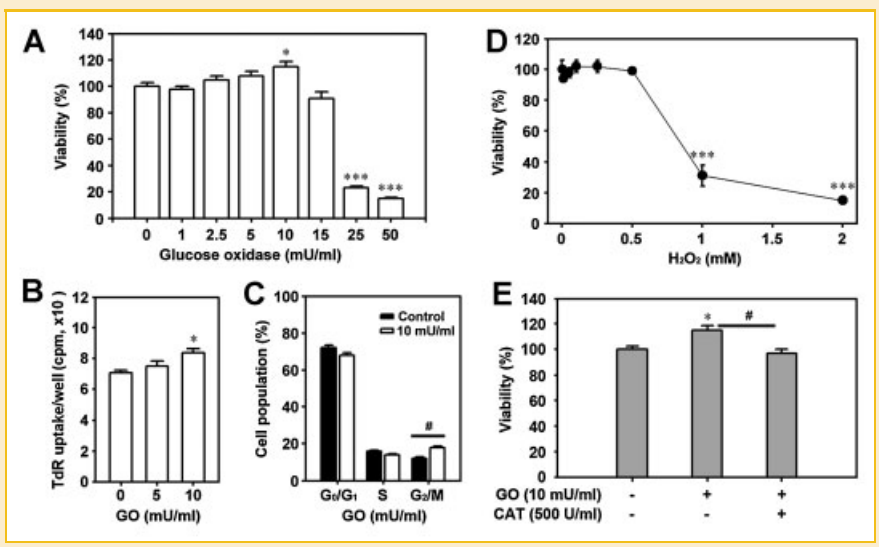

Fig. 2. Viability and proliferation of PLFs are increased by the addition of GO but not by H_2O_2. PLFs were exposed to the indicated GO concentrations (0–50 mU/ml) for 24 h and then processed for the WST-8 assay (A), tritium uptake assay (B), and flow cytometric analysis after PI staining (C). D: The cells were also exposed to H_2O_2 itself with increasing concentrations (0–2 mM) for 24 h and then processed for the cell viability assay. E: PLFs were exposed to 10 mU/ml GO in the presence and absence of 500 U/ml catalase for 24 h prior to the WST-8 assay. In the experiment of C, cell cycle progression for each experiment was analyzed using the WinMDI 2.9 program. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the untreated control values. $^{\#}P < 0.05$ versus the experimental values.

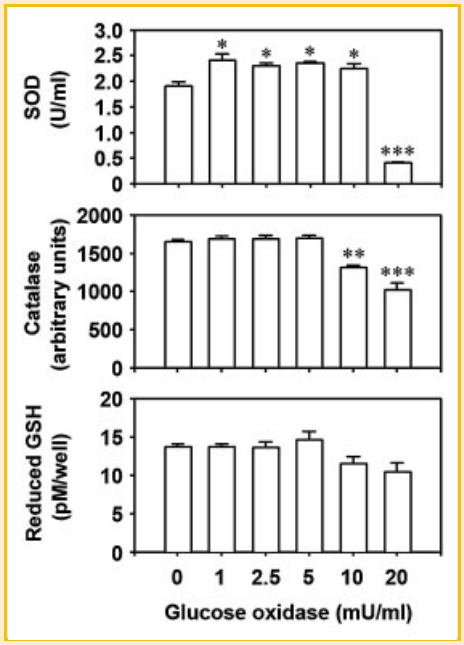

Fig. 3. The activities of intracellular antioxidant systems depend on the concentration of GO. PLFs were exposed to various GO concentrations (0–20 mU/ml) for 24 h. The activities of SOD and catalase and the levels of reduced GSH were then determined. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the untreated control values.

