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Journal of Cellular Biochemistry 114:2551–2561 (2013)

Journal of Cellular Biochemistry

bFGF and JAGGED1 Regulate Alkaline Phosphatase Expression and Mineralization in Dental Tissue-Derived Mesenchymal Stem Cells

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ABSTRACT

Basic fibroblast growth factor (bFGF) and Notch signaling play critical roles in various cell behaviors. Here, we investigated the influence of bFGF and Notch signaling in alkaline phosphatase (ALP) expression and mineralization process in human periodontal ligament-derived mesenchymal stem cells (PDLSCs) and stem cells isolated from human exfoliated deciduous teeth (SHEDs). PDLSCs and SHEDs were cultured in osteogenic medium supplemented with bFGF or on the immobilized Notch ligands, JAGGED1. The ALP mRNA and protein expression were measured by quantitative reverse transcriptase polymerase chain reaction and enzymatic activity assay, respectively. Mineral deposition was determined using alizarin red S staining. The results showed that the addition of bFGF resulted in the decrease of ALP mRNA expression and enzymatic activity. In addition, the attenuation of mineralization was noted. These phenomenons were blocked by the addition of a fibroblast growth factor receptor inhibitor (SU5402) or a MEK inhibitor (PD98059). Interestingly, bFGF supplementation also decreased the Notch signaling component mRNA levels. Thus, to evaluate effect of Notch signaling in mineralization process, PDLSCs and SHEDs were exposed to JAGGED1 modified surface. The ALP mRNA and protein expression were significantly upregulated and the mineral deposition was markedly increased. These results could be reversed by the addition of a γ-secretase inhibitor. In addition, bFGF could attenuate the Notch-signaling-induced mineralization in both PDLSCs and SHEDs. These results suggest that mineralization was enhanced by Notch signaling but attenuated by bFGF signaling. This knowledge can be further utilized to control PDLSCs and SHEDs mineralization for tissue regeneration purpose. J. Cell. Biochem. 114: 2551–2561, 2013.

KEY WORDS: NOTCH SIGNALING; BASIC FIBROBLAST GROWTH FACTOR; ALKALINE PHOSPHATASE; MINERALIZATION

Alkaline phosphatase (ALP) is a membrane bound ectoenzyme. It participates in several processes of bone formation and regeneration. ALP degrades inorganic pyrophosphate, which is an inhibitor of hydroxyapatite formation, resulting in an increase of inorganic phosphate (P_i) concentration at extracellular site [Addison et al., 2007]. Further, P_i is transferred into cytoplasm and subsequently regulates osteoblast marker gene expression [Beck et al., 2000; Wu et al., 2003]. P_i also regulates the mineralization process by direct participating in hydroxyapatite formation [Osathanon et al., 2009]. In addition, it has been shown that an in vitro mineralization was altered in primary calvarial cell isolated from *ALP* knockout and heterozygous mice [Wennberg et al., 2000]. In this regard, primary calvarial cells isolated from *ALP* knockout mice has significantly decreased in mineral

Conflict of interest: None.

Grant sponsor: Integrated Innovation Academic Center IIAC; Grant sponsor: Genetic and Anatomical Analyses of Craniofacial Structures, Faculty of Dentistry, Chulalongkorn University; Grant sponsor: Chulalongkorn University Centenary Academic Development Project; Grant sponsor: Higher Education Research Promotion and National Research University Project, Office of the Higher Education Commission, Thailand; Grant sponsor: Asahi Glass Foundation; Grant sponsor: Research Chair Grant 2012, National Science and Technology Development Agency (NSTDA), Thailand.

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Manuscript Received: 12 March 2013; Manuscript Accepted: 17 May 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 June 2013

DOI 10.1002/jcb.24602 • © 2013 Wiley Periodicals, Inc.

deposition and cells from *ALP* heterozygous mice has a delayed mineralization process compared to those of wild type mice [Wennberg et al., 2000]. The potential application of ALP in bone tissue engineering was also reported. The ALP immobilized fibrin scaffolds enhance mineralization in vitro and bone regeneration in vivo [Osathanon et al., 2009]. These results indicate the tight relation between alkaline phosphatase and mineralization in osteogenic differentiation and bone regeneration process.

Several reports have been investigated the regulation of ALP and mineralization. Basic fibroblast growth factor (bFGF) is one of the molecules that has been investigated as it has substantial role in the regulation of cell behaviors that is proliferation and differentiation [Emmenegger et al., 2012; Perez et al., 2013]. The role of bFGF in regulation of *ALP* expression and mineralization is still controversy. Our previous study in human dental pulp stem cells illustrated that bFGF inhibited ALP enzymatic activity and mineralization in vitro [Osathanon et al., 2011]. However, it has been reported that bFGF enhanced osteogenic differentiation of bone marrow-derived mesenchymal stem cells in collagen hydrogel as determined by the induction of *ALP* expression and the mineral deposition in vitro [Oh et al., 2012]. Thus, these results indicate that the regulation of ALP and mineralization may be cell type specific.

Similarly, Notch signaling has also shown to regulate *ALP* and mineralization in cell-type specific manner. It has been shown that overexpression of Notch intracellular domains (NICDs) promoted ALP enzymatic activity and mineralization in vascular smooth muscle cells [Shimizu et al., 2009]. On the contrary, Notch signaling has been demonstrated as negative regulation of osteogenic differentiation as the abolishment of Hey1, a direct Notch target gene, resulted in the increase of mineralization in human mesenchymal stem cells [Zamurovic et al., 2004]. Together, the investigation of specific regulation in particular cell type is indeed necessitated.

Stem cells isolated from human exfoliated deciduous teeth (SHEDs) and human periodontal ligament stem cells (PDLSCs) are of interest due to the ease to obtain the cells and their potential applications that is dental pulp tissue regeneration [Cordeiro et al., 2008], alleviating Parkinson′s disease [Wang et al., 2010], spinal cord contusion injury therapy [Taghipour et al., 2012] and, particularly on, bone regeneration [Tour et al., 2012]. In addition, the immunomodulatory properties of these stem cells could be beneficial in future clinical therapeutic utilization [Wada et al., 2009; Yamaza et al., 2010]. Various studies have been investigated the control mechanism of osteogenic differentiation and potential bone regeneration application of these cells. Currently, the evidences revealing the ALP and mineralization in PDLSCs and SHEDs are yet lacking. In the present study, we aimed to investigate the role of bFGF and Notch signaling in the regulation of ALP expression and mineral deposition in PDLSCs and SHEDs.

MATERIALS AND METHODS

CELL CULTURE

The PDLSCs and SHEDs were isolated under protocol approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Teeth removal due to required treatment reasons that is prolong deciduous teeth and third molar impaction were collected for cell isolation using methods previously described [Govitvattana et al., 2012; Osathanon et al., 2013]. The isolated cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B in 100% humidity, 37°C and 5% carbon dioxide. Medium was changed every 48 h. After reaching confluence, the cells were sub-cultured at a 1:3 ratio. The cells from passage 3 to 6 were employed in this study.

