

Original Article

BMP-2 downregulation is involved in the inhibition of cementoblast differentiation by lithium

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Abstract: Cementum, which is formed by cementoblast, plays an important role in periodontal regeneration. Wnt signalling and BMP-2 are involved in the regulation of cementoblast differentiation, but little is known about the effect of lithium, an agonist of Wnt signalling, on cementoblast behaviour. In this research, OCCM-30 cementoblast was employed to investigate the influence of various concentrations of lithium on proliferation and differentiation. Cell metabolic assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, cell staining, alizarin red staining and alkaline phosphatase assay were performed. BMP-2 expression was also compared among different groups. Results showed no significant difference in proliferation, whereas differentiation was inhibited in the presence of lithium. Given these results, along with previous reports, BMP-2 downregulation is involved in the inhibition of cementoblast differentiation by lithium.

Keywords: Lithium, cementoblast, BMP-2

Introduction

Cementum is a type of special mineralized tissue covering tooth root that protects dental pulp from external stimulation. Cementum also attaches the tooth root to the alveolar bone [1, 2]. Thus, cementum is regarded as one of the most important periodontal tissues. Cementum regeneration is seldom observed under physiological conditions. However, when it is influenced by lasting mechanical force [3] or bacterial infection [4], cementum undergoes a series of processes, including resorption and formation, called regeneration. Based on the essential role of cementum, its regeneration is considered as the gold standard of success during orthodontic or periodontal treatment. Cementoblasts are cells responsible for cementum regeneration, which is similar to osteoblasts in some aspects. For example, both types of cells express Runx2, Col1, Ocn, Opn and Bsp genes. The expression of these genes is altered during cementoblast differentiation [5]. A number of studies have been conducted, but the exact mechanisms involved in cementoblast differentiation have not been elucidated [6].

Bone morphogenetic protein 2 (BMP-2) is an autocrine and paracrine growth factor that is essential for the growth of mineralized tissue. In osteoblasts, BMP-2 promotes the differentiation of bone precursor cells, and its expression is closely related to the status of osteoblast maturation [7]. However, the effect of BMP-2 on cementoblasts is contradictory. Zhao [8] reported that exposure of rh-BMP-2 to mature cementoblasts inhibits BSP and Col1 gene expression, as well as mineral nodule formation in a dose-dependent manner. By contrast, Lee and colleagues presented that rh-BMP-2 can stimulate human cementoblast growth and differentiation [9]. All evidence suggests the involvement of BMP-2 protein in cementum metabolism.

Wnt signalling is critical for the normal development of periodontal tissues through regulating cell proliferation, differentiation and apoptosis [10, 11]. In the presence of canonical Wnt agonist, β -catenin is accumulated in the cytoplasm and then translocated into nucleus, followed by the interaction with LEF/TCF family of transcription factors. Through this method, the tran-

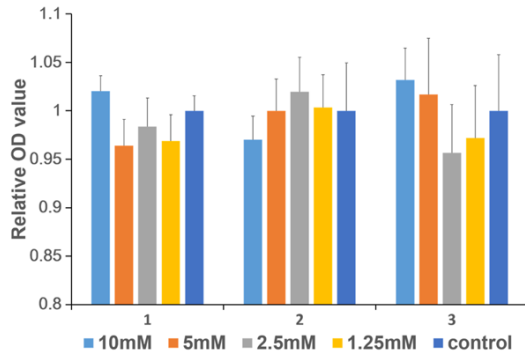


Figure 1. Proliferation analysis among various concentrations of lithium at 24, 48 and 72 h.

scription of target genes is altered and induces a series of downstream biological effects [12]. Previous studies reported the expression and regulation of Wnt signalling in various periodontal cells, including osteoblasts, periodontal ligament (PDL) cells and cementoblasts. According to published literature, activation of Wnt signalling causes different outcomes in cementum. Wnt signalling inhibits cementoblast differentiation and promotes proliferation in vitro [13]. By contrast, the canonical Wnt signalling pathway induces in vivo cementum regeneration and in vitro cementogenic differentiation of PDLs [14, 15]. Root resorption is also speculated to be related to the downregulation of Wnt signalling [16]. These data indicate the complicated regulatory effect of Wnt signalling in cementum metabolism.

