Effect of Jagged1 on the expression of genes in regulation of osteoblast differentiation and bone mineralization ontology in human dental pulp and periodontal ligament cells

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ABSTRACT

Objective: To define the effect of Jagged1 on the gene expression in osteoblast differentiation, regulation of osteoblast differentiation and regulation of bone mineralization ontology.

Methods: Human dental pulp and periodontal ligament cells were isolated using explant method. Cells were seeded on Jagged1 immobilized surface. The mRNA expression was determined using real-time polymerase chain reaction. Mineral deposition was evaluated using alizarin red s staining. Publicly available database of gene expression profiles (GSE126249 and GSE94989) were downloaded and performed bioinformatic analysis to identify gene expression in osteoblast differentiation and regulation of osteoblast differentiation and mineralization ontology.

Results: Both human dental pulp cells and human periodontal ligament cells expressed higher Notch target gene (*HES1* and *HEY1*) when cells were seeded on Jagged1 immobilization surface. Jagged1 significantly increased an *in vitro* mineral deposition in both cell types after maintaining in osteogenic induction medium for 14 days. Correspondingly, the significant increase of *ALPL* mRNA expression was observed, while there was no significant change in *ANKH* and *ENPP1* mRNA expression in Jagged1 treated condition. From bioinformatic analysis of genes in osteoblast differentiation ontology, *IARS*, *COL1A1*, *ALPL*, *COL6A1*, *CREB3L1*, and *SNAI2* mRNA levels were upregulated while *GJA1* mRNA levels were decreased upon Jagged1 exposure. In the regulation of bone mineralization ontology, evidences supported that *TGFB1* and *TGFB3* were upregulated in Jagged1 treated condition.

Conclusion: Jagged1 promote the mRNA expression of several genes in osteoblast differentiation related gene ontology. This could be further employed to identify the mechanism by which Jagged1 promoted osteogenic differentiation in human dental pulp and periodontal ligament cells.

1. Introduction

Mineralization is controlled by the balance of phosphate (Pi) and pyrophosphate (PPi). In this regard, PPi functions as an inhibitor of mineral deposition.⁵ This molecule is generated by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1). ENPP1 breaks down adenosine triphosphate (ATP) at extracellular site, resulting in adenosine monophosphate (AMP) and PPi. PPi can be cleaved by alkaline phosphatase (ALP) enzyme encoded by *ALPL* gene.⁵ ALP brakes PPi into Pi and subsequently Pi stimulates osteogenic differentiation and promotes mineral deposition.⁵ Previous studies demonstrated that PPi supplementation in osteogenic induction markedly inhibited mineral

deposition while the supplement of Pi increased mineralization *in vitro*, confirming the role of these molecules in the regulation of mineralization.^{2,9}

Notch signaling pathway is known for inducing osteogenic differentiation and mineralization.^{8,10,11} Notch pathway requires Notch receptors and ligands which are transmembrane proteins that activate Notch signaling through cell-to-cell contact.⁷ After binding of Notch receptors and ligands, Notch receptor is cleaved at intracellular site by γ -secretase enzyme and release of notch intracellular domain (NICD).⁷ This NICD then moves to the nucleus to bind with transcriptional complex proteins resulting in activation of Notch target genes including Hairy/Enhancer of Split (HES) and related genes (HEY, CHF,

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HRT,HESR).⁷ Jagged1 is the Notch ligand which previously reported that it promoted mineralization in various cell types.^{8,10,11} However, the regulation of phosphate/pyrophosphate regulatory genes has not yet been identified. In addition, the potential genes related to osteogenic differentiation that controlled by Jagged1 has not yet been reported. The current study focused to investigate the effect of Jagged1 on phosphate/pyrophosphate regulatory gene and to determine the influence of Jagged1 on osteoblast differentiation and mineralization gene expression using bioinformatic analysis.

