


รายงานวิจัยฉบับสมบูรณ์

# โครงการบทบาทของนอชท์ลิแกนต่อการควบคุมสภาพของเซลล์เอ็นยึดปริทันต์ของมนุษย์

โดยผู้ช่วยศาสตราจารย์ ทันตแพทย์ ดร. ธนภูมิ โอสถานนท์

มิถุนายน 2556

สัญญาเลขที่ MRG5480112

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

# บทคัดย่อ

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**ชื่อโครงการ :** บทบาทของนอชท์ลิแกนต่อการควบคุมสภาพของเซลล์เอ็นยึดปริทันต์ของมนุษย์

**ชื่อนักวิจัย :** ผู้ช่วยศาสตราจารย์ ทันตแพทย์ ดร. ธนภูมิ โอสถานนท์

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สัญญาณนอชท์มีบทบาทในเซลล์หลายชนิดโดยเกี่ยวข้องกับการควบคุมการคงสภาพและการแปรสภาพของเซลล์ การศึกษานี้ผู้วิจัยศึกษาความสามารถในการควบคุมการแปรสภาพของเซลล์ต้นกำเนิดเมเซนไคม์ที่แยกได้จากเนื้อเยื่อเอ็นยึดปริทันต์มนุษย์ด้วยการใช้พื้นผิวที่มีการตรึงนอชท์ลิแกนเจกเก็ต1 ภายหลังจากการหว่านเซลล์ลงบนพื้นผิวที่มีการตรึงเจกเก็ต 1 เซลล์มีการแสดงออกของอาร์เอ็นเอนำรหัสของยีนเป้าหมายของสัญญาณนอชท์ เฮส 1 และ เฮ1 มากขึ้นเมื่อเทียบกับกลุ่มควบคุมและกลุ่มที่กระตุ้นด้วยนอชท์ลิแกนในอาหารเลี้ยงเซลล์ปกติ การเพิ่มขึ้นของอาร์เอ็นเอนำรหัสของยีนเป้าหมายของสัญญาณนอชท์นี้จะถูกยับยั้งด้วยสารยับยั้งเอนไซม์แกมม่าซีครีเทส เมื่อทำการหว่านเซลล์ลงบนพื้นผิวที่มีการตรึงเจกเก็ต 1แล้วเลี้ยงต่อในอาหารเลี้ยงเซลล์ที่กระตุ้นการแปรสภาพไปเป็นเซลล์สร้างกระดูก พบว่ามีการทำงานของเอนไซม์อัลคาไลฟอสฟาเทสและมีการสะสมตะกอนแคลเซียม รวมทั้งการแสดงออกของอาร์เอ็นเอนำรหัสของ อัลคาไลน์ฟอสฟาเทส คอลลาเจนชนิดที่ 1 และออสทิโอพอนทิน เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุม อย่างไรก็ตามพบว่ามีการแสดงออกของอาร์เอ็นเอนำรหัสของออสทิโอแคลซินลดลง นอกจากนี้พบว่ามีการลดลงของอาร์เอ็นเอนำรหัสของทวิส 2 ด้วย แสดงให้เห็นว่าเจกเก็ต 1 กระตุ้นเซลล์ต้นกำเนิดเมเซนไคม์ที่แยกได้จากเนื้อเยื่อเอ็นยึดปริทันต์ให้มีการแปรสภาพไปเป็นเซลล์สร้างกระดูกผ่านทางการกระตุ้นสัญญาณนอชท์และยับยั้งทวิส นอกจากนี้ยังพบว่าเมื่อทำการหว่านเซลล์ลงบนพื้นผิวที่มีการตรึงเจกเก็ต 1 ในภาวะที่ปราศจากซีรั่ม เซลล์จะมีการแปรสภาพไปเป็นเซลล์ประสาท โดยพบมีการเพิ่มการแสดงออกของเบต้า 3-ทูบลูลินเมื่อยับยั้งการแสดงออกของสัญญาณนอชท์ด้วยสารยับยั้งเอนไซม์แกมม่าซีครีเทส หรือการใช้โดมิแนนเนกกาทีฟของมาสเตอร์มายไลค์โปรตีน จะสามารถยับยั้งการแปรสภาพของเซลล์ต้นกำเนิดจากเนื้อเยื่อเอ็นยึดปริทันต์มนุษย์ได้ ดังนั้นการปรับปรุงพื้นผิวชีววัสดุด้วยนอชท์ลิแกนสามารถใช้ในการกระตุ้นการแปรสภาพของเซลล์ต้นกำเนิดเมเซนไคม์ที่แยกได้จากเนื้อเยื่อเอ็นยึดปริทันต์ให้แปรสภาพไปเป็นเซลล์สร้างกระดูกและเซลล์ประสาทได้ ในภาวะการเลี้ยงที่เหมาะสม