In experiments, cells were seeded at a density of 25,000 cells/wells in a 24-well-plate and maintained in a growth medium or an osteogenic medium (growth medium supplemented with ascorbic acid (50 μg/ml), Dex (100 nM) and β-glyceraphosphate (10 mM). The medium was changed every 48 h. In some case, bFGF (Invitrogen) was added into the culture at concentration 20 ng/ml. For activate Notch signaling, cells were seeded on JAGGED1/Fc (R&D systems) immobilization tissue culture surface according to our previous published protocol [Osathanon et al., 2013]. The human Fc portion was used as the control immobilization surface. In the inhibition study, a fibroblast growth factor receptor inhibitor (SU5402; Calbiochem), MEK inhibitor (PD98059; Calbiochem), or a gamma-secretase inhibitor (DAPT; Sigma) was added into culture condition at concentration 10, 40, or 20 μM, respectively.

ALKALINE PHOSPHATASE ACTIVITY ASSAY

The cells were cultured in osteogenic medium under various treating condition. Then, the cells were lyzed in alkaline lysis buffer. Aliquots were incubated at 37°C in a solution containing 2 mg/ml *p*-nitrophenol phosphate, 0.1 M 2-amino-2methyl-1-propanol and 2 mM $MgCl_2$. After 15 min, 50 mM NaOH was added to stop the reaction. The presence of *p*-nitrophenol was measured at an absorbance of 410 nm. Total cellular protein was determined using a BCA assay. The enzyme activity was normalized to total cellular protein.

MINERALIZATION ASSAY

The cells were cultured in osteogenic medium under various treating condition. Then, the cells were fixed with cold methanol for 10 min, washed with deionized water and stained with 1% Alizarin Red S solution for 3 min at room temperature on a shaker. The amount of calcium deposition was quantified by destaining with 10% cetylpyridinium chloride monohydrate in 10 mM sodium phosphate at room temperature for 15 min. The absorbance was measured at 570 nm. To determine the influence of alkaline phosphatase enzyme (ALP) and inorganic phosphate on mineralization, levamisole (Sigma), an inhibitor of ALP, or foscarnet (Sigma), an inhibitor of sodium-dependent phosphate cotransporters, were added to the osteogenic differentiation medium at final concentration 1 M and 0.1 mM, respectively.

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

Cells were cultured for designated timepoints and the total cellular RNA was extracted with Trizol reagent. RNA samples (1 μg) were converted to cDNA by reverse transcriptase reaction (Invitrogen). The polymerase chain reaction (PCR) was performed in a LightCycler® Nano system (Roche Diagnostic) with LightCycler® 480 SYBR Green I Master kit (Roche Diagnostic). The amplification profile was: 95°C/10 s, 60°C/10 s, and 72°C/20 s for 40 cycles. Reaction product

TABLE I. Primer Sequences

Gene	Primer sequences (forward)	Primer sequences (reward)
ALP	5′ CGAGATACAAGCACTCCCACTTC 3′	5′ CTGTTCAGCTCGTACTGCATGTC 3′
HES1	5′-AGGCGGACATTCTGGAAATG 3′	5′ CGGTACTTCCCCAGCACACTT 3′
HEY1	5′-GGAGAGGCGCCGCTGTAGTTA 3′	5′ CAAGGGCGTGCGCGTCAAAGTA 3′
NOTCH1	5′-GCCGCCTTTGTGCTTCTGTTC-3′	5′-CCGGTGGTCTGTCTGGTCGTC-3′
NOTCH2	5′-CCAGAATGGAGGTTCCTGTA 3′	5′ GTACCCAGGCCATCAACACA 3′
NOTCH3	5′-TCTTGCTGCTGGTCATTCTC 3′	5′ TGCCTCATCCTCTTCAGTTG 3′
NOTCH4	5′-AGCCGATAAAGATGCCCA 3′	5′ ACCACAGTCAAGTTGAGG 3′
DLL1	5′-AGACGGAGACCATGAACAAC 3′	5′ TCCTCGGATATGACGTACAC 3′
JAGGED1	5′-AGTCACTGGCACGGTTGTAG 3′	5′ TCGCTGTATCTGTCCACCTG 3′
MMP2	5′-CAAGAAGTATGGCTTCTGCC 3′	5′ GCACCCTTGAAGAAGTAGCT 3′
MMP13	5′-GGCGACTTCTACCCATTTGA 3′	5′ ATACGGTTGGGAAGTTCTGGC 3′
MT1-MMP	5′-CATCGCTGCCATGCAGAAGT 3′	5′ GTCATCATCGGGCAGCAC 3′
GAPDH	5′-CACTGCCAACGTGTCAGTGGTGT-3′	5′-GTAGCCCAAGGATGCCCTTGAG-3′

was quantified with GAPDH as the reference gene and further normalized to the control of each experiment. The oligonucleotide sequences of the primers were shown in Table I [Muller et al., 2002; MacKenzie et al., 2004; Xu et al., 2004; Patel et al., 2005; Ma et al., 2007; Chuenjitkuntaworn et al., 2010; Phi et al., 2010].

STATISTICAL ANALYSES

Data were reported as mean ± standard deviation. Statistical analyses were performed using two-independent Student *t*-test for two-group comparison. A one-way analysis of variance (ANOVA) followed by Dunnett test was employed to compare in experiments containing three or more groups. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

ALP AND Pi REGULATED MINERALIZATION IN SHEDs AND PDLSCs

To determine the role of ALP and P_i on mineralization in SHEDs and PDLSCs, levamisole or foscarnet was added to the osteogenic differentiation medium and the cells were cultured for 14 days. The results illustrated that, upon maintaining cells in osteogenic medium, the mineralization was significantly increased (Fig. 1). The addition of levamisole or foscarnet in osteogenic medium resulted in a marked reduction of mineral deposition in both cell types. These results suggest that both ALP and P_i substantially regulate mineralization in SHEDs and PDLSCs.

bFGF INHIBITED ALP EXPRESSION AND MINERALIZATION

To determine the influence of bFGF on *ALP* expression in both SHEDs and PDLSCs, cells were cultured in normal growth medium or osteogenic medium for 7 days. The results illustrated that the *ALP* mRNA levels were decreased after exposed cells with bFGF in both normal growth medium and osteogenic medium (Fig. 2A and B). Correspondingly, the ALP enzymatic activity was significantly decreased upon treating cells with bFGF under osteogenic medium at 7 and 14 days (Fig. 2C,D). Further, the mineral deposition was significantly reduced in the bFGF treated groups compared to that of osteogenic medium control at 14 days (Fig. 2E,F).