2. Materials and methods

2.1. Cell culture

Human periodontal ligament and dental pulp cell isolation protocol was approved by the Human Research Ethic Committee, Faculty of Dentistry, Chulalongkorn University (No.052/2018 and No.079/2018). Cells were explanted from remaining periodontal and dental pulp tissues of healthy teeth extracted conforming with the patients' treatment plan (for example impacted teeth and orthodontic reason). After explantation, cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Invitrogen, USA), 100 Units/ml Penicillin (Invitrogen), 100 µg/ml Streptomycin (Invitrogen), and 250 ng/ml Amphotericin B (Invitrogen) at 37 °C in a humidified 5% CO₂ atmosphere. Cell culture medium was replaced every 2 days.

2.2. Jagged1 immobilization

Recombinant human Jagged1/Fc protein (R&D system) was indirectly immobilized on tissue culture plates conforming with previous publish arrangement.¹⁰ In brief, tissue culture plates were soaked with recombinant protein G (50 µg/mL) for 16 h and subsequently washed with sterile PBS for three times. Then, the surfaces were coated with bovine serum albumin solution (10 mg/mL) for 2 h. After washing with sterile PBS, tissue culture plates were incubated with recombinant human Jagged1/Fc protein (Jagged1) for 2 h. In the control condition, human IgG Fc fragment (hFc) was engaged instead of Jagged1.

2.3. Mineralization assay

After cells were seeded on Jagged1 and preserved in osteogenic induction medium (growth medium supplemented with 5 mM β-glycerophosphate (Sigma), 50 µg/mL L-ascorbic acid (Sigma) and 250 nM dexamethasone(Sigma)) for 14 days, cells were fixed by cold methanol and gently rinsed by deionized water. Cells were stained with 1% alizarin red s solution with gently agitation for 5 min at room temperature. The surplus staining was washed out using deionized water.

2.4. Polymerase chain reaction

RNA extraction was conducted using RiboEx™ solution (GeneAll®, Korea). Nanodrop2000 was employed to determine the quality and quantity of the isolated RNA. One microgram of RNA was turned to complimentary DNA using ImProm-II™ Reverse Transcription System (Promega). Real-time polymerase chain reaction was conducted on the MiniOpticon real-time PCR system (Bio-Rad) with FastStart Essential DNA Green Master kit (Roche Diagnostics). Product specificity was evaluated using melting curve analysis. The mRNA expression levels of target genes were normalized with the expression of *GAPDH*. Primer oligonucleotide sequences were shown in Table 1.

2.5. Bioinformatic analysis

Publicly available database of gene expression profiles, GSE126249 and GSE94989, were download from Gene Expression Omnibus

database. GSE126249 described the mRNA expression of Jagged1 treated human periodontal ligament cells as determined by microarray analysis.¹⁴ GSE94989 described mRNA expression of Jagged1 treated human dental pulp cells as determined by a high throughput RNA sequencing technique.⁸ Gene expression pattern of osteoblast differentiation (GO:0001649), regulation of osteoblast differentiation (GO:0045667), and regulation of bone mineralization (GO:0030500) ontology was evaluated using a network-based visual analytics for gene expression profiling, meta-analysis and interpretation (NetworkAnalyst).¹⁶

2.6. Statistical analysis

Data were presented as scattered plot. Each dot represented the individual data value and the lines indicated median values of the conditions. Statistical analysis was performed using Prism8 (GraphPad, USA). Two sample comparison was performed using Mann Whitney U test. The statistical significance was indicated when *p* value < 0.05.

3. Results

3.1. Effect of Jagged1 on phosphate/pyrophosphate regulatory related gene expression

Cells were seeded on Jagged1 immobilized surface or the hFc immobilized control surface and maintained in normal growth medium for 24 h. Subsequently, culture medium was turned into osteogenic induction medium. Similar to previous reports,^{8,10} Jagged1 promoted mineral deposition human periodontal ligament (PDL) and human dental pulp cells at day 14 after osteogenic induction (Fig. 1A).