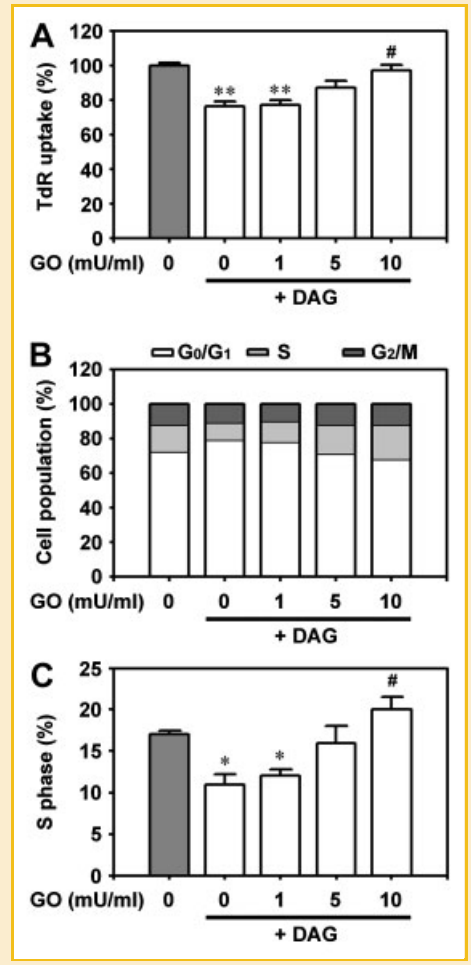

Fig. 4. Effects of GO on the proliferation and cell cycle progression of DAG-treated PLFs. PLFs were exposed to the indicated GO concentrations (0–10 mU/ml) in the presence of DAG. After 7 days of incubation, the levels of DNA synthesis and the cell populations in each stage of cell cycle progression were determined by tritium uptake assay (A) and flow cytometric analysis after PI staining (B and C), respectively. The data in B show representative results from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the untreated control values. $^{\#}P < 0.05$ versus DAG treatment alone.

supplemented with DAG. Incubation of PLFs for 7 days in the medium resulted in a decrease in DNA synthesis in the cells compared to that in untreated control cells (Fig. 4A). This decrease was not inhibited by the addition of 1 mU/ml GO, but the combination with 10 mU/ml GO for the same amount of time blocked the DAG-mediated decrease in TdR uptake level in the cells. Flow cytometric analysis after PI staining revealed a significant reduction of the cell population in the S phase in DAG-treated cells and its suppression by the combined treatment with 10 mU/ml GO (Fig. 4B,C). Treatment of PLFs with more than 20 mU/ml GO caused cytotoxic effects with the attendant migration into the sub-G_1 phase of cell cycle progression (data not shown). The levels of cyclin D1 and CDK2 proteins in PLFs were not affected by incubation in osteoblast-inducing medium regardless of the presence of GO ranging from 1 to 10 mU/ml (Fig. 5A). In contrast, a significant reduction in cyclin E protein level was found in cells incubated in DAG-containing medium for 7 days (Fig. 5A,B). In addition, combined treatment with GO prevented the DAG-mediated decrease in cyclin E level in a dose-dependent manner. When the DAG-treated PLFs were co-incubated with 10 mU/ml GO for 7 days, an approximately fivefold increase in cyclin E level was observed compared to that of the untreated control cells (Fig. 5B).

GO TREATMENT AT LOW DOSES STIMULATES OSTEOBLASTIC DIFFERENTIATION OF PLF

Since it is known that the differentiation of cells into osteoblasts is accompanied by the expression and activity of ALP, we determined the effects of GO on ALP activity in PLFs during osteoblastic differentiation. Three days after incubation, DAG treatment itself increased ALP activity in the cells, whereas the increase was significantly attenuated by co-incubation with GO (Fig. 6A). Such GO-mediated suppression in ALP activity was observed when the DAG-treated cells were cultured for 7 days in the presence of 1 or 2.5 mU/ml GO (Fig. 6B). However, a combined treatment with 10 mU/ml GO for the same time period augmented the ALP activity that had increased in DAG-treated PLFs. This augmentation was further apparent when the cells were incubated in combination with 10 mU/ml GO for 10 days (Fig. 6C). Figure 6D shows a time-dependent increase and acceleration in ALP activity in DAG-treated PLFs after combined treatment with 10 mU/ml GO during osteoblastic induction. We next evaluated the effects of GO on the mineralization of DAG-treated PLFs using Alizarin red staining. Figure 7A represents the transparent appearance of Alizarin red stained-PLFs and the increase in the number of stained cells due to

