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Molecules and Cells

Wnt/β-Catenin Signaling Enhances Osteoblastogenic Differentiation from Human Periodontal Ligament Fibroblasts

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Wnt/β-catenin signaling has been known to influence bone formation and homeostasis. In this study, we investigated the canonical Wnt signaling regulation of osteogenic differentiation from periodontal ligament (PDL) fibroblasts. Stimulating PDL fibroblasts with lithium chloride (LiCl), a canonical Wnt activator, significantly increased mineralized nodule and alkaline phosphatase (ALP) activity in a time- and dose-dependent manner. LiCl up-regulated protein expression of osteogenic transcription factors, including the runt-related gene 2, Msx2, and Osterix 2, in the PDL fibroblasts. Treatment of these cells with LiCl also increased the mRNA levels of ALP, FosB, and Fra1 in a dose-dependent manner. Blockage of canonical Wnt signaling by treating the cells with DKK1 inhibited Wnt1-stimulated mRNA expression of these osteogenic factors. Furthermore, pretreatment with DKK1 reduced the ALP activity and matrix mineralization stimulated by Wnt1. Collectively, these results suggest that canonical Wnt signaling leads to the differentiation of PDL fibroblasts into osteogenic lineage with the attendant stimulation of osteogenic transcription factors.

INTRODUCTION

The human periodontal ligament (PDL) is a highly specialized fibrous connective tissue that attaches the tooth root to the surrounding alveolar bone to maintain tooth stability and function. The PDL consists of a heterogeneous cell population including fibroblasts, osteoblasts, cementoblasts, epithelial cells, and endothelial cells (Bordin et al., 1984; McCulloch and Bordin, 1991). Among these types of cells, the fibroblasts are the predominant cell type in the periodontium and play a central role in normal function and pathological alterations. It has been suggested that fibroblastic cells located in the PDL are a source of osteoblasts required for the continuous remodeling of alveolar bone, and play key roles in the process of periodontal tissue regeneration (Roberts et al., 1982). Various approaches for periodontal regeneration have been developed using cells derived from the PDL and demonstrated the potential of PDL fibroblasts in regulating both osteoblastic and osteoclastic differentiation occurring within the periodontal tissue. However, the molecular mechanisms by which osteogenic differentiation of PDL fibroblasts is controlled have not been sufficiently clarified.

The processes involved in tissue regeneration and repair are controlled by various signaling pathways. Wnt/β-catenin signaling controls the differentiation of progenitor cells into osteoblasts (Hartmann, 2007). Wnt molecules are a family of secreted proteins that are highly important in multiple cellular functions. Several Wnt proteins, such as Wnt1, 3a, 4, 5, 10b, and 13, have significant roles in osteoblast formation (Krishnan et al., 2006; Liu et al., 2008; Westendorf et al., 2004). Wnt signals are mediated through β-catenin which plays a pivotal role in the canonical Wnt signaling pathway (Nusse, 2005). The interaction of Wnt proteins with frizzled (Fz) receptors and low-density lipoprotein-receptor-related protein 5/6 (LRP5/6) co-receptors results in the destruction of a complex that consists of axin, adenomatous polyposis coli (APC), and GSK3β (He et al., 2004). The destruction of this complex leads to the stabilization and nuclear translocation of active dephosphorylated β-catenin, which, in turn, activates the lymphoid enhancer factor-1 (LEF)/T-cell factor (TCF) transcription system involved in the regulation of cell cycle progression and differentiation (Gordon and Nusse, 2006).

It was previously found that Wnt signaling plays essential roles in osteoblastogenesis by directly stimulating runt-related transcription factor 2 (Runx2) gene expression promoting osteoblastogenesis (Bradbury et al., 1994; Gaur et al., 2005). Similarly, the over-expression of Wnt10b in mice inhibits bone loss and shifts mesenchymal stem cells (MSCs) towards osteoblastic lineage by inducing Runx2 (Bennett et al., 2005). Stimulating MSCs with lithium chloride (LiCl), as an activator of the Wnt signaling pathway, induces the differentiation of MSCs into osteoblasts *in vitro* and *in vivo* (Clément-Lacroix et al., 2005). Conversely, Dickkopfs 1 (DKK1), which can bind to LRP5