Lithium has been used to treat human bipolar disease for many years and widely used as a mood-stabilizing drug. Recent clinical studies have reported that lithium reduces fracture risk in patients, enhancing the possibility of using lithium to facilitate periodontal regeneration [17, 18]. Following these discoveries, the molecular mechanisms involved were clarified. To date, most researchers agree that lithium plays an important role in activating Wnt signalling by inhibiting glycogen synthase kinase-3 β . We previously reported that lithium can attenuate root resorption during orthodontic tooth movement in cementum metabolism [19]. Han and his colleagues [20] presented the improved cementogenic differentiation of PDLs while incorporating lithium ions into bioactive scaffolds. Furthermore, lithium is regarded as a substitute for Wnt3a, which can simulate canonical activation in many studies. However, the effect and mechanism of lithium on cementoblast behav-

iour are less understood. Thus, this research aims to determine the influence of lithium on cementoblasts.

Materials and methods

Cell culture

Based on previously published methods, immortalized murine cementoblast cells (OCCM-30) were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Carlsbad, CA, USA), supplemented with 10% foetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) containing 100 U/mL penicillin and 100 μ g/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every 2 days and subcultured when confluence was 80%. Lithium chloride was purchased from Sigma (Shanghai, China).

MTT analysis

Cells were seeded in 96-well plates at a density of 5×10^3 cells per well for adherence. Afterward, the medium was changed to lithium-conditioned medium at various concentrations. After incubation for 24, 48 and 72 h, 10 μ L of MTT (5 g/L) was added to each well, and the cells were cultured for another 3 h. The medium was then aspirated, and the remaining formazan was dissolved in 150 μ L of dimethyl sulfoxide. Absorbance was measured at 590 nm using a micro-ELISA reader (Synergy2; Biotek, Winooski, VT, USA). Proliferation rate was calculated relative to the control group.

Cell staining

Cementoblasts were seeded in six-well plates containing 4×10^3 cells and 1 mL of serum-free medium. After reaching 80% confluence, the cells were treated with serum-free medium containing lithium (10 mM) and then incubated for 24 h. Subsequently, 1 μ L of calcein AM (1 mg/mL) was added, and the morphology of the cells was observed under a fluorescence microscopy (Olympus, Tokyo, Japan).

Alizarin red staining

Cells were grown to 60% confluency in DMEM/F12 containing 10% FBS. Medium was then changed to α -MEM conditioned media containing 5% FBS, 50 mg/mL ascorbic acid and 10 mmol/L β -glycerophosphate, plus lithium at 5