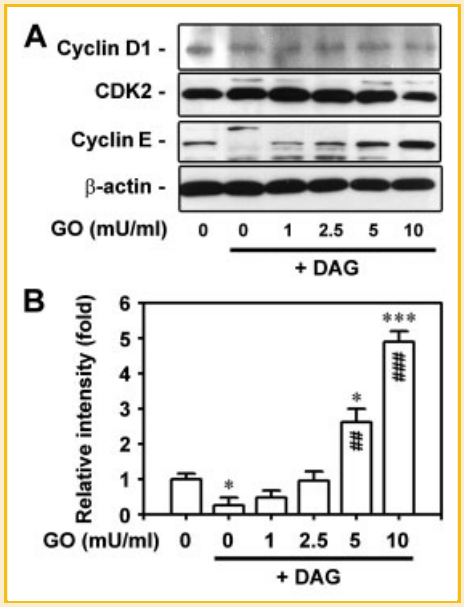

Fig. 5. Effects of GO on the induction of cell cycle regulatory proteins in DAG-treated PLFs. A: The cells were incubated in the DAG-containing osteogenic medium with and without the indicated doses (0–10 mU/ml) of GO for 7 days and then processed for Western blot analysis using total protein lysates. B: The values represented are the mean ± SD of three independent experiments, where actin was used as the control protein. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the untreated control values. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus DAG treatment alone.

GO in a dose-dependent manner when the cells were analyzed after 14 days of incubation. Results from colorimetric analysis showed that combined treatment with 5 and 10 mU/ml GO led to a significant increase in the optical density up to 175% and 195%, respectively, compared to that of the control (Fig. 7B). In parallel with these findings, GO-mediated augmentation of collagen content was observed in the cells co-incubated with DAG for 10 days (Fig. 8A). The combination with 10 mU/ml GO also augmented the osteocalcin levels that had increased in the DAG-treated PLFs (Fig. 8B).

GO-MEDIATED FACILITATION OF OSTEOGENIC DIFFERENTIATION IS INHIBITED BY CATALASE

In order to determine whether GO-mediated stimulation of osteoblastic differentiation in PLFs is related to the effects of H_2O_2, the DAG-treated PLFs were incubated in the presence and absence of SOD and catalase. GO-mediated acceleration of ALP activity in DAG-treated PLFs was significantly prevented with the addition of 500 U/ml catalase (Fig. 9A). Catalase also inhibited GO-mediated augmentations of collagen content (Fig. 9B), mineralization (Fig. 9C), and osteoclacin level (Fig. 9D). However, the addition of 500 U/ml SOD failed to suppress GO-mediated facilitation on the induction of osteogenic markers in the cells. None of these antioxidants alone significantly influenced cell viability or DNA synthesis (data not shown).

GO-MEDIATED FACILITATION OF OSTEOBLASTIC DIFFERENTIATION IN PLFS IS ACCOMPANIED BY THE INCREASED INDUCTIONS OF OSTERIX AND RUNX2

Transcription factors such as osterix and Runx2 play a key role during osteoblastic differentiation by affecting a diverse array of