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and block Wnt/β-catenin signaling, decreases bone formation (Morvan et al., 2006). However, the potential relationship between the canonical Wnt pathway and osteogenic differentiation of PDL fibroblasts has yet to be clarified. In the present study, we demonstrate that canonical Wnt signaling enhances osteogenic differentiation from the PDL fibroblast, while DKK1 inhibits this process.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was purchased from Gibco-BRL (USA). Lithium Chloride (LiCl), Wnt1, and Dickkopf-1 (DKK1) were obtained from the Sigma Chemical Company (USA). The β-catenin, actin, Ref-1, Runx2, Msx2, Osx2, and goat-anti rabbit antibodies were supplied by Santa Cruz Biotechnology (USA). Unless otherwise specified, the chemicals and laboratory items were purchased from Sigma Chemical Company and Falcon Labware (USA), respectively.

Cell culture

PDL fibroblasts were obtained from three male healthy individuals, aged 20-30 years who underwent molar extraction. These cells were cultured according to the methods described elsewhere with minor modifications (Howard et al., 1998). Written informed consent for the use of the periodontal tissues was obtained from all donors. This study was approved by the Review Board of Chonbuk National University Hospital (CNUH). All cultures were maintained at 37°C with a humidified gas mixture of 5% CO_2/95% air and switched to a fresh batch of medium every 3 days. All of the experiments were performed at passages 4-7. Cells were then grown in an osteogenic medium that consisted of α-MEM containing 5% FBS, 50 μg/ml ascorbic acid, and 3 mM β-glycerophosphate for 7 days in the presence and absence of LiCl, Wnt1, or DKK1.

Alizarin red staining

Alizarin red staining was performed using a slight modification of the method reported by Han et al. (2009). The culture medium was discarded and cells were briefly fixed for 30 min in 4% paraformaldehyde fluid, washed three times with ice-cold phosphate-buffered saline (PBS), and stained for 5 min with alizarin red prior to light microscopic observation.

Alkaline phosphatase activity

Cells were washed twice with PBS and lysed in 50 mM Tris-HCl buffer (pH 7.0) containing 1% (v/v) Triton X-100 and 1 mM PMSF. The total protein was then quantified using the Bradford method (1976). The Alkaline Phosphatase activity was measured by a slightly modified method from a previous report (Han et al., 2009). The whole cell lysate was assayed by adding 200 μl of *p*-nitrophenylphosphate (pNPP) as a substrate (Sigma, USA) for 30 min at 37°C. The reaction was stopped by adding 3 N NaOH, and the absorbance was read spectrophotometrically at 405 nm. The enzyme activity was expressed as μM/mg of protein.

RNA isolation and real time RT-PCR

The total RNA was extracted from the cells treated with each of the designated agents using STAT-60, which is a monophasic solution of phenol and guanidine isothiocyanate (Tel-Test Inc., USA). The real-time quantification of RNA targets was then performed in the Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, Australia) using a QuantiTect SYBR Green RT-PCR kit (QIAGEN, USA). The reaction mixture (20 μl) contained 200 ng of the total RNA, 0.5 μM of each primer, the appropriate amounts of enzymes, and fluorescent dyes, as recommended by the supplier. The Rotor-Gene 2000 cycler was programmed as follows: 30 min at 50°C for reverse transcription; 15 min at 95°C for DNA polymerase activation; 15 s at 95°C for denaturing; and 45 cycles of 15 s at 94°C, 30 s at 55°C, 30 s at 72°C. Data collection was carried out during the extension step (30 s at 72°C). The PCR reaction was followed by melting cure analysis to verify the specificity and identity of the RT-PCR products, which can distinguish the specific PCR products from the non-specific PCR product resulting from primer-dimer formation. The primers used were 5′-GGA CAT GCA GTA CGA GCT GA-3′ (sense), 5′-GCA GTG AAG GGC TTC TTG TC-3′ (antisense) for ALP, 5′-TCCAGGCGGAGA CAGATCAGTTG-3′ (sense), 5′-TCTTCGTAGGGGATCTTGC AGCC-3′ (antisense) for FosB, and 5′-CCCTGCCGCCCTG TACCTTGTATC-3′ (sense), 5′-AGACATTGGCTAGGGTGGC ATCTGCA-3′ (antisense) for Fra1. The temperature of the PCR products was increased from 65 to 99°C at a rate of 1°C/5 s, and the resulting data was analyzed using software provided by the manufacturer.