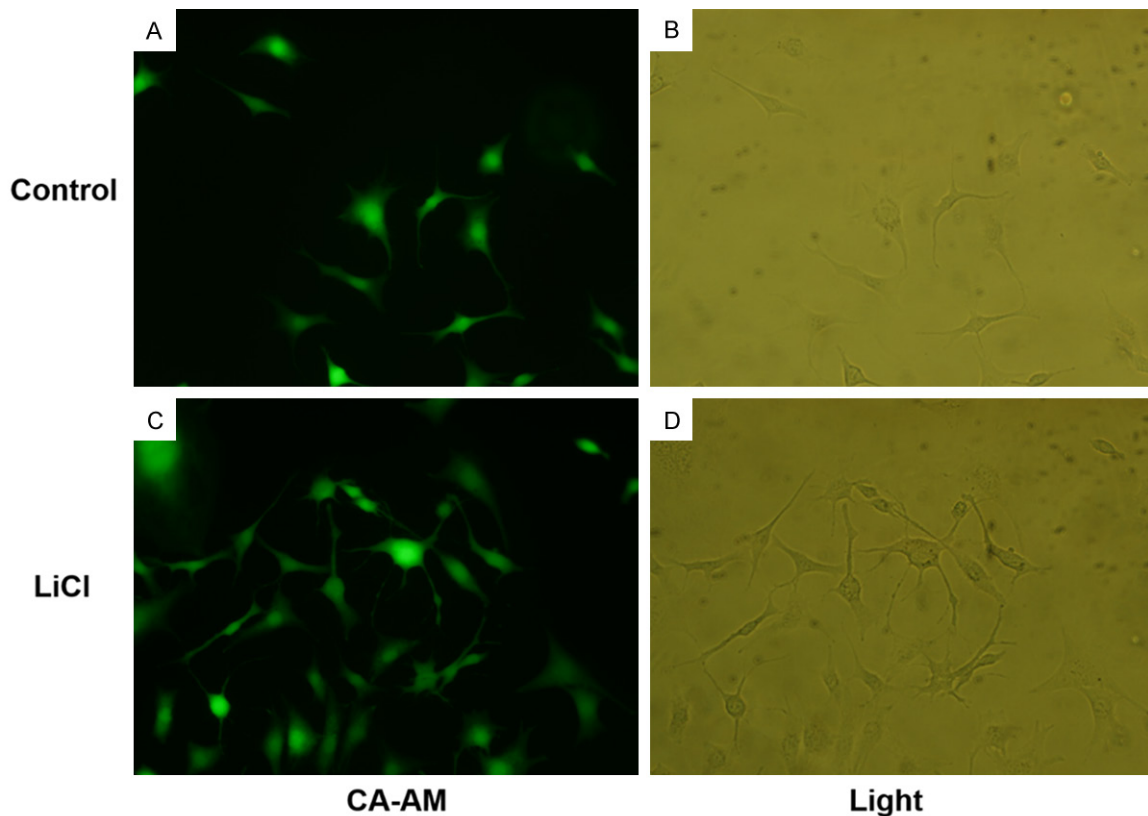


Figure 2. Calcein AM staining showing the morphology of cementoblast after incubation with 10 mM lithium for 24 h. No difference was observed (200 ×).

and 10 mM concentrations. The growth medium was replaced every 3 days up to 10 days. The cells were then fixed in formalin for 20 min at room temperature and washed with distilled water. Alizarin red solution (2%) was added to fixed cells and incubated for 10-20 min. The cells were then rinsed with distilled water and imaged. After photographs were obtained, 1 mL of cetylpyridinium chloride (1%) was added into the plate, and the OD540 values were measured.

ALP activity

Cementoblasts were exposed to lithium in differentiation medium for 3 days before ALP activity assessment. Cells were washed with phosphate-buffered saline (PBS) and then incubated in a mixture of 150 μ L of alkaline buffer solution (67 mM 4-nitrophenyl phosphate; Fluka, Buchs, Switzerland) and 10 μ L of 1.5 mM $MgCl_2$ solution for 30 min at 37°C. Afterward, 0.5 mM NaOH was added to stop the reaction, and absorbance values were measured at 405 nm. ALP activity was calculated using enzyme activity units per microgram of protein. Protein

quantitation of each well was performed with a bicinchoninic acid protein assay kit (Jiancheng Biotechnology; Nanjing, China).

Western blot

Equal loading (30 μ g) was run on a sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membranes. After blocking for 1 h, rabbit primary antibodies against mouse BMP-2 or GAPDH (Beyotime, Shanghai, China) at a ratio of 1:500 were added on the membranes and left overnight at 4°C. Primary antibody-bound membranes were washed three times in PBS-T and incubated at room temperature for 1 h with secondary antibody conjugated with alkaline phosphatase (Beyotime, Shanghai, China), with a working ratio of 1:2000. Immunosignals were visualized according to the manufacturer's instructions.

Immunofluorescence staining

After incubation with or without lithium at 10 mM, cells were fixed with 10% formalin in PBS, pH 7.3 for 10 min, and then permeabi-