In time course experiments, the addition of exogeneous bFGF significantly attenuated the *ALP* mRNA expression at day 3 and 5 in osteogenic culture condition (Fig. 2G,H). The role of bFGF on the decrease of *ALP* mRNA expression was confirmed by the addition of FGFR inhibitor (SU5402). The results illustrated that the FGFR inhibitor (SU5402) treatment was able to attenuate the reduction of ALP mRNA expression upon exposed to bFGF for 3 days, confirming the influence of bFGF on *ALP* expression (Fig. 2I,J).

bFGF INHIBITED ALP EXPRESSION AND MINERAL DEPOSITION VIA FGFR AND MEK PATHWAY

SHEDs and PDLSCs were treated with SU5402 and PD98059 to inhibit FGFR and MEK, respectively. The cells maintained in osteogenic medium were employed as the control. The cells were cultured for 7 and 14 days, for ALP expression analyses and mineralization assay, respectively. The results illustrated that treating cells with FGFR and MEK inhibitor could attenuate the inhibition effect of bFGF. In this regard, the *ALP* mRNA expression, ALP enzymatic activity and mineral deposition of FGFR and MEK inhibitor treatment groups were

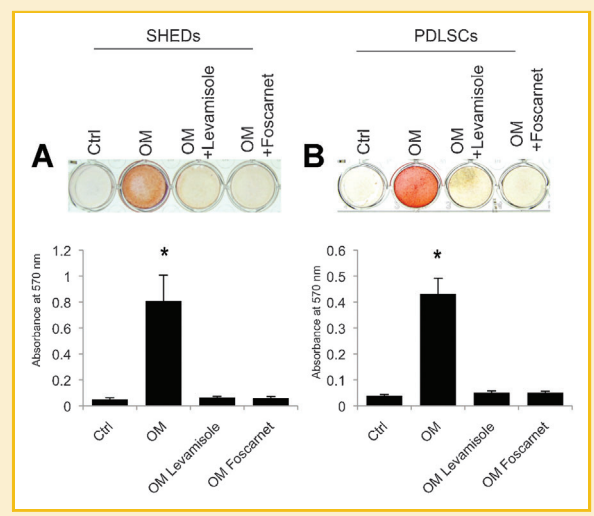

Fig. 1. ALP and P_i regulated mineralization in SHEDs and PDLSCs. The mineralization was evaluated using alizarin red s staining at 14 days after osteogenic induction in SHEDs (A) and PDLSCs (B) (Ctrl; normal growth medium, OM; Osteogenic medium). The graphs illustrated the absorbance of eluted staining at 570 nm. The asterisk indicated the statistical significance compared to the control.

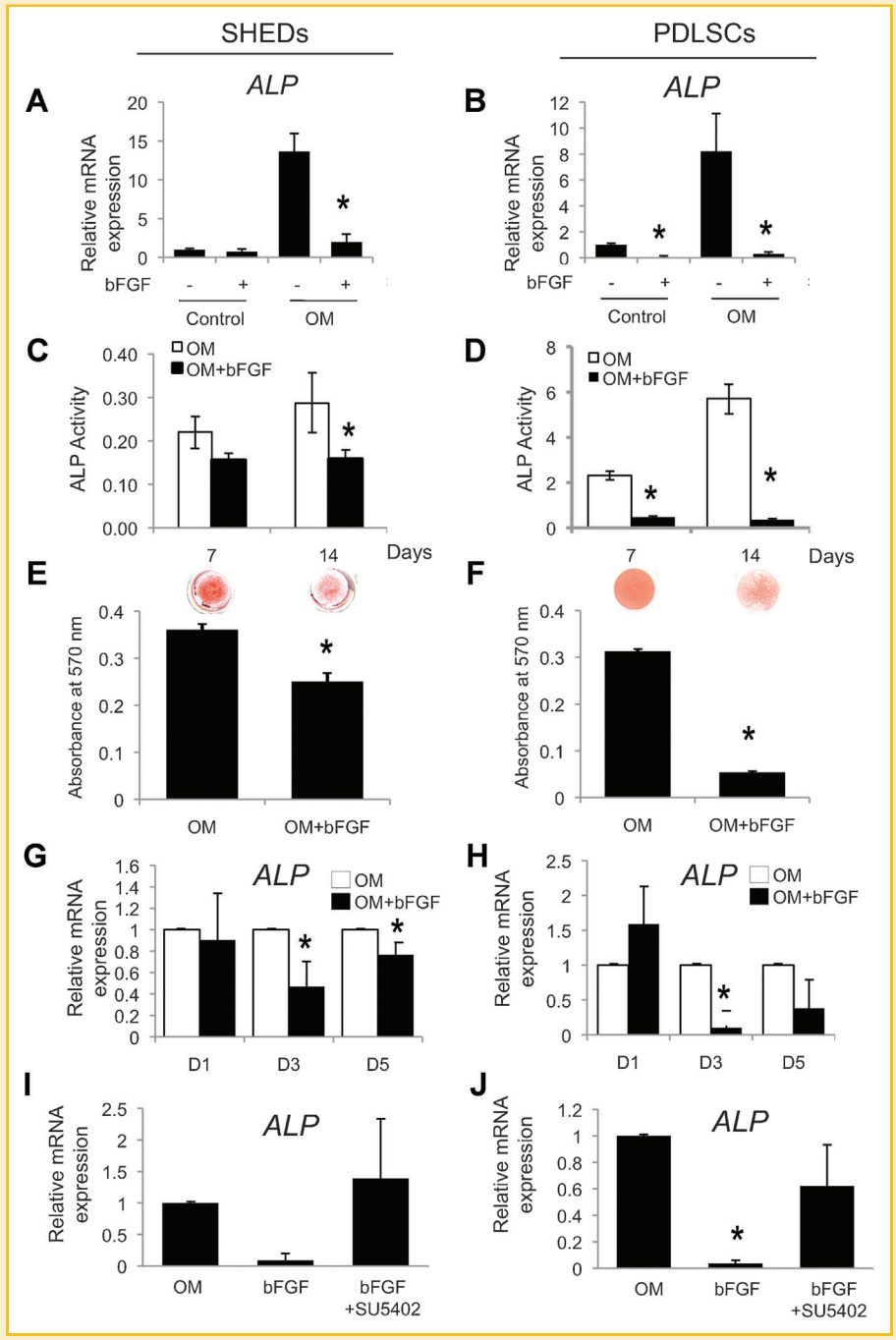

Fig 2. bFGF inhibited *ALP* expression and mineral deposition. The attenuation of *ALP* mRNA expression by bFGF at 7 days in normal and osteogenic medium was illustrated (A and B). The ALP enzymatic activity in osteogenic medium was shown at 7 and 14 days (C and D). Mineral deposition was determined at 14 days after maintaining in osteogenic medium (E and F). The time course experiments illustrated that bFGF attenuated *ALP* mRNA expression at 1, 3, and 5 days after cultured in osteogenic induction condition (G and H). The influence of bFGF was confirmed using FGFR inhibitor (SU5402). The *ALP* mRNA expression at 3 days was evaluated in osteogenic condition (OM), osteogenic condition supplemented with bFGF (bFGF) and osteogenic condition supplemented with bFGF and SU5402 (bFGF + SU5402) (I and J). The asterisk indicated the statistical significance compared to the control.

higher than bFGF treating group (Fig. 3). However, the *ALP* mRNA expression, ALP enzymatic activity and mineral deposition were still significantly lower in the inhibitor treated groups compared to the control in PDLSCs (Fig. 3B,D, and F). Taken together, these results suggesting that bFGF inhibits *ALP* mRNA expression, ALP enzymatic activity and mineral deposition, partly, via FGFR and MEK signaling pathway. The influence of selected inhibitor concentration on native cell behavior was illustrated in Supplementary Figures 1–3.