**คำหลัก :** สัญญาณนอชท์ เจกเก็ต1 เซลล์ต้นกำเนิดที่แยกได้จากเนื้อเยื่อเอ็นยึดปริทันต์ การแปรสภาพ เซลล์สร้างกระดูก

## Abstract

**Project Code :** MRG5480112

**Project Title:**Role of Notch Ligand on Controlling of Human Periodontal Ligament Cell Fate.

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**Project Period :**June 2011 – June 2013

Notch signaling plays critical roles in various cell types by regulating cell fate determination and differentiation.Here, we investigated the ability to control differentiationof human periodontal ligament derived mesenchymalstem cells using modified surfaces containing the affinityimmobilized Notch ligand, JAGGED1. After seeding humanperiodontal ligament derived mesenchymal stem cells(HPDLs) on JAGGED1 modified surfaces, expression of Notchsignaling target genes, *HES1* and *HEY1*, was higher thanthose exposed to soluble JAGGED1 or control surfaces. Upregulationof Notch signaling target genes was attenuated after treatment with the gamma-secretase inhibitor. Upon seedingthe cells on Jagged-1 immobilized surface and maintained inosteogenic medium, alkaline phosphatase enzymatic activityand mineralization as well as mRNA expression of alkalinephosphatase (*ALP*), collagen type I (*COL I*) and osteopontin(*OPN*) were significantly increased compared to those ofcontrols. However, osteocalcin (*OCN*) mRNA expressionlevel was decreased when cells were exposed to Jagged-1modified surfaces. HPDLs on JAGGED1 modified surfacesexpressed lower *TWIST2* mRNA levels than the control, suggestingthat the mechanism whereby JAGGED1 enhancesosteogenic differentiation of HPDLs may occur through Notchsignaling and TWIST regulation. In addition, neurogenic differentiation was observed upon seeding cells on the JAGGED1 bounded surface in the absence of serum. Inhibition of Notch signaling using gamma-secretase inhibitor or dominant negative mastermind like protein resulted in the attenuation of neurogenic differentiation. In summary, an alteration ofbiomaterial interface using Notch ligands illustrates a promisingsystem to control HPDLs differentiation toward osteogenicand neurogenic lineage under specific culture condition.



**Keywords :** Notch signaling, human periodontal ligament-derived mesenchymal stem cells, differentiation, osteoblasts

# Executive Summary

Roles of Notch Ligands on Controlling of Human Periodontal Ligament Cell Fate

## 1. Background and Significance (ความสำคัญและที่มาของปัญหา)

Periodontal disease is worldwide oral health problem. In United States, more than 50% of elderly patients have gingivitis and about 30% of population have periodontal pocket (Oliver et al., 1998). The prevalence of healthy gingiva was nearly zero in a sample group from Lao People's Democratic Republic (Chuckpaiwong et al., 2000). Periodontitis has been diagnosed in over 40% of sampled Thai adults (Chaisupamongkollarp et al., 2008). High prevalence of missing teeth and edentulous are noted and the mean number of remaining teeth is 18.6 teeth per person in elderly Thai (Ploysangngam et al., 2008). These situations lead to destruction of tooth supporting structure, impair masticatory function and subsequently alter quality of life. Without a doubt, effective treatments for regeneration of periodontal tissues are needed.

Early periodontal healing process is similar to that of normal incisional wound and bone healing. It is divided into three phases; 1) inflammation, 2) granulation tissue formation and 3) matrix formation and remodeling (Wikesjo and Selvig, 1999). Uninterrupted, stable fibrin clots positioned between the gingival flap and periodontal compromised root are crucial for periodontal regeneration (Baker et al., 2005). The fibrin clot supports cell migration, proliferation and formation of newly regenerated tissues. However, periodontal healing differs from wound and bone healing in the maturation and remodeling phase since it requires the attachment of collagen fibers to cementum or root dentin and alveolar bone (Wikesjo and Selvig, 1999). It is hypothesized that the healing connective tissue recognizes the exposed root dentin as an inert foreign body, then subsequently forms the collagen fibers parallel to the root surface (Wikesjo and Selvig, 1999). The exposed root dentin also stimulates cementoblast differentiation and forms cementum into which collagen fibers are anchored (Wikesjo and Selvig, 1999). The process of periodontal ligament formation is slower than bone. This could lead to ankylosis of the treated tooth in some cases. Together, these data indicate that periodontal tissue regeneration is distinct. Therefore, specific design criteria for periodontal treatment materials and procedure need to be carefully identified and evaluated.