BMP-2 inhibited by lithium

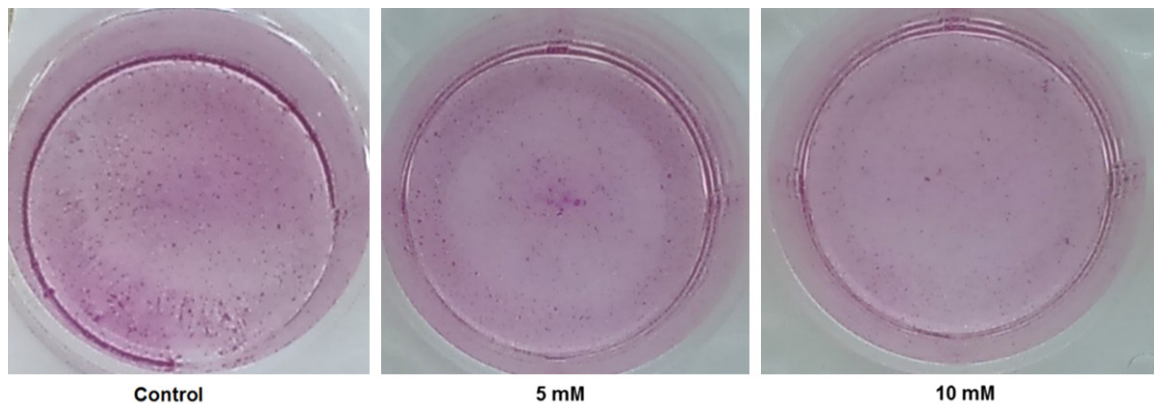


Figure 3. Alizarin red staining of cementoblasts after incubation with different lithium concentrations for 10 days.

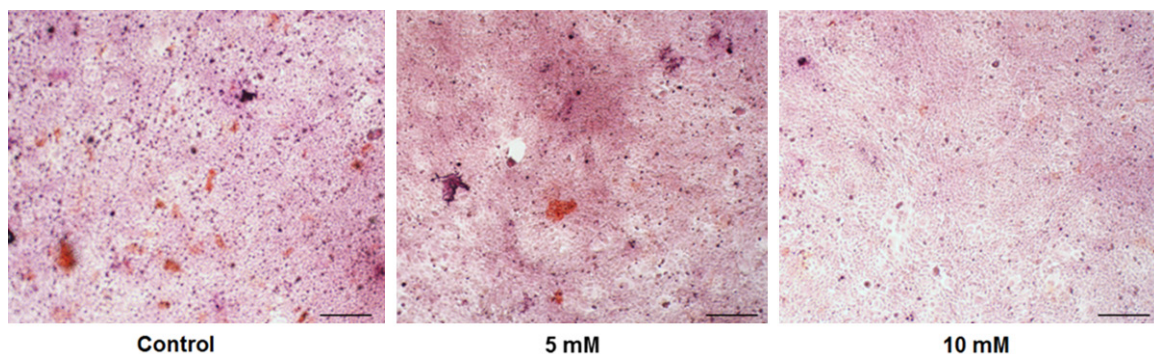


Figure 4. Microscope images showing less mineral nodules after alizarin red staining in lithium treatment groups (40 ×).

lized with 0.2% Triton X-100 in PBS for 5 min at room temperature. After blocking the cells with 1% skimmed milk for 1 h at room temperature, the cells were incubated with polyclonal mouse anti-BMP-2 (Beyotime, Shanghai, China) antibodies at a 1:100 dilution overnight at 4°C. FITC-anti-mouse IgG (Santa Cruz, CA, USA) was used as a secondary antibody at a 1:200 dilution for 1 h at room temperature. Images were obtained with a fluorescent microscope connected to a digital camera (Olympus, Tokyo, Japan).

Statistical analysis

All experiments were performed in triplicate, and the data were expressed as means \pm SD. The differences between mean values were evaluated using ANOVA, and $P < 0.05$ was considered statistically significant.

Results and discussion

Cementum is an important periodontal tissue that anchors teeth in the alveolar bone. The ori-

gin of cementoblast is unclear. Recent studies have presented the diverse origins of cementoblasts. In general, cementoblasts that form acellular cementum are from the epithelial root sheath, whereas cementoblasts that form cellular cementum are generated by dental follicle cells [1]. The differentiation of these cells into cementoblasts is regulated by various factors, such as Wnt signalling. However, the effect of Wnt signalling on cementoblasts is poorly understood. In this study, we investigated the function of lithium on the proliferation and differentiation of cementoblast.

The effect of lithium on the growth of cementoblast was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). We selected concentrations of lithium ranging from 1.25 mM to 10 mM according to previously published studies [13]. Results showed no significant differences among the groups with or without lithium treatment, which is consistent with previous research [13] (**Figure 1**). Wnt signalling activation is reported to pro-

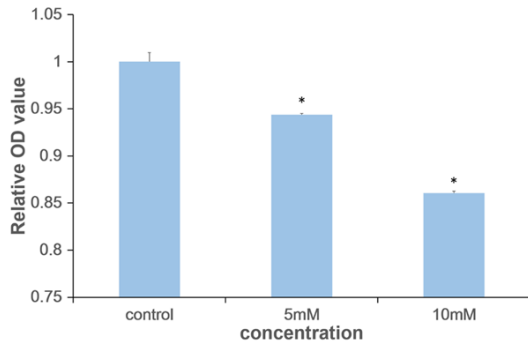


Figure 5. Quantitative analysis of alizarin red staining showing the inhibitory effect of lithium on cementoblast mineralization.