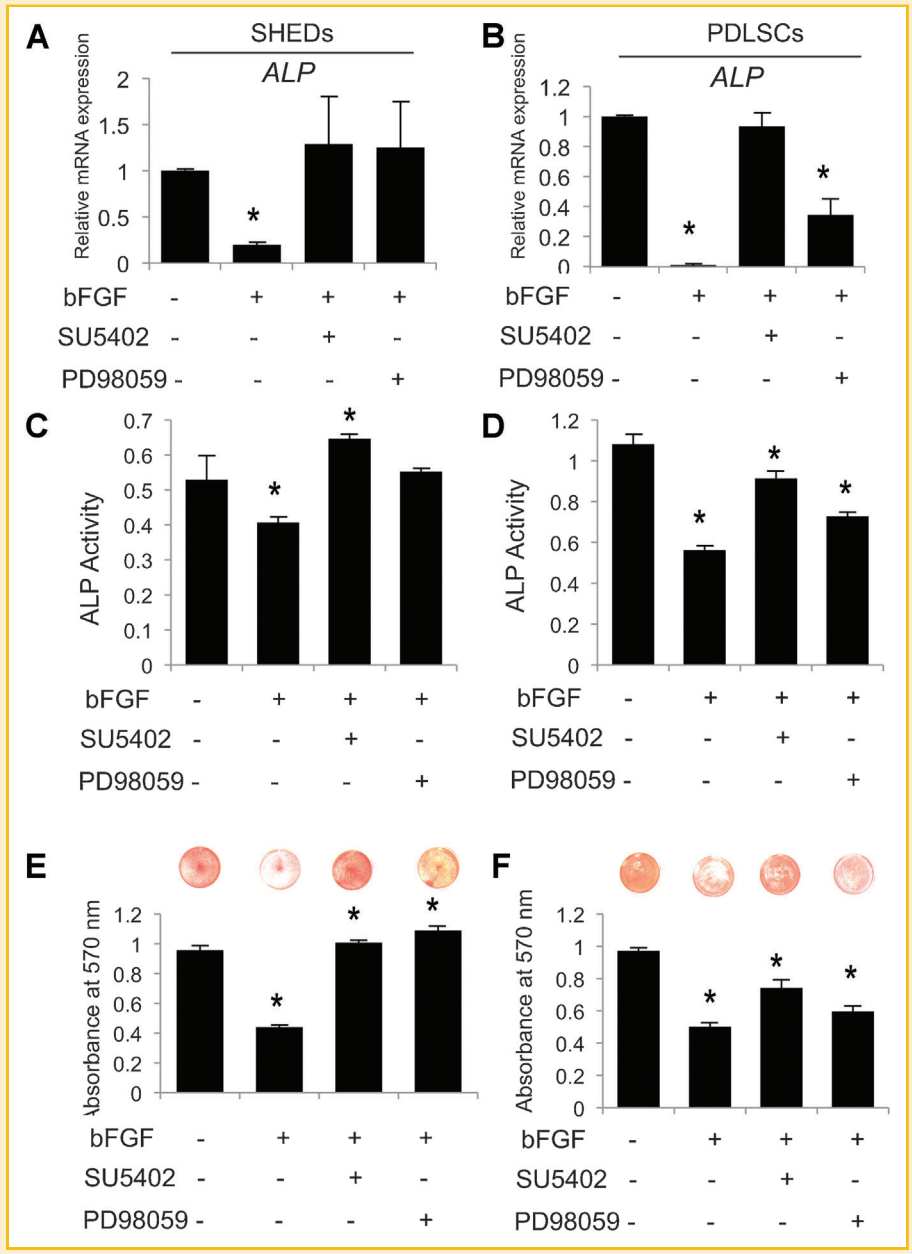

Fig. 3. bFGF attenuating *ALP* expression and mineralization occurred via FGFR and MEK signaling pathway. Cells were treated with bFGF alone or bFGF and FGFR inhibitor (SU5402) or bFGF and MEK inhibitor (SB98059) compared to untreated control. The ALP mRNA levels were determined at day 7 after osteogenic induction (A and B). The ALP enzymatic activity was also evaluated at day 7 (C and D). In addition, the mineralization was determined using alizarin red s staining (E and F). The asterisk indicated the statistical significance compared to the control.

bFGF INHIBITED NOTCH SIGNALING COMPONENTS' EXPRESSION

SHEDs and PDLSCs were treated with bFGF for 7 days. NOTCH receptors, ligands and its target mRNA expression were evaluated. The baseline mRNA expression of NOTCH receptors and ligands in SHEDs and PDLSCs was evaluated (Supplementary Fig. 4). These cells exhibited high expression of *NOTCH1* and *NOTCH2*. Similar *DLL1* and *JAGGED1* mRNA levels was also noted. Upon exposing to bFGF, *NOTCH1*, *NOTCH2*, *NOTCH3*, *JAGGED1*, *DLL1*, and *HES1* mRNA levels was decreased compared to the control culture in SHEDs. However, the significant decrease was noted for *NOTCH1*, *NOTCH2*, *JAGGED1*, and *HES1*mRNA expression (Fig. 4A). In PDLSCs, *NOTCH1*, *NOTCH2*, *NOTCH3*, *JAGGED1*, and *HES1* mRNA expression was significantly decreased upon treating cells with bFGF (Fig. 4B). *NOTCH4* mRNA levels was not change comparing between the control and bFGF treated group in both cell types. In addition, upon treating cells with bFGF, the *DLL1* mRNA expression slightly decreased in SHEDs while the increase was noted in PDLSCs. However, no statistical significance was noted.