Preparation of cell fractions

Nuclear proteins were prepared as described in a previous study (Maulik et al., 1998) and quantified according to the Bradford method. In addition, the PDL fibroblasts were incubated in a lysis buffer (250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml each of leupeptin, aprotinin, and pepstatin A) on ice for 30 min and centrifuged at 750 × *g* for 10 min at 4°C. The supernatants were further centrifuged at 10,000 × *g* for 25 min at 4°C and used as a cytosolic fraction.

Western blot analysis

Western blot was conducted as previously reported (Kook et al., 2009). Protein extract samples (20 μg) were separated by 8-10% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. The blots were washed with TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], blocked with 5% skim milk for 1 h, and incubated with the appropriate primary antibody at the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. The blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, USA) and exposed to X-ray film (Eastman-Kodak, USA).

Statistical analysis

All data are expressed as mean ± standard deviation (S.D.). One-way ANOVA was used for multiple comparisons (Duncan's multiple range test) using SPSS software ver. 17.0. A P values < 0.05 were considered significant.

RESULTS

Nuclear translocation of β-catenin by LiCl in PDL fibroblasts

To investigate the effect of Wnt/β-catenin signaling on the osteogenesis of PDL fibroblasts, we initially examined whether LiCl treatment changes the subcellular localization of β-catenin in the cells. Treating the cells with LiCl for 24 h increased the levels of β-catenin in both cytosolic and nuclear fraction dose-dependently (Fig. 1). This result indicates that LiCl stimulation leads to the activation of Wnt/β-catenin pathway in PDL fibro-

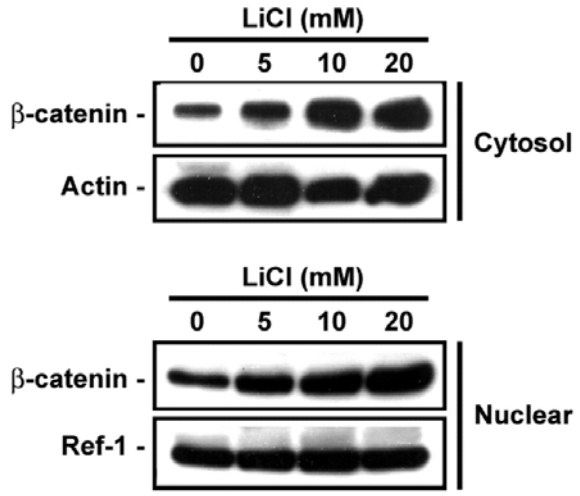

Fig. 1. LiCl-mediated nuclear translocation of β-catenin in PDL fibroblasts. Cells were incubated with and without 20 mM LiCl for 24 h and adjusted for immunoblotting analysis. A representative result from three independent experiments is shown. Actin and Ref-1 were used as cytosolic and nuclear control proteins, respectively.

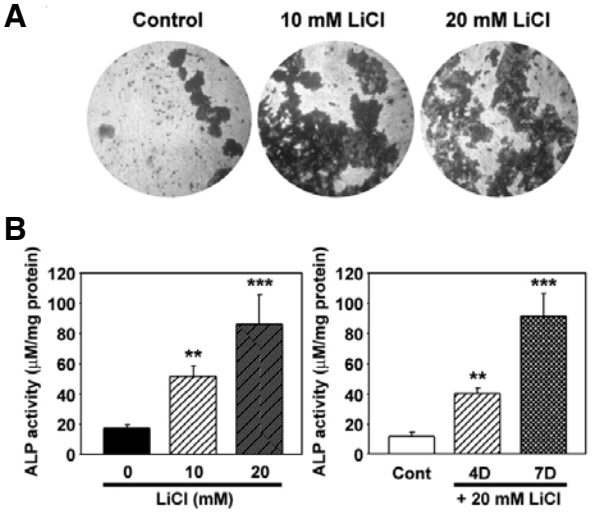

Fig. 2. Stimulating effect of LiCl on osteogenic differentiation of PDL fibroblasts. (A) Cells were cultured with an osteogenic medium in the presence of LiCl (10 or 20 mM) for 7 days. The resulting mineralization was assessed by Alizarin red staining. Each microscopic image shown is a representative of five separate experiments. (B) Cultures were also treated with LiCl (10 or 20 mM) for 7 days or with 20 mM LiCl for 4 and 7 days. ALP activity was determined, and the values represented are the mean ± S.D. of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the untreated control values.

blasts.