Bioengineering approaches and tissue engineering may lead to novel treatments for this disease. Growing evidence supports an important roles Notch ligands in controlling cell fate and behaviors (Beckstead et al., 2006; Beckstead et al., 2009). He et al reported that over-expression of Delta-1, a Notch ligand, in dental pulp stem cells enhanced proliferation and differentiation into odontoblast-like cells (He et al., 2009). On the contrary, Zhang et al showed that over-expression of another notch signaling ligand, Jagged-1, in dental pulp stem cells resulted in inhibition of odontoblast differentiation in vitro and mineralization in vivo (Zhang et al., 2008). The different function of Notch ligands, Delta-1 and Jagged-1, on cell behaviors was also reported in other cell types such as human hematopoietic progenitor cells (Jaleco et al., 2001). Moreover, Brooker et al reported that knockout of different Notch ligands exhibited distinct phenotypes in mice (Brooker et al., 2006). Our studies are designed to determine the roles of Notch ligands, Delta-1 and Jagged-1, in controlling human periodontal ligament cell fate and behaviors. These studies will further our understanding of specific responses of periodontal tissue via cell-cell interaction molecules of Notch signaling, and allow us to evaluate the potential approach of immobilized Notch ligands surface for periodontal tissue regeneration. Moreover, it may lead to the development of an easily manufactured and cost-effective therapy for periodontal tissue regeneration and, subsequently, bridge the gap of basic biology and bioengineering end to clinical dentistry.

## 2. Research Aims (วัตถุประสงค์)

a. To determine the expression pattern of Notch signaling ligands and their requirements in human periodontal ligament cell activities/differentiation.
b. To fabricate and characterize the immobilized Notch ligands surface.
c. To analyze human periodontal ligament cell behaviors on the bioengineered Notch ligands surface.

## 3. Research Strategies (ระเบียบวิธีวิจัย)

*Please find further detail on materials and methods in the appendix 1 and 2.*

## 4. Results

After seeding humanperiodontal ligament derived mesenchymal stem cells(HPDLs) on Jagged-1 modified surfaces, expression of Notchsignaling target genes, Hes-1 and Hey-1, was higher thanthose exposed to soluble Jagged-1 or control surfaces. Upregulationof Notch signaling target genes was attenuated after treatment with the gamma-secretase inhibitor. Upon seedingthe cells on Jagged-1 immobilized surface and maintained inosteogenic medium, alkaline phosphatase enzymatic activityand mineralization as well as mRNA expression of alkalinephosphatase (ALP), collagen type I (COL I) and osteopontin(OPN) were significantly increased compared to those ofcontrols. However, osteocalcin (OCN) mRNA expressionlevel was decreased when cells were exposed to Jagged-1modified surfaces. HPDLs on Jagged-1 modified surfacesexpressed lower TWIST2 mRNA levels than the control, suggestingthat the mechanism whereby Jagged-1 enhancesosteogenic differentiation of HPDLs may occur through Notchsignaling and TWIST regulation.

Further study on the role of Notch signaling in human periodontal ligament stem cells was investigated in regard of neurogenic differentiation. Neurogenic induction of hPDLSCs was performed via neurosphere formation. Cells were aggregated and form spheres as early 1 day in culture. In addition, the induced cells exhibited increased mRNA and protein expression of neuronal markers that is, $\beta$3-tubulin and neurofilament. During neuronal differentiation, a significant increase of Hes1 and Hey1 mRNA expression was noted. Using pharmacological inhibition ($\gamma$-secretase inhibitor) or genetic manipulation (overexpression of dominant negative mastermind-like transcription co-activators), neurosphere formation was attenuated and a marked decrease in neurogenic mRNA expression was observed. To confirm the role of Notch signaling in neuronal differentiation of hPDLSCs, the Notch ligand, Jagged-1, is bound to the surface using an affinity immobilization technique. The hPDLSC cultured on a Jagged-1-modified surface had increased expression of Notch signaling target genes, Hes-1 and Hey-1, confirming the activity and potency of surface-bound Jagged-1. Further, hPDLSC on surface-bound Jagged-1 under serum-free conditions showed multiple long and thin neurite-like extensions, and an increase in the expression of neurogenic mRNA markers was observed. Pretreatment of the cells with $\gamma$-secretase inhibitor, DAPT, before seeding on the Jagged-1-modified surface blocked development of the neurite-like morphology.