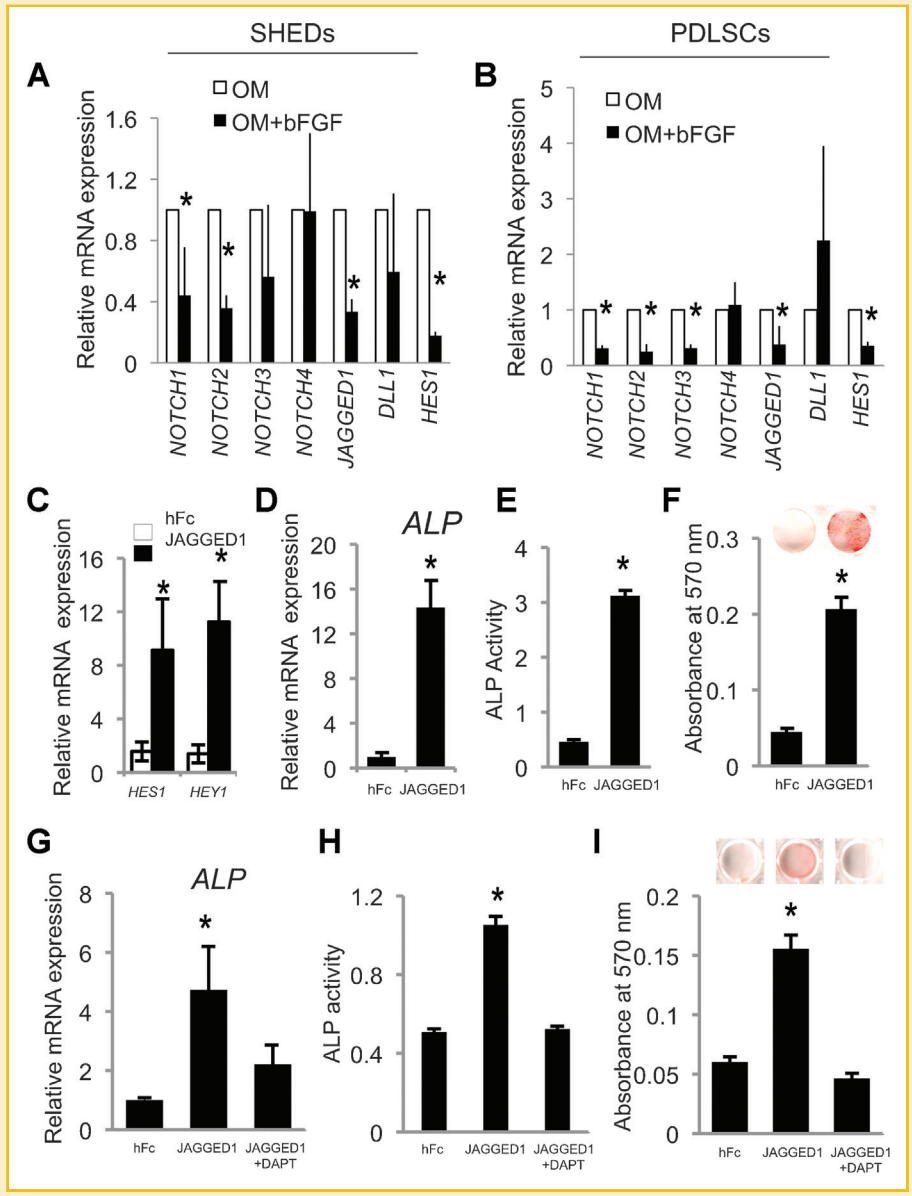

Fig. 4. Notch signaling promoted *ALP* expression and mineral deposition. After treatment SHEDs and PDLSCs with bFGF for 7 days, the mRNA levels of Notch receptors, ligands and target genes were determined (A and B). The activity of immobilized JAGGED1 was determined by the upregulation of *HES1* and *HEY1* mRNA expression at 24 h after exposed to JAGGED1 bounded surface (C). JAGGED1 immobilized surface promoted *ALP* mRNA expression (D), ALP enzymatic activity (E), and mineral deposition (F) in SHEDs. The involvement of Notch signaling was confirmed using gamma-secretase inhibitor (DAPT). Cells pretreated with DAPT could attenuate the ALP mRNA expression (G), ALP enzymatic activity (H), and mineral deposition (I). The asterisk indicated the statistical significance compared to the control.

NOTCH SIGNALING ENHANCED ALP EXPRESSION AND MINERALIZATION

As described above, the exogeneous bFGF inhibited mRNA expression of Notch signaling components, implying the role of Notch signaling in regulation of *ALP* expression and mineralization in both cell types. To further determine the effect of Notch signaling, Notch ligand, JAGGED1, was immobilized on the surface of tissue culture dishes. The activation of Notch signaling in SHEDs was evaluated by measuring mRNA expression of Notch target genes. Upon seeded SHEDs on JAGGED1 immobilized surface, the increase mRNA levels of Notch target genes, *HES1* and *HEY1*, was noted at 24 h (Fig. 4C). Cells on JAGGED1 immobilized surface exhibited higher *ALP* mRNA expression and ALP enzymatic activity than those on the control surface upon cultured in osteogenic medium for 7 days (Fig. 4D,E). Further, the mineral deposition was markedly enhanced when SHEDs were exposed to JAGGED1 bounded surface for 14 days in osteogenic medium (Fig. 4F). To further confirm the influence of Notch signaling, a gamma-secretase inhibitor (DAPT) was employed. The DAPT inhibited the cleavage of Notch receptors, resulting in the decrease of notch intracellular domain (NICD) levels. The enhancement of *ALP*