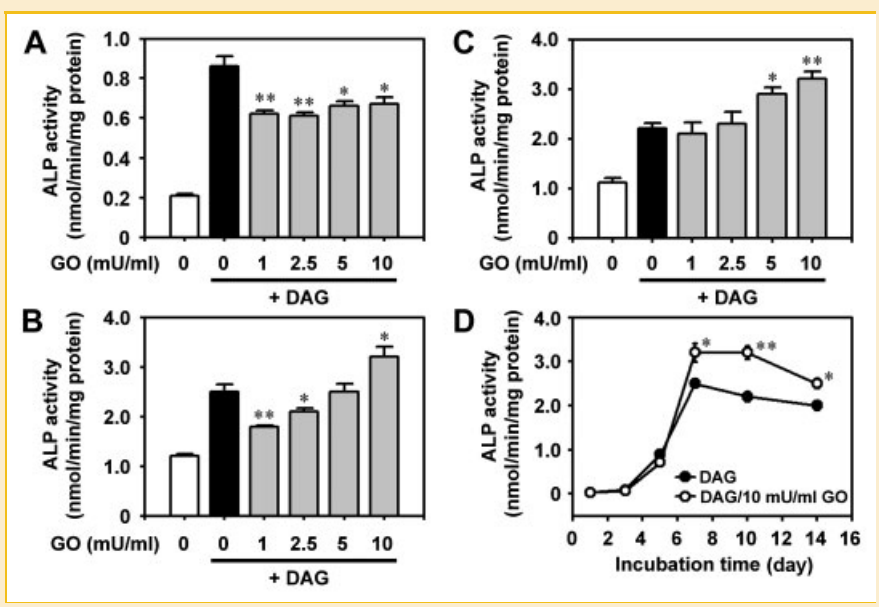

Fig. 6. GO augmentation of the DAG-mediated increase in ALP activity in PLFs. Cells were exposed to the indicated GO concentrations (0–10 mU/ml) in the presence of DAG and processed for the analysis of ALP activity after 3 (A), 7 (B), and 10 days (C) of incubation. D: PLFs were also incubated in DAG-containing medium with and without 10 mU/ml GO. At various times, ALP activity was determined. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the untreated control values.