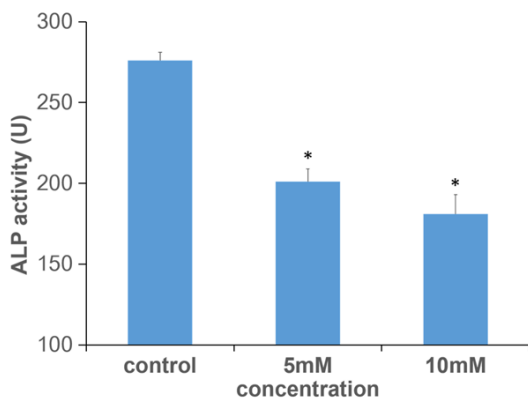


Figure 6. Alkaline phosphatase activity analysis after cementoblast treatment with lithium for 3 days.

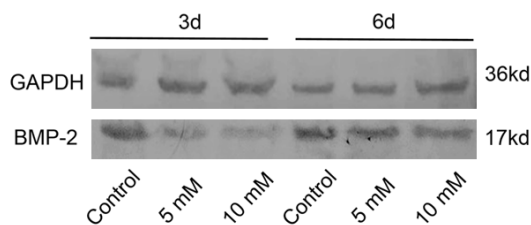


Figure 7. Expression of BMP-2 protein of cementoblasts incubated with various lithium concentrations.

mote proliferation; however, we did not observe any differences during the 3-day experiment. In the previous report, Wnt3a was used as the agonist of Wnt signalling, but not lithium. To investigate the toxicity of lithium toward cementoblasts further, we performed calcein AM staining. After incubation with 10 mM lithium for 24 h, cell morphologies were analysed and no differences were found. Cementoblasts in both groups presented a typical triangular shape with densities (Figure 2). Given these

results, along with previous studies, lithium seems to have no significant effect on cementoblast proliferation. Based on this result, we selected 10 and 5 mM as the experimental concentrations for the following studies.

The effect of lithium on cementoblast mineralization was further clarified through alizarin red staining. As shown in Figure 3, gross observation of mineralization is different in each group. The mineral nodules decreased with increasing lithium concentrations after incubation for 10 days. The images taken under a microscope showed less mineralized nodules in lithium-treated groups (Figure 4). After photographs were obtained, the mineralized nodules were dissolved for quantitative analysis, and the results are presented in Figure 5. The relative OD540 values verified a dose-dependent inhibition. Alkaline phosphatase (ALP) activity, another marker of cementoblast differentiation, was investigated on the third day of incubation with lithium. ALP is regarded as an early marker for cementoblast differentiation during culture. The results showed that ALP activity of cementoblasts decreased in the presence of lithium (Figure 6), indicating that lithium evidently inhibits ALP activity. The results suggested that Wnt signalling may be a key factor regulating cell differentiation. Previous studies showed that Wnt activation can inhibit expression of genes encoding ALP, as well as ALP activity [13], which is consistent with the current results. Thus, we confirm the inhibitory effect of lithium on cementoblast differentiation.

Although a few publications have reported the inhibition of lithium on differentiation is caused by Wnt signalling activation [21, 22], the exact molecular mechanisms involved are still unclear. We further investigated the change in BMP-2 protein levels in the presence or absence of lithium. As shown in Figure 7, the protein expression of cementoblasts was tested by Western blot. Cementoblasts expressed more BMP-2 protein with longer incubation times whether lithium was present or not. Previous reports investigated conflicting functions of BMP-2 toward cementoblasts. A study indicated the promotion of BMP-2 in cementoblast differentiation, whereas another indicated inhibition of BMP-2 [8, 9]. Current results showed that BMP-2 increased with cementoblast maturation, suggesting a positive effect