Effect of canonical Wnt signaling on mineralization and ALP activity

To evaluate whether the LiCl-mediated Wnt/β-catenin pathway can stimulate osteogenic differentiation from PDL fibroblasts, we examined mineralization using Alizarin red staining and ALP

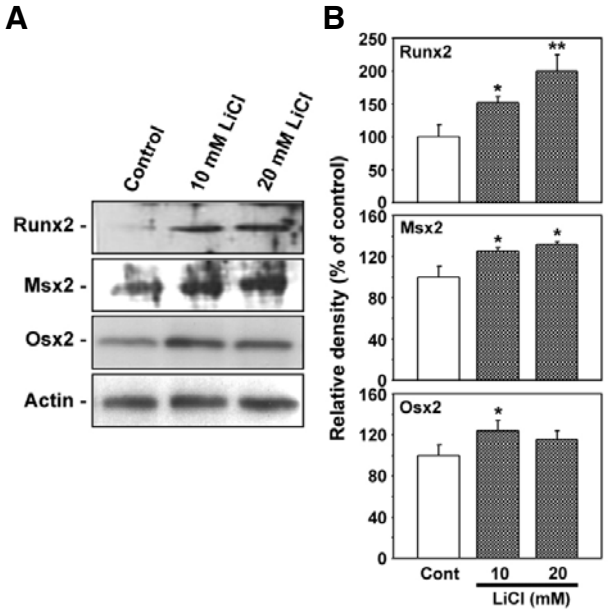

Fig. 3. Effects of LiCl on the expression of osteogenic transcription factors in PDL fibroblasts. (A) Cells were treated with LiCl (10 or 20 mM) for 7 days and then the levels of Runx2, Msx2, and Osx2 were determined by Western blot analysis using total protein lysates. (B) The data show a relative expression pattern of these proteins from triplicate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the untreated control values.

activity. As shown in Fig. 2A, the cultures incubated with LiCl for 7 days represented a transparent augmentation of Alizarin red staining, where a dose-dependent increase of calcium nodule formation and matrix mineralization was shown after LiCl treatment. In parallel with this result, LiCl increased ALP activity in a time and dose-dependent manner (Fig. 2B).

Stimulating effect of canonical Wnt signaling on osteogenic differentiation factors

The osteogenic potential of Wnt signaling in PDL fibroblasts was supported by evaluating changes in protein expression of osteoblast specific transcription factors, such as Runx2, Msx2, and Osterix 2 (Osx2). Western blot analysis showed that the expression of each protein was increased by incubation with LiCl for 7 days (Fig. 3). To confirm the osteoblastogenesis facilitating effect of LiCl, we employed a real time RT-PCR using ALP, FosB, and Fra1 as examples of other osteogenic target genes. A dose-dependent increase in each mRNA level was observed after LiCl treatment (Fig. 4). An increase in the mRNA levels was higher in FosB and Fra1 than ALP. These results suggest that canonical Wnt pathway encourages the differentiation of PDL fibroblasts into osteogenic lineage.

Inhibitory effect of DKK1 on Wnt1-induced osteogenic differentiation from PDL fibroblasts

In order to confirm whether canonical Wnt signaling is a positive regulator for osteogenesis from PDL fibroblasts, these cells were incubated with DKK1, which binds to LRP5/6 co-receptors and inhibits canonical Wnt signaling, just before treatment with Wnt1. Similar to the results where LiCl increased the mRNA levels of several factors, the addition of 500 ng/ml Wnt1 to the cultures significantly augmented mRNA expression of ALP, FosB, and Fra1 (Fig. 5). Pretreatment of cells with DKK1 re-