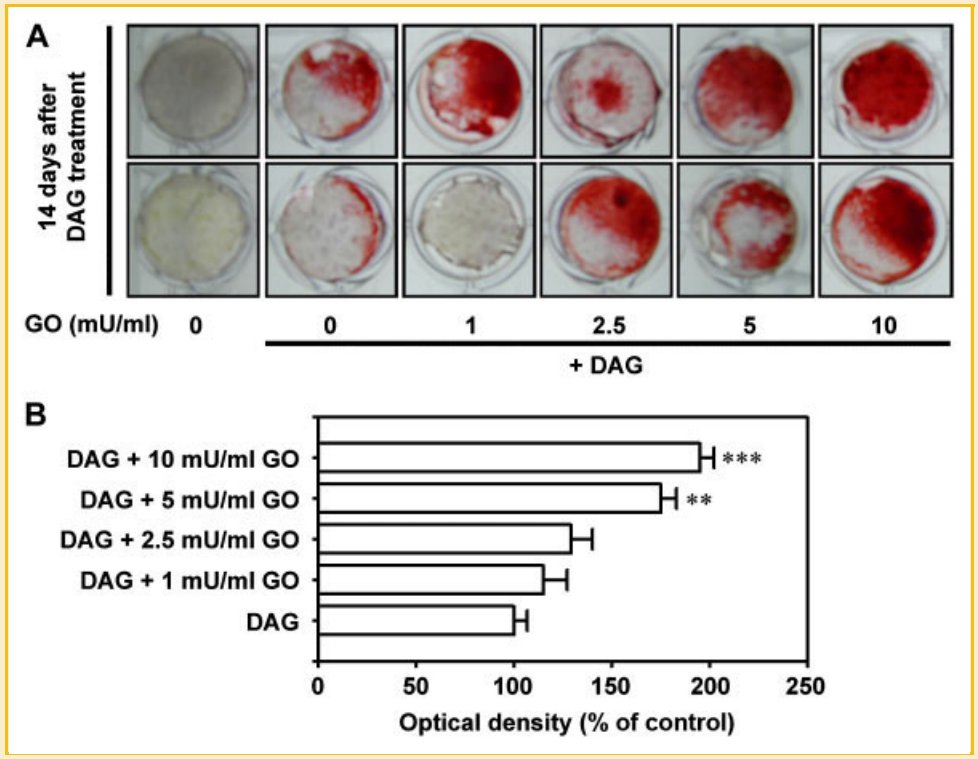

Fig. 7. Stimulating effect of GO on mineralization in DAG-treated PLFs. A: Cells were cultured with the DAG-containing osteogenic medium in the presence and absence of GO for 14 days. The resulting mineralization was assessed by Alizarin red staining. Each microscopic image shown is a representative of five separate experiments. B: Absorbance specific for Alizarin red was measured, and $^{**}P < 0.01$ and $^{***}P < 0.001$ represent significant differences between the cells treated with DAG only and those treated in combination with GO. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

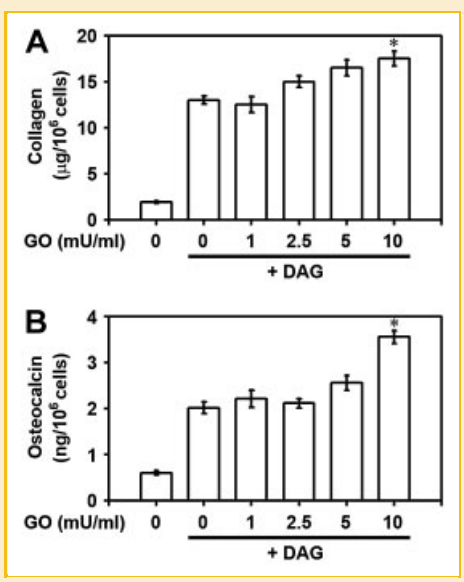

Fig. 8. GO treatment increases the contents of collagen and osteocalcin in DAG-treated PLFs. Cells were incubated in the osteogenic medium with and without the indicated GO concentrations (0–10 mU/ml). The cellular levels of collagen (A) and osteocalcin (B) were determined after 10 days of incubation. $^{*}P < 0.05$ versus the untreated control values.

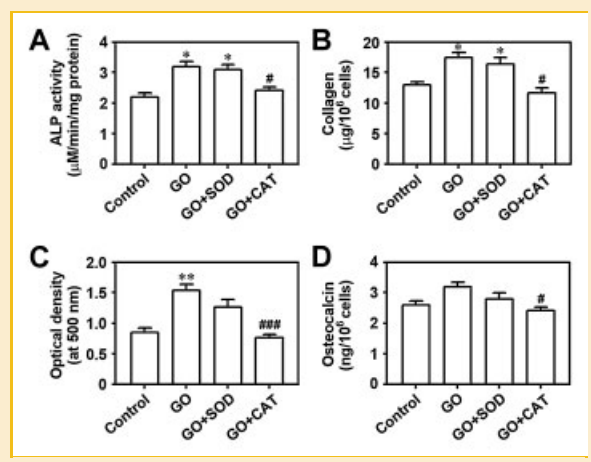

Fig. 9. Inhibitory effect of catalase on the GO-mediated facilitation of osteogenic differentiation in PLFs. Cells were incubated in DAG-containing osteogenic medium in the presence of 10 mU/ml GO, 500 U/ml SOD, and/or 500 U/ml catalase for 10 days and then processed for the ALP activity (A), collagen synthesis (B), mineralization (C), and osteocalcin content. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus DAG treatment alone. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus GO treatment.

down-stream effectors required for bone formation. We investigated the effects of GO in the induction of these factors and osteopontin during osteoblastic differentiation using Western blot and real time RT-PCR analyses. The protein levels of osterix and Runx2 in DAG-treated PLFs barely increased after 3 days of addition of 10 mU/ml GO (Fig. 10A,B). This increase became prominent when the cells were incubated for 7 days in the presence of GO (Fig. 10C,D). Combined incubation with GO for 7 days also augmented the levels of osteopontin in DAG-treated cells. In parallel with this, mRNA levels of Runx2 and osterix were significantly increased in the cells exposed to 10 mU/ml GO, compared to the untreated controls (Fig. 10E,F). The GO-mediated increase of Runx2 and osterix at mRNA levels was significantly diminished by treating the cells with 500 U/ml catalase.