At 24 h after exposing to Jagged1, *HES1* and *HEY1* mRNA levels were significantly increase in both cell types, indicating the activation of Notch signaling (Fig. 2B and C). The expression of phosphate/pyrophosphate regulatory genes was also investigated using real-time polymerase chain reaction (Fig. 2D-F). *ALPL* mRNA levels were markedly increased in Jagged1 treated condition for both cell types. However, there was no statistically significant difference regarding mRNA expression of *ANKH* and *ENPP1*.

3.2. Jagged1 regulated genes in osteoblast differentiation and mineralization related ontology

Gene expression profile of Jagged1 treated human dental pulp cells was downloaded and bioinformatic analysis was performed to identify significantly differential expression of genes in three ontology namely osteoblast differentiation (GO:0001649), regulation of osteoblast differentiation (GO:0045667), and regulation of bone mineralization (GO:0030500). Results demonstrated that, in osteoblast differentiation ontology, *IARS*, *COL1A1*, *ALPL*, *COL6A1*, *CREB3L1*, and *SNAI2* mRNA levels were upregulated while *GJA1* mRNA levels was down-regulated in Jagged1 treated condition compared with the hFc control (Fig. 2). Further, three significant upregulated genes, *JAG1*, *ACVR1*, and *SNAI2*, were identified in the regulation of osteoblast differentiation ontology of Jagged1 treated cells (Fig. 2). For the regulation of bone mineralization ontology, mRNA levels of *SRGN* and *GJA1* were decreased while *TGFB1*, *ACVR1*, and *TGFB3* mRNA levels were increased in Jagged1 condition (Fig. 2).

Raw microarray data of human periodontal ligament cells and raw read counts of human dental pulp cells were downloaded from publicly available database and four selected genes were identified and compared the fold change between hFc and Jagged treated conditions. Similarly, *ALPL*, *TGFB1*, and *TGFB3* raw expression were significantly increased in both cell types (Fig. 3). However, *GJA1* mRNA expression was downregulated in human dental pulp cells but not in human periodontal ligament cells after Jagged1 treatment (Fig. 3).

Table 1
Oligonucleotide sequences.

Gene	Accession Number	Primer sequences
<i>HES1</i>	NM_005524.2	(Forward) 5'AGGCGGACATTCTGGAAATG 3' (Reverse) 5' CGGTACTTCCCAGCACACTT3'
<i>HEY1</i>	NM_012258.3	(Forward) 5' CTGCAGATGACCGTGGATCA3' (Reverse) 5' CCAAACTCCGATAGTCCATAGCAA3'
<i>ALPL</i>	NM000478.5	(Forward) 5' GACCTCCTCGGAAGACACTC 3' (Reverse) 5' TGAAGGGCTTCTTGCTGTG 3'
<i>ANKH</i>	NM054027.4	(Forward) 5' GAGGTGACAGACATCGTGG 3' (Reverse) 5' CCTTTAAATCAAGGCCTCTTTCATTAC 3'
<i>ENPP1</i>	NM006208.2	(Forward) 5' AAATATGCAAGCCTCTTGT 3' (Reverse) 5' TTTAGAAGGTGGTTAAGACTTCCATGA 3'
<i>GAPDH</i>	NM_002046.3	(Forward) 5' TCATGGGTGTGAACCATGAGAA3' (Reverse) 5' GGCATGGACTGTGGTCATGAG3'

4. Discussion

The present study confirmed previous reports demonstrating that Jagged1 activated Notch signaling and enhanced mineralization in several cell types, including human mandibular and iliac bone cells, human periodontal ligament cells, and human dental pulp cells.^{8,10,11,13} Further, in the present study, we observed that Jagged1 also regulated phosphate/pyrophosphate regulatory genes; *ALPL*, *ANKH*, *ENPP1*. *ALPL* is known to cleave PPI at the extracellular site, generating free Pi.⁵ Pi then further control mineralization by co-precipitated with calcium ion. *ANKH*, a membrane transporter, functions to transfer PPI from intracellular to extracellular area.⁵ While *ENPP1* generates PPI at the extracellular site by breaking down adenosine triphosphate.⁵ The accumulation of PPI leads to the attenuation of mineral deposition. Hence, the balance of Pi/PPI ratio could control the mineralization. Although, previous reports have been shown the upregulation of *ALPL* expression by Jagged1.^{8,10,11,13} The influence of Jagged1 on *ENPP1* and *ANKH* is not yet been identified. In the present study, we reported that