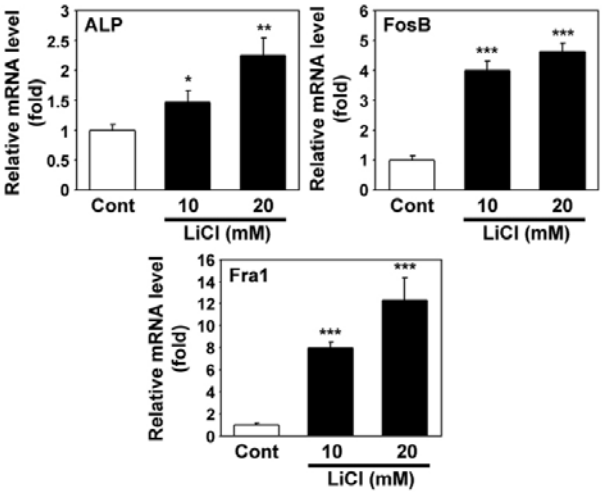

Fig. 4. Effect of LiCl on mRNA expression of ALP, FosB, and Fra1. PDL fibroblasts were treated with LiCl (10 or 20 mM) for 7 days, and mRNA levels of the factors were measured using the real-time RT-PCR technique. The values reported are the mean ± S.D. of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. the untreated control values.

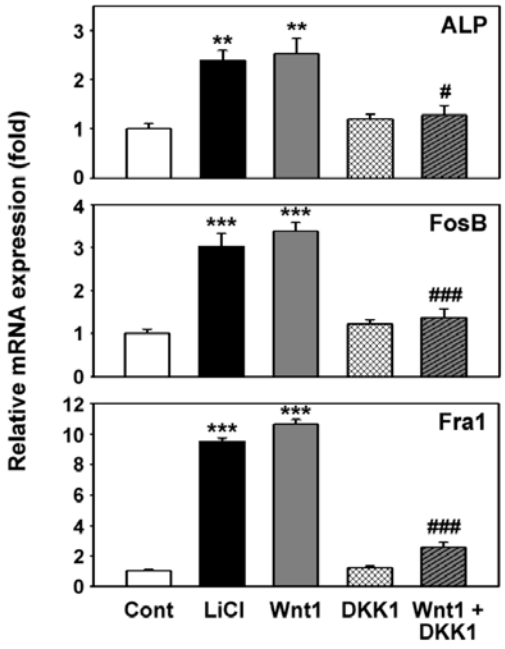

Fig. 5. Inhibitory effect of DKK1 on Wnt1-induced mRNA expression of osteogenic regulatory factors. PDL fibroblasts were pretreated with 0.5 μg/ml DKK1 for 30 min before the addition of 500 ng/ml Wnt1. LiCl (20 mM) was also used as a counterpart stimulator of Wnt signaling. After 48 h of incubation, the mRNA levels of ALP, FosB, and Fra1 were measured by real-time-PCR. The results shown are the mean ± S.D. from three separate experiments. **$P < 0.01$ and ***$P < 0.001$ vs. the untreated control values. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs. Wnt1 treatment alone.

duced the Wnt1-increased mRNA levels of these osteogenic factors to the control levels. In addition, DKK1 almost completely diminished the ALP activity that had increased after Wnt1 treatment in the cells (Fig. 6).

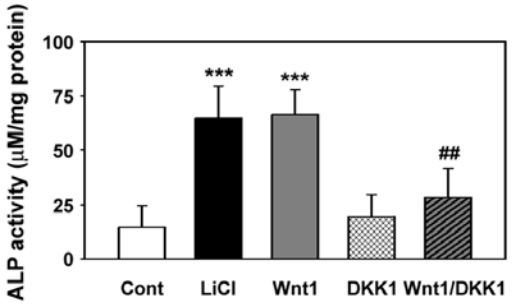

Fig. 6. Inhibition by DKK1 of Wnt1-mediated increase of ALP activity in PDL fibroblasts. Cells were pretreated with 0.5 μg/ml DKK1 30 min before the addition of 500 ng/ml Wnt1 and processed for the analysis of ALP activity after 7 days of incubation. ***$P < 0.001$ vs. the untreated control values. $^{\#\#}P < 0.01$ vs. Wnt1 treatment alone.

DISCUSSION

Human periodontal ligament-derived cells have different tendencies for osteogenesis, chondrogenesis, adipogenesis, or proliferation. These cellular characteristics can be controlled in part by the addition of cytokines, growth factors, or drugs. Therefore, identifying the biochemical pathways related to these soluble factors can drive a balanced cellular differentiation. In the present study, we present the experimental evidence describing the stimulating effect of canonical Wnt/β-catenin signaling on osteogenic differentiation of the PDL fibroblasts.