DISCUSSION

The present findings demonstrate for the first time that GO at low concentrations increases the viability of PLFs and their ability to synthesize DNA in cultures supplemented with and without DAG. We also showed that DAG-stimulated osteoblastic differentiation in cells was augmented by combined treatment with GO. In addition, the PLF DNA synthesis elicited by GO was not induced by direct addition of H_2O_2 into the cultures. These results are in part consistent with previous reports that showed that the proliferation rate in bovine aortic endothelial cells was facilitated by GO but not by H_2O_2 [Ruiz-Ginés et al., 2000]. Burdon et al. [1996] also reported that exposure to 1 μM H_2O_2 stimulated the proliferation of BHK-21 fibroblasts, but this agent at 0.5 and 1 mM caused growth inhibition and apoptotic cell death. However, there have been many reports demonstrating the opposite action of H_2O_2 on cells. The direct addition of H_2O_2 inhibited viability as well as osteoblastic differentiation in primary bone marrow stromal cells [Bai et al., 2004; Liu et al., 2004], MC3T3-E1 preosteoblastic cells [Choi et al., 2009; Kim et al., 2010; Xu et al., 2011], and primary rabbit calvarial osteoblasts [Bai et al., 2004]. Many studies have shown that active components with antioxidant potential protect cells against the H_2O_2-induced inhibition of proliferation and osteoblastic differentiation [Liu et al., 2004; Choi et al., 2009; Kim et al.,