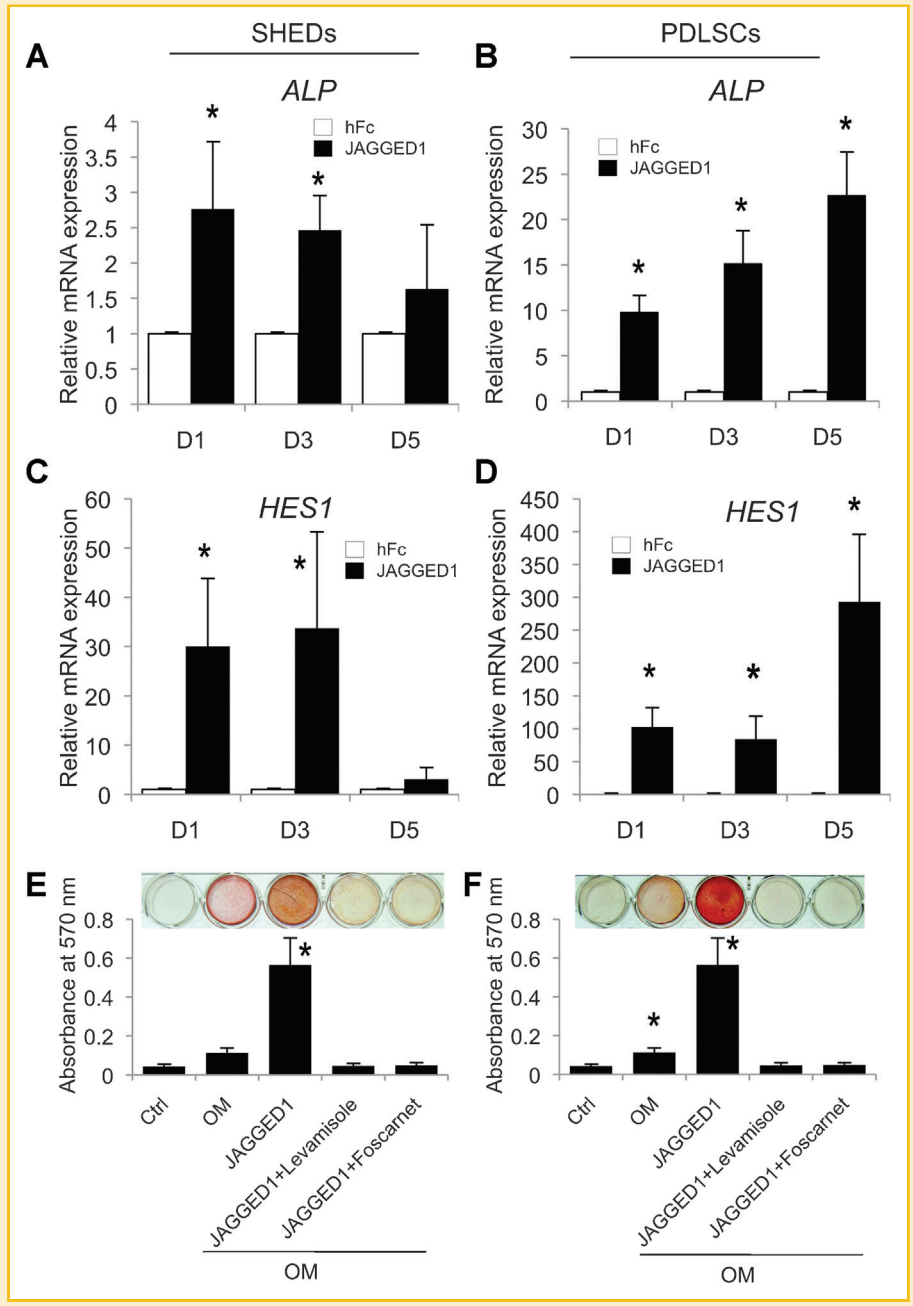

Fig. 5. JAGGED1 bounded surface promoted *ALP* and *HES1* mRNA expression. In time course experiments, JAGGED1 inducing *ALP* mRNA expression was illustrated at 1, 3, and 5 days after cultured in osteogenic induction condition (A and B). The upregulation of *HES1* mRNA levels was also illustrated (C and D). The mineralization was evaluated using alizarin red s staining at 14 days (E and F). The asterisk indicated the statistical significance compared to the control.

mRNA expression, ALP enzymatic activity and mineral deposition on JAGGED1 surface were attenuated in those groups treated with DAPT (Fig. 4G–I), confirming the effect of Notch signaling on the *ALP* expression and mineralization in SHEDs. The similar results were also noted in PDLSCs (data not shown).

In time course experiment, *ALP* mRNA levels were significantly increased after the cells were seeded on JAGGED1 bounded surface and maintained in osteogenic medium for 1 day (Fig. 5A,B). Similar trend was observed in *HES1* mRNA expression (Fig. 5C,D). However, it was noted that both *ALP* and *HES1* mRNA expression in SHEDs was decreased at day 5, while in PDLSCs, the mRNA levels were increased compared to day 1 and 3. Interestingly, the *ALP* and *HES1* mRNA levels were upregulated in corresponding direction in both cell types.

Further, the levamisole or foscarnet addition in the condition, in which cells were exposed to JAGGED1 bounded surface and maintained in osteogenic medium for 14 days, resulted in the

mineralization inhibition (Fig. 5E,F). These results imply that JAGGED1 induced *ALP* expression and ALP further regulate mineralization via generating P_i.

bFGF ATTENUATED THE JAGGED1-ENHANCED ALP EXPRESSION AND MINERALIZATION

The increase *ALP* mRNA expression of cells on JAGGED1 bounded surface was significantly attenuated when the osteogenic medium was supplemented with bFGF for 7 days (Fig. 6A,B). The ALP enzymatic activity was also significantly decreased in those cells treated with bFGF in both hFc control surface and JAGGED1 immobilized surface at day 7 in osteogenic medium (Fig. 6C,D). In addition, the corresponding results were markedly noted for the mineral deposition at day 14 in both the control and JAGGED1 mediated Notch signaling induction condition (Fig. 6E,F). These results may imply that bFGF regulating ALP expression and mineralization in SHEDs and PDLSCs may involve other additional intracellular pathway. In time course study, both *ALP* and *HES1* mRNA levels was significantly reduced in bFGF treated group compared to the control (Fig. 6G–J), implying that bFGF alters Notch signaling and further influences *ALP* expression and mineralization in both SHEDs and PDLSCs.

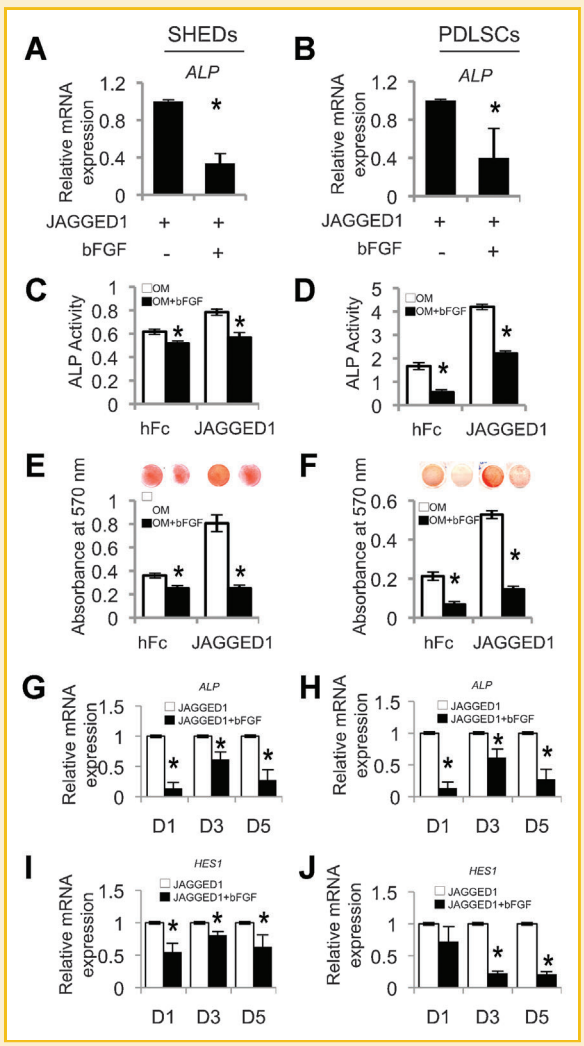

Fig. 6. bFGF attenuated JAGGED1-enhanced *ALP* expression and mineralization. The *ALP* mRNA expression was determined upon cultured cells in osteogenic medium for 7 days (A and B). The ALP enzymatic activity was also evaluated at day 7 (C and D). Further, the mineral deposition was determined using alizarin red s staining at day 14 (E and F). In time course experiments, the attenuation of *ALP* mRNA expression was illustrated at 1, 3, and 5 days after cultured in osteogenic induction condition (G and H). The downregulation of *HES1* mRNA levels was also illustrated (I and J). The asterisk indicated the statistical significance compared to the control.

bFGF ATTENUATED MATRIX METALLOPROTEINASE (MMP) EXPRESSION

MMPs were recently shown to involve in osteogenic differentiation. We further examined *MMP2*, *MMP13*, and *MT1-MMP* mRNA expression upon bFGF treatment. The results illustrated that bFGF significantly attenuated *MMP2*, *MMP13*, and *MT1-MMP* mRNA levels in both SHEDs and PDLSCs at day 7 (Fig. 7A,B). This information may imply another regulatory mechanism of bFGF on mineralization of these cells.