The effects of Wnt signaling on osteogenesis *in vitro* and *in vivo* have been widely reported. The enhancement of Wnt signaling either by Wnt over-expression (Bennett et al., 2007) or deficiency of Wnt antagonists (Morvan et al., 2006; ten Dijke et al., 2008) increased bone formation in mice and humans. Wnt/β-catenin signaling also stimulates differentiation of mouse MSCs toward the osteoblastic lineage (Gaur et al., 2005; Gong et al., 2001). In this study, we mimicked the effect of Wnt by adding LiCl to PDL fibroblasts and examined whether canonical Wnt signaling positively affects the differentiation of these cells into osteoblasts. LiCl treatment strongly augmented ALP activity and mineralization in PDL fibroblasts cultured in the osteoinductive medium. This result suggests that the use of agents that up-regulate the canonical Wnt pathway can enhance osteogenic differentiation of PDL fibroblasts.

It has been known that the osteogenic transcription factors including Runx2, Msx, and Osx are essential for bone formation and osteoblast differentiation (Ichida et al., 2004; Komori, 2005; Nakashima et al., 2002). In agreement with these reports, the present study demonstrates that LiCl increased the levels of Runx2, Msx2, and Osx2 in a dose-dependent manner. Runx2 is the major transcription factor regulating osteoblast commitment and osteogenic differentiation of mesenchymal cells (Deng et al., 2008). It was previously reported that the up-regulation of β-catenin protein levels by Wnt signaling is enough to promote Runx2 expression, which leads to osteoblast differentiation (Day et al., 2005). In addition, Wnt10b induced mesenchymal cells toward the osteoblast lineage with an attendant increase in Runx2 and the suppression of adipogenic transcription factors (Bennett, 2005). It has been known that Osx acts downstream of Runx2 (Franceschi et al., 2007).

Considerable evidence involved in the relationship between Wnt signaling and Osx revealed that the Wnt molecule up-regulates the expression of both Runx2 and Osx (Bennett, 2005). There is a report emphasizing that β-catenin is an essential signal for the specification of an $Osx1^+$ osteoblast to a bone-secreting osteoblast (Rodda and McMahon, 2006). It was also reported that Msx2 promoted the osteogenic differentiation of skeletal progenitors in part by reducing DKK1 expression and enhancing Wnt signaling (Cheng et al., 2008). Consequently, the activation of transcription factors, such as Runx2, Msx2, and Osx2, is closely related to LiCl-mediated osteogenic differentiation in PDL fibroblasts.

Notably, members of the Fos family of activator protein-1, FosB, and Fra1, play important roles in osteoblast differentiation and bone formation. A previous study showed that the up-regulation of FosB expression by cyclic stretch stimulated osteogenic differentiation of human mesenchymal precursor cells (Haasper et al., 2008). Fra1 also led to osteosclerosis, which was likely due to the accelerated differentiation of osteoprogenitors into mature osteoblasts (Jochum et al., 2006). Based on our present findings and previous studies, we suggest that FosB and Fra1 might have a physiological importance for osteogenic differentiation stimulated by Wnt/β-catenin signaling.

Several molecules, including the DKK family negatively regulate canonical Wnt signaling. Among the four DKK proteins, DKK1 and DKK2 have been well defined as Wnt antagonists (Mao et al., 2001; 2002). The inhibition of Wnt/β-catenin signaling by DKK1 blocked osteoblastogenic differentiation in bone marrow stromal cells (Amantea et al., 2008). Over-expression of DKK1 decreased endogenous β-catenin and ALP activity in osteoblasts (Qiang et al., 2008), whereas deletion of DKK1 led to a significant increase of bone formation (Morvan et al., 2006). Consistent with these previous studies, the addition of DKK1 completely prevented the Wnt1-mediated osteogenic events from occurring in the PDL fibroblasts.

In summary, this study demonstrates that the activation of the Wnt/β-catenin signaling pathway is involved in osteogenic differentiation from PDL fibroblasts. Our findings suggest that a pharmacological modulation of canonical Wnt signaling can increase the osteogenic capacity of PDL cells, improving the cells' efficacy for the reparation of periodontal tissue defects.

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