Jagged1 significantly enhanced *ALPL* mRNA expression while *ANKH* and *ENPP1* mRNA levels were not affect by the Jagged1 treatment. Hence, Pi/PPI ratio should be increased at the extracellular site, corresponding with the increase of mineralization in Jagged1 treated condition.

From bioinformatic analysis, it was noted that Jagged1 promoted several gene expression in gene ontology; osteoblast differentiation, regulation of osteoblast differentiation and regulation of bone mineralization. However, it was noted that *GJA1* and *SRGN* mRNA levels were downregulated in Jagged1 treated condition. *GJA1*, also known as connexin 43, is a gap junction regulating various cell functions. It has been shown that high expression of *GJA1* was observed in human dental follicle cells, implying the function of this molecule during tooth eruption.¹⁵ Osteoblasts from *Gja1* deficient mice exhibited the reduction of late osteogenic differentiation marker expression.¹ Induced pluripotent stem cells derived from *GJA1* mutated cells exhibited a delay osteogenic differentiation.⁴ The relationship of Notch signaling and *GJA1* has been described. In this regard, Notch signaling inhibition

Fig. 1. (A) Mineral deposition was evaluated using alizarin red s staining at day 14 after maintaining in osteogenic induction medium. (B–C) Genes expression was determined using real-time polymerase chain reaction at 24 h after Jagged1 exposure. Bars indicated the statistically significant difference. PDL; human periodontal ligament cells, Pulp; human dental pulp cells.

Fig. 2. Heatmap demonstrated the significantly differential expression of genes in 3 ontology; osteoblast differentiation (GO:0001649), regulation of osteoblast differentiation (GO:0045667), regulation of bone mineralization (GO:0030500) in Jagged1 treated human dental pulp cells compared with the hFc control.

in human embryonic stem cell differentiation enhanced GJA1 expression and promoted cardiac differentiation.⁶ Taken these evidences together, GJA1 participates in osteogenic differentiation and bone homeostasis. The underlining mechanism why Jagged1 inhibiting GJA1 expression in human dental pulp cells is yet unknown. Further study is indeed required to elucidate this issue.

SRGN encodes an intracellular proteoglycan. The expression was

associated with inflammation. In human chondrocytes, SRGN expression was increased after stimulating with interleukin- β .¹² It bound to CD44 and stimulated inflammatory process in these cells.¹² Similar functions were reported in lipopolysaccharide-induced murine chondrocytes.³ Role of SRGN in cancer progression was also reported.¹⁷ Influence of Notch signaling on SRGN expression has never been announced. We observed in the current study that Jagged1 inhibited

Fig. 3. Graph illustrated the raw read counts (pulp) and expression values (PDL) of the selected differentially expressed genes from osteoblast differentiation (GO:0001649), regulation of osteoblast differentiation (GO:0045667), regulation of bone mineralization (GO:0030500) ontology in Jagged1 treated condition compared with the hFc control. PDL; human periodontal ligament cells, Pulp; human dental pulp cells.

SRGN expression in human dental pulp cells. Mechanism and function of Jagged1 attenuated SRGN expression required further investigation.

Author contribution

K.H. contributes in microarray data interpretation and bioinformatic analysis. T.O. contributes in data acquisition, interpretation and manuscript revision. J.S. experimental design, data interpretation, and critical manuscript revision. All authors gave final approval for publication.

Declaration of competing interest

Authors declare no conflict of interest.

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