DISCUSSION

In the present study, we described the role ALP and P_i in mineralization as well as the influence of bFGF and Notch signaling on *ALP* expression and mineralization in SHEDs and PDLSCs. The ALP and P_i dramatically regulated mineralization in these cells. The bFGF inhibited *ALP* mRNA expression, ALP enzymatic activity, and mineral deposition via FGFR and MEK signaling pathway. On the contrary, Notch signaling-initiation using surface bounded Notch ligands, JAGGED1, enhanced *ALP* mRNA expression, ALP enzymatic activity, and mineral deposition. We also found that bFGF attenuated the mRNA expression of Notch receptors, ligands and target genes. Moreover, the results in the present study illustrated that bFGF were able to attenuate JAGGED1-induced *ALP* expression and mineral deposition in both cell types.

The characterizations of SHEDs and PDLSCs were previously reported by our group. These cells expressed both embryonic and mesenchymal stem cell markers. In addition, they had multipotent differentiation ability. In this regards, we previously reported that these cells had the differentiation ability toward osteogenic, adipogenic and neurogenic lineages [Govitvattana et al., 2012; Osathanon et al., 2013]. In the present study, we illustrated that the inhibition of ALP enzymatic activity (using levamisole) resulted in the marked reduction of mineral deposition in both SHEDs and PDLSCs. These results were consistent with previous studies in other cell types. In this regard, levamisole was shown to inhibit mineralization in murine pre-osteoblast cells (MC3T3), calvarial cells, human osteoblasts and bovine aortic smooth muscle cells [Anagnostou et al., 1996; Wada et al., 1999; Osathanon et al., 2009; Lencel et al., 2011]. In addition, it has been shown that overexpression of ALP in non-osteoblast cells (fibroblastic cells, renal epithelial cells, and capillary endothelial cells) resulted in the increase of mineralization of these cells. Further, foscarnet was employed to further confirm the downstream mechanism of ALP enhancing mineral deposition in SHEDs and PDLSCs. Blocking P_i uptake using foscarnet (a competitive sodium dependent phosphate co-transporter) resulted in dramatic

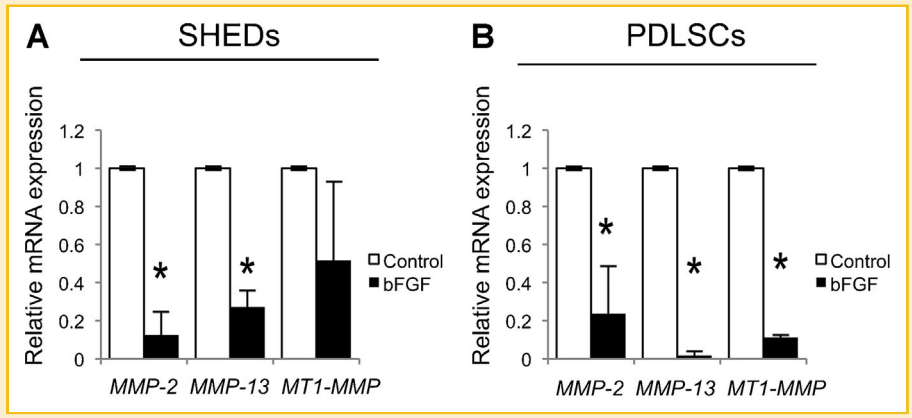

Fig. 7. bFGF attenuated *MMP* expression. The *MMP2*, *MMP13*, and *MT1-MMP* mRNA expression was determined upon cultured cells in osteogenic medium for 7 days (A and B). The asterisk indicated the statistical significance compared to the control.

decrease of mineralization in both cell types, similar to those treated with levamisol. Together, the results imply that ALP generates P_i, which further promotes mineralization process in these cells, similar to those previous reports showing that P_i regulated osteogenic differentiation in osteoblasts [Beck et al., 2000; Wu et al., 2003].

The role of bFGF on osteogenic differentiation was still controversy. Several studies reported that bFGF enhanced ostegenic differentiation in vitro and bone formation in vivo. In this regard, it has been shown that the use of control bFGF release in combination with adipose derived mesenchymal stem cells promoted calvarial bone regeneration in vivo [Kwan et al., 2011]. In addition, pre-osteoblast treated with bFGF had higher expression of collagen type I, ALP, and osteocalcin compared to the control untreated cells [Jeong et al., 2010]. On the contrary, the bFGF-inhibited mineralization and bone formation has also been reported. Our previous study showed that bFGF inhibited ALP enzymatic activity and mineralization in human dental pulp stem cells [Osathanon et al., 2011]. Correspondingly, bFGF was demonstrated to participate in pre-osteoblast proliferation but inhibit mineralization by inducing the proliferation associated gene and attenuating mineralization associated gene expression [Hughes-Fulford and Li, 2011]. Moreover, it has previously been reported in SHEDs that bFGF inhibited WNT/β-catenin pathway via ERK1/2 signaling and further inhibited osteogenic differentiation [Li et al., 2012]. In the present study, we confirmed that bFGF inhibited *ALP* expression and mineralization in both SHEDs and PDLSCs. Beside that it was shown that bFGF induced the expression of pyrophosphate generatine enzyme (*PC-1*) and pyrophosphate transporter (*ank*) but inhibited the expression of *alp* in pre-osteoblastic cell line (MC3T3-E1) [Hatch et al., 2005]. Together, these results indicate that there are several mechanisms in which bFGF inhibit mineralization process. Therefore, the specific mechanism for particular interest cell types (SHEDs and PDLSCs) should be further determined.