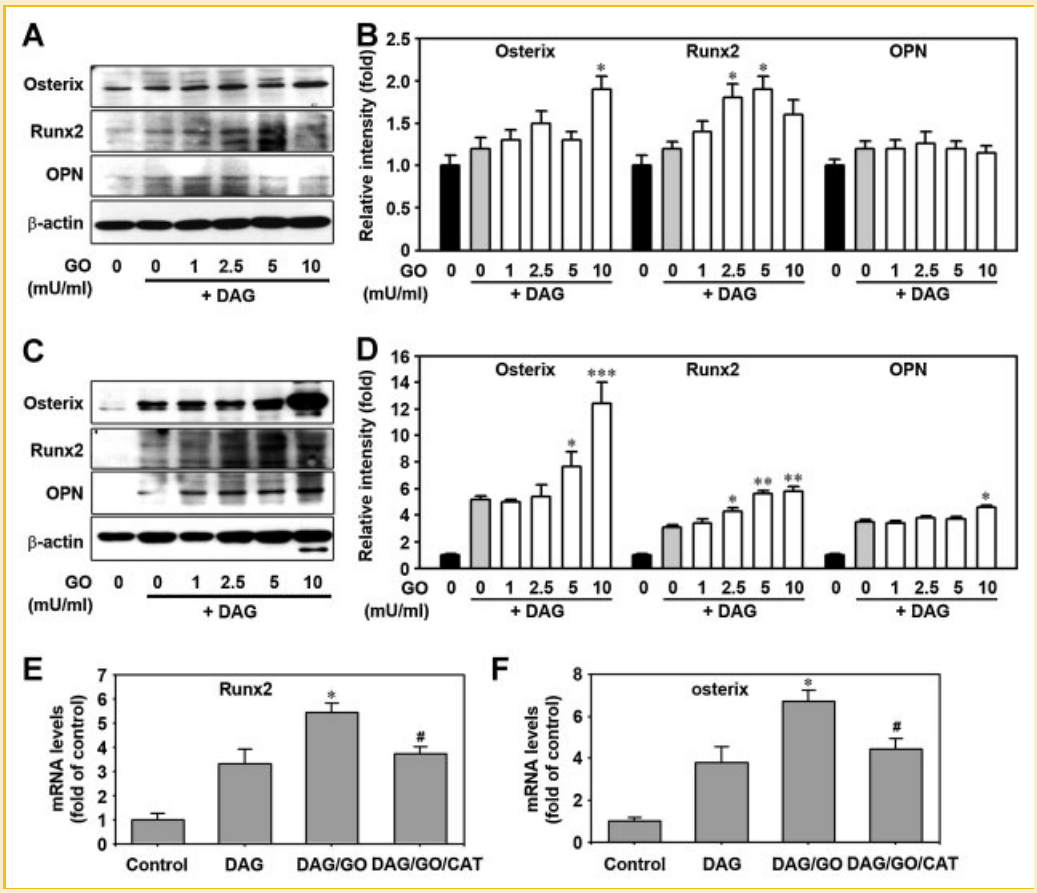

Fig. 10. Combined treatment with GO accelerates the expressions of osteogenic transcription factors in DAG-treated PLFs. Cells were incubated in DAG-containing medium with and without the increasing GO concentrations (0–10 mU/ml). The levels of osterix, Runx2, and osteopontin proteins were determined by Western blot analysis using total protein lysates after 3 (A) and 7 days (C) of incubation. The expression patterns of these proteins were analyzed using a densitometer from three independent experiments after normalizing the bands to the level of actin. The values represented in B and D are the mean ± SD corresponding to A and C, respectively. The mRNA levels of Runx2 (E) and osterix (F) in the cells exposed to 10 mU/ml GO and/or 500 U/ml catalase were also analyzed after 7 days of osteoblastic differentiation. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus DAG treatment only. $^{\#}P < 0.05$ versus GO + DAG treatment.

2010]. These reports suggest that H_2O_2-mediated oxidative stress acts predominantly as an inhibitory mediator on cell proliferation and differentiation. Although we cannot explain the exact reasons involved in the opposing roles of H_2O_2 on cells, accumulating evidence supports three main explanations. One is that the cellular responses to H_2O_2 are affected according to the origins of cells examined. Second, the concentration of H_2O_2 might affect cellular responses to the agent, regardless of the method of exposure, that is, GO versus H_2O_2 itself. Third, the cellular responses to H_2O_2 could depend on the time at which the cells are exposed to the agent and whether it is in a continuous or temporary manner. When cells are incubated with GO, H_2O_2 is generated continuously in small amounts, whereas the direct addition of H_2O_2 to the cultures leads to a high but temporary stimulus.

Studies have shown that differentiated osteoblastic cells, not proliferating cells, are sensitively affected by H_2O_2. This is believed to be due to the subsequent decrease in the intracellular antioxidant defense system according to the differentiation. For example, MC3T3-E1 cells co-incubated with ascorbic acid and β-glycerophosphate exhibited lower expressions and activities of catalase, GSH peroxidase, total SOD, and Cu/Zn SOD compared to those of the untreated control cells [Chaves Neto et al., 2011]. It was also reported that MnSOD activity regulated cellular proliferation and quiescence. Thus, its decrease facilitated a superoxide signal-mediated proliferation, but its increase induced quiescence [Sarsour et al., 2008]. Similarly, the expression of MnSOD in MC3T3-E1 cells was higher in cells grown in osteoblast-inducing medium than it was in cells grown in growth medium [Chaves Neto et al., 2011]. These findings support a correlation between the changes in intracellular antioxidant activities and the susceptibility of bone cells to H_2O_2. However, our present data showed that the activity of total SOD increased in PLFs exposed to GO ranging from 1 to 10 mU/ml, although volumes greater than 5 mU/ml GO decreased the activity of catalase. The levels of reduced GSH were not changed in PLFs even when the cells were treated with 20 mU/ml GO. Moreover, exogenous SOD did not affect the differentiation of PLFs into osteoblasts. This suggests that the cellular antioxidant defense system is not directly related to the proliferation elicited in GO-exposed PLFs. Rather, the induction of cyclin E protein in PLFs is thought to be related to GO-stimulated proliferation. This is supported by the roles of cyclin E on cell cycle progression, the concentration of which increases in the late G_1 phase, decreases in the early S phase and induces the initial processes of DNA replication.