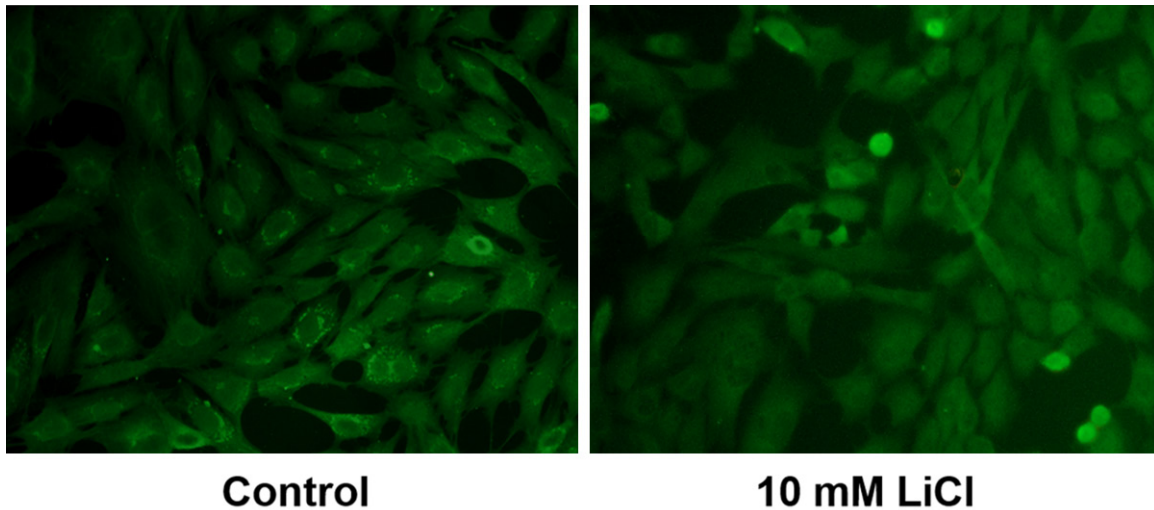


Figure 8. Immunofluorescence images of BMP-2 protein in cementoblasts under different incubation conditions (200 \times).

of BMP-2. The amount of BMP-2 protein evidently decreased with the increase in lithium concentration at the third and sixth days. To the best of our knowledge, this study is the first to report the effect of Wnt activation on BMP-2 expression in cementoblasts. Wnt signalling can also activate BMP-2 expression in osteoblasts, a type of cell similar to cementoblasts [23]. The discrepancy between the two cell types indicates the possible differences in cell origin.

Further inspection was carried out using immunofluorescence staining with anti-BMP-2 protein. The images are presented in **Figure 8**, showing results similar to the previous experiment. BMP-2 belongs to the transforming growth factor family, and its expression is regulated by other bone-related factors and signalling pathways. Hedgehog/Gli, PGE2, PTH/CR-EB, NF- κ B, oestrogen receptor and microtubule signalling pathways all participate in the regulation of BMP-2 expression. BMP-2 is also self-regulated through the autocrine method [24, 25]. In this study, we reported that the inhibition of BMP-2 is induced by lithium in mineralization medium, which may suggest a novel method in regulating BMP-2 expression.

Lithium has been widely investigated because it facilitates bone regeneration. However, few studies, together with the present study, reported the inhibition of lithium in cementum, which may be attributed to the distinct characteristics of cementoblasts compared with osteoblasts. Recently, gene expression of these two cell

types was compared in osteocalcin-green fluorescent protein transgenic mice, confirming the different expression levels of Wnt signalling [26]. Although cementum and bone share some similarities, more investigations are needed before considering the application of agents for cementum regeneration. In our previous studies, lithium attenuates root resorption during orthodontic treatment. Given all the evidence, investigating the effect of lithium in osteoclasts is necessary, as this may account for the differences in the results of the previous and current studies.

In summary, lithium exhibited no influence on cementoblast proliferation and inhibition on differentiation. BMP-2 downregulation was also involved in the process, suggesting a potential regulatory mechanism aside from Wnt signalling. Our results also indicated the distinct reaction of cementoblasts compared with osteoblasts toward the same agent. The results warrant further investigations.

Acknowledgements

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Disclosure of conflict of interest

None.

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