We previously reported that Notch signaling initiation using JAGGED1-bounded surface resulted in the significant enhancement of *ALP* mRNA expression, ALP enzymatic activity and mineralization in PDLSCs [Osathanon et al., 2013]. In the present study, we also found that Notch signaling promoted *ALP* expression and mineralization in SHEDs, implying the involvement of Notch signaling in osteogenic differentiation in both cell types. In addition, we demonstrated that bFGF regulated Notch signaling component mRNA expression which may further effect osteogenic differentiation. The interaction of bFGF and Notch signaling is yet unknown. However, it was demonstrated that bFGF downregulated *JAGGED1* and *NOTCH4* mRNA expression in human umbilical vascular endothelial cells (HUVECs) [Kiec-Wilk et al., 2010]. In the present study, we also observed the downregulation of *JAGGED1* in bFGF treated SHEDs and PDLSCs, while *NOTCH4* mRNA lelvels were not changed. On the contrary, other Notch receptor mRNA levels (*NOTCH1*, *NOTCH2*, and *NOTCH3*) were dramatically decreased. Similar trend was noted on *HES1* expression. Together, these data indicate the effect of bFGF on Notch signaling component expression. However, the exact mechanism should be further investigated and the direct physical interaction between Notch and bFGF signaling pathway should be further elucidated.

The involvement of MMP on osteogenic differentiation process was demonstrated. It was shown that MMP2 cleaved dentin matrix protein 1 and then the cleaved protein influenced dental pulp stem cell differentiation [Chaussain et al., 2009]. In addition, osterix, a key osteoblast marker gene, directly regulated MMP13 expression via the direct binding to its promotor [Zhang et al., 2012]. MMP2, MMP13, and MT1-MMP were shown to participate in the mechanical force induced osteoblast differentiation [Barthelemi et al., 2012]. However, some studies illustrated that the MMP negatively regulated osteogenic differentiation. In this regard, the osteogenic differentiation was associated with the MMP1 downregulation [Hayami et al., 2007]. In addition, the inhibition of MMP expression resulted in the enhancement of ostegenic marker gene expression [Hayami et al., 2011]. In the present study, we noted the attenuation effect of bFGF on MMP expression, implying another potential mechanism of bFGF on mineralization. Thus, further investigation is still required to elucidate this issue in SHEDs and PDLSCs.

In the present study, we observed similar effects of bFGF and Notch signaling comparing between SHEDs and PDLSCs, regarding the *ALP* expression and mineral deposition. However, several dissimilarities were noted between SHEDs and PDLSCs. First, the baseline ALP enzymatic activity in SHEDs was markedly lower than those of PDLSCs, implying the inherit potency toward osteogenic lineage. Corresponding with a

previous study, Chadipiralla et al. [2010] investigated the osteogenic differentiation potency of human periodontal ligaments cells and human dental pulp cells of exfoliated deciduous teeth. The results demonstrated that human periodontal ligament cells could be a better cell source for osteoblast differentiation. Second, the *ALP* mRNA expression, ALP enzymatic activity and mineral deposition were still lower in the FGFR or MEK inhibitor treated groups compared to the control in PDLSCs. While the bFGF-inhibited *ALP* expression and mineralization was rescued upon treatment with those inhibitors in SHEDs. Together, these results may imply the involvement of other signaling pathway in the regulation of *ALP* expression and mineralization in PDLSCs. Further investigation is indeed necessitated. Third, it was noted that the fold reduction of ALP mRNA expression and ALP activity was lower in SHEDs than PDLSCs. Moreover, SU5402 treatment in SHEDs resulted in the significant enhancement of ALP activity and mineralization compared to the control. These phenomenons may be due to the high endogeneous bFGF levels in SHEDs compared to PDLSCs (unpublished data). Our group is currently investigated the role of endogeneous bFGF on SHEDs and PDLSCs behaviors, including mineralization mechanism. Finally, the mineralization levels between SHEDs and PDLSCs were relatively similar, although, the marked difference in ALP enzymatic activity was noted. This could be occurred due to the complex regulation of ALP and P_i as well as their role in mineralization. As described previously, ALP enzymatic activity generates P_i from pyrophosphate [Addison et al., 2007]. P_i subsequently regulates osteogenic differentiation and mineralization [Beck et al., 2000; Wu et al., 2003]. However, several factors are involved in this process, for example, ANK (progressive ankylosis gene), NPP1 (nucleotide pyrophosphatase phosphodiesterase-1), P_iT1 (type III sodium-dependent phosphate transporter1), and PHOSPHO1 [Foster et al., 2008]. ANK and NPP1 inhibit mineralization through the increase of mineral deposition inhibitor, pyrophosphate [Foster et al., 2008]. P_iT1 overexpression results in the mineral deposition enhancement while the inhibition of P_iT1 results in the attenuation of mineralization [Yoshiko et al., 2007]. Thus, the different endogenous expression levels of these factors may be participated in the mineralization process of SHEDs and PDLSCs as well. Further investigation should be performed to clarify this phenomenon.

In summary, bFGF inhibited, while, Notch signaling promoted *ALP* mRNA expression, ALP enzymatic activity, and mineralization in SHEDs and PDLSCs. The bFGF treatment resulted in the decrease of Notch signaling component mRNA expression. These data indicate the possible interaction of bFGF and Notch in the regulation of mineralization process in both cell types. Further elucidation of specific mechanisms is certainly required.

ACKNOWLEDGMENTS

This study was supported by Integrated Innovation Academic Center: IIAC Chulalongkorn University Centenary Academic Development Project (PP), The Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (PP) and in part by Asahi Glass Foundation (TO). TO was supported by DRU in Genetic and Anatomical Analyses of Craniofacial Structures, Faculty of Dentistry, Chulalongkorn University. PP was supported by the Research Chair Grant 2012, the National Science and Technology Development Agency (NSTDA), Thailand.

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SUPPORTING INFORMATION

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Fig. S1. The efficiency of various inhibitor concentrations was determined. PDLSCs were treated with bFGF for 48 h in the presence of SU5402 or PD98059. *VEGF* mRNA expression was inhibited by SU5402 in dose-dependent manner (A). The influence of bFGF on *ALP* mRNA levels was rescued upon treatment with PD98059 in dose-dependent manner (B). To evaluate the potency of DAPT, Notch target gene, *HES1*, was evaluated. DAPT attenuated JAGGED1 induced *HES1* expression in dose-dependent manner (C). The efficiency of levamisole and foscarnet on mineralization was measured. Levamisole (D) and foscarnet (E) were able to inhibit mineral deposition in PDLSCs in dose-dependent manner.

Fig. S2. The selected dose of each inhibitor was not toxic to PDLSCs as illustrated by calcein AM/EthD-1 staining at 24 and 48 h after treatment. The graph illustrated the cell viability using MTT assay at 24 h after inhibitor treatment. No statistical significance was noted.

Fig. S3. PDLSCs were treated with SU5402 (A), PD98059 (B), and DAPT (C) at 10, 40, and 20 μM, respectively. The influence of inhibitor alone on *ALP* mRNA expression was evaluated. At 48 h, no significant difference was noted in PD8059 and DAPT treated cells. However, the significant increase of *ALP* mRNA expression was observed in SU5402 treated cells. This may be occurred due to the inhibition of endogenous bFGF action.

Fig. S4. The base line mRNA expression of Notch receptors (A–B) and ligands (C–D) was investigated in both SHEDs and PDLSCs.