*For further detail in the results, please find in the appendix 1, 2, and 3.*

## 5. Discussion and conclusion

A role of Jagged-1 in cell fate decision control has been reported in several cell types. Jagged-1 promoted neuroectodermal commitment of embryonic stem cells (Ramasamy et al., 2010). We illustrated that exposing hPDLSC to Jagged-1 modified surface promoted neurogenic commitment in serum free culture medium as determined by the expression of neurogenic markers, *Sox2* and $\beta$*3-tubulin*. However, we also observed that Jagged-1 enhanced osteogenic differentiation in hPDLSCs in normal growth medium as well as in osteogenic medium. These contrary results may have occurred for several reasons. First, the activation of Notch signaling prompts hPDLSCs to differentiate. However, the specific lineage determination is regulated by other external stimuli in the culture environment. In this respect, it has been reported that the activation of Notch target genes was differently regulated by extrinsic factor. Different culture medium supplementation influenced other intracellular signaling, which further controlled and interacted with Notch signaling (Meier-Stiegen et al., 2010). Moreover, a withdrawal of trophic factors resulted in the reversible change of Numb isoforms that further controlled the activation of Notch signaling target genes (Kyriazis et al., 2010). Together, these results suggest that culture environment has considerable influences on the different regulation of Notch signaling target gene expression and cell's behaviors. Corresponding to our observation, Jagged-1 promoted neurogenic commitment of hPDLSCs in serum free condition but, in the present of ten percents serum, osteogenic differentiation was enhanced, implying the role of culture condition of Notch signaling-influenced cell function. Second, the difference in cell fate determination upon exposure to Notch ligand might have occurred due to different Notch receptor interactions as well as downstream intracellular signaling cascade. In this regard, it has been illustrated that an activation of canonical Notch signaling resulted differences in regulation and cell behavior compared to the non-canonical pathway (Le Gall et al., 2008). In addition, it has been demonstrated that an alteration of Notch receptor expression may impact control of specific cell lineages as shown in olfactory epithelium (Carson et al., 2006). However, the mechanism of Notch signaling regulating human periodontal ligament stem cell differentiation is indeed required further investigation.

*For further detail in the discussion and conclusion, please find in the appendix 1, 2, and 3.*

## 6. Suggestion and future study

Notch signaling is control by various mechanisms such as glycosylation of Notch protein, and cleavage of Notch protein. Ligand-independent Notch signaling also play role in Notch signaling and cell behavior. Thus, further study should be performed to evaluated the regulation mechanism in this particular cell type.

The immobilization of protein on material surface has a potential pitfall in term of the ability to control the orientation of protein domain on surface, which might result in limited activity of immobilized protein. In this study, we used indirect method to immobilize Notch ligand through Fc chimera domain of protein, so that Notch ligand will be immobilized in the disired orientation. Although, we also use the ELISA to detect the presence of immobilized protein, yet the activity of protein to cell may not directly predicted. Luciferase assay should be applied to determine the activity of immobilized protein, when questionable.

In this report, we showed the influence of Notch signaling in osteogenic and neurogenic differentiation. The control of Notch signaling in the differentiation of other lineages should also be further elucidated.

In addition, the regulation of cell differentiation and stemness is complex mechanism. The interaction of Notch signaling and other pathway should be further investigated to elucidate the controlling mechanism of human periodontal ligament stem cell differentiation.

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**8. Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.**

1. **Osathanon T**, Ritprajak P, Nowwarote N, Manokawinchoke J, Giachelli C, Pavasant P. Surface-bound orientated Jagged-1 enhances osteogenic differentiation of human periodontal ligament-derived mesenchymal stem cells. J Biomed Mater Res A. 2013;101(2):358-67.

   **Impact factor 2011: 2.625**
2. **Osathanon T,** Manokawinchoke J, Nowwarote N, Aguilar P, Palaga T, Pavasant P. Notch signaling is involved in neurogenic commitment of human periodontal ligament-derived mesenchymal stem cells. Stem Cells Dev. 2013;22(8):1220-31.

   **Impact factor 2011: 4.459**
3. **Osathanon T,** Giachelli CM, Pavasant P. Directing differentiation of human periodontal-ligament-derived stem cells using Notch-ligand modified surfaces. International Association for Dental Research-Southeast Asia division 2011, Singapore.

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# Surface-bound orientated Jagged-1 enhances osteogenic differentiation of human periodontal ligament-derived mesenchymal stem cells

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**Abstract:** Notch signaling plays critical roles in various cell types by regulating cell fate determination and differentiation. Here, we investigated the ability to control differentiation of human periodontal ligament derived mesenchymal stem cells using modified surfaces containing the affinity immobilized Notch ligand, Jagged-1. After seeding human periodontal ligament derived mesenchymal stem cells (HPDLs) on Jagged-1 modified surfaces, expression of Notch signaling target genes, Hes-1 and Hey-1, was higher than those exposed to soluble Jagged-1 or control surfaces. Up-regulation of Notch signaling target genes was attenuated after treatment with the γ secretase inhibitor. Upon seeding the cells on Jagged-1 immobilized surface and maintained in osteogenic medium, alkaline phosphatase enzymatic activity and mineralization as well as mRNA expression of alkaline phosphatase (ALP), collagen type I (COL I) and osteopontin (OPN) were significantly increased compared to those of controls. However, osteocalcin (OCN) mRNA expression level was decreased when cells were exposed to Jagged-1 modified surfaces. HPDLs on Jagged-1 modified surfaces expressed lower TWIST2 mRNA levels than the control, suggesting that the mechanism whereby Jagged-1 enhances osteogenic differentiation of HPDLs may occur through Notch signaling and TWIST regulation. In summary, an alteration of biomaterial interface using Notch ligands illustrates a promising system to control HPDLs differentiation toward osteogenic lineage. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 358–367, 2013.

**Key Words:** notch signaling, Jagged-1, human periodontal ligament-derived mesenchymal stem cells, osteoblast differentiation, TWIST

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## INTRODUCTION

Notch signaling occurs through cell–cell interaction. It is classically activated after binding of the extracellular domain of Notch receptors on one cell with membrane bound Notch ligands on another cell.[1] After the binding, Notch receptor is cleaved by a disintegrins and metalloproteinase (ADAM)/tumor necrosis factor-α-converting enzyme (TACE) metalloproteinases, followed by γ-secretase, resulting in translocation of Notch intracellular domain (NICD) into the nucleus and transcriptional activation of downstream target genes, that is, Hes and Hey family.[1] Four Notch receptors (Notch-1, -2, -3, and -4), three Delta-like ligands (Delta-1, -3, and -4) and two Jagged ligands (Jagged-1 and -2) have been identified in mammals.[1] Notch signaling plays crucial functions in various stages of development and tissue regeneration.[2–4] Growing evidence supports an important role of Notch ligands in controlling cell fate and behaviors. For example, it has been shown that Notch receptors and ligands were expressed in response to the application of protective agent to an exposed dental pulp tissues (pulp capping).[5] The expression of Notch ligands was observed around the capping and stromal area of the injured dental pulp.[5] Thus, these data suggest the potential role of notch signaling in healing of dental pulp tissues.

Among various types of Notch ligands, Jagged-1 was shown to relate to osteoblast differentiation. Bone marrow stromal cell subpopulations with high expression levels of Jagged-1 had higher alkaline phosphatase activity in

Additional Supporting Information may be found in the online version of this article.
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culture.[6] Correspondingly, a human mesenchymal stem cell clone expressing high levels of Jagged-1 had higher mineralization potential under osteogenic induction conditions.[7] In addition, forced expression of Jagged-1 in human bone marrow-derived stromal cells resulted in an enhancement of osteogenic differentiation.[8]

Previous studies described a method to immobilize Jagged-1 on a material surface.[9,10] Using indirectly immobilized Jagged-1, the activity of C-promotor binding factor-1 (CBF-1) luciferase reporter, an indicator of Notch signaling, was significantly increased in epithelial cells.[10] In addition, immobilized Jagged-1 modified surfaces were able to direct the differentiation of epithelial cells.[9] Cells plated on a Jagged-1 surface were rapidly stratified forming a multilayer of cells in tight clusters compared to the control surface,[9] suggesting an intact bioactivity of surface bound Jagged-1.

Periodontal ligament is a fibrous connective tissue that locates