Osteogenic transcription factors including Runx2 and osterix are essential for bone formation and osteoblast differentiation [Ichida et al., 2004; Komori, 2005]. Runx2, known as core-binding factor 1 (Cbfa1), is located in the promoter regions of all the osteoblast-specific genes and controls their expressions. The results from this study show that osteoblastic differentiation in DAG-treated PLFs is augmented by combined treatment with GO, as demonstrated by the increases in ALP activity, mineralization, and collagen and osteocalcin contents. GO-mediated increases in these osteoblastic markers are correlated with the inductions of Runx2 and osterix. We previously found that stimulating PLFs with lithium chloride significantly elicited mineralized nodule formation and ALP activation, which is accompanied by the up-regulation of Runx2 and osterix [Heo et al., 2010]. Therefore, it is suggested that the activations of the transcription factors Runx2 and osterix are required for the osteogenic differentiation of PLFs. Combined treatment with GO at low concentrations facilitates the osteoblastogenesis of the cells by stimulating these transcription factors.

As it has been suggested that H_2O_2 sensitively down-regulates osteoblastic differentiation rather than proliferation, our current results demonstrated a contradictory action of H_2O_2 on the proliferation and osteoblastic differentiation of PLFs. One of the possible mechanisms that can explain these conflicting results is increased protein phosphorylation according to H_2O_2 exposure [Ruiz-Ginés et al., 2000; Takada et al., 2003]. It has been documented that signaling pathways including bone morphogenetic protein (BMP)-Smad signaling, mitogen-activated protein kinases (MAPKs), and phosphatidylinositol 3 kinase are involved in osteoblast differentiation [Ghosh-Choudhury et al., 2002; Bai et al., 2004]. Accumulating evidence also suggests that the transcriptional activity of Runx2 is controlled through phosphorylation by cellular kinases such as MAPKs and protein kinase A [Ge et al., 2009; Greenblatt et al., 2010]. This indicates that the osteoblastic differentiation of cells is affected by the statuses of cellular protein kinases. There is a report showing that fibroblast growth factor-2 induces the osteoblastic differentiation of vascular smooth muscle cells by activating Runx2 through MAPK-dependent and oxidative stress-sensitive signaling pathways [Nakahara et al., 2010]. It is also known that H_2O_2 induces the tyrosine phosphorylation of various growth factor receptors and oncogens, eventually activating MAPKs [Guyton et al., 1996; Rao, 1996]. More detailed experiments will be needed to elucidate the mechanisms by which GO accelerates the osteoblastic differentiation of PLFs. Investigation to verify the up-stream and down-stream effectors of Runx2 and osterix are also required in future studies.

In conclusion, PLFs are capable of differentiating into osteoblasts and regulating the balanced activation of osteoblasts and osteoclasts during orthodontic tooth movement. Although the precise mechanisms involved in the GO-mediated stimulation of proliferation and osteoblastic differentiation in PLFs are not clearly defined, our present findings suggest that H_2O_2 at relatively low concentrations elicits the functions of PLFs in maintaining the dimensions between the tooth root and alveolar bone, as well as in mediating a balanced metabolism in alveolar bone remodeling. It should also be noted that ROS are generated in a small amount around and/or in the tension or compression side of the PDL after mechanical stimuli. Collectively, this study demonstrates that GO treatment stimulates the proliferation of PLFs through the up-regulation of cyclin E and augments their potential to differentiate into osteoblasts through the induction of bone-specific transcription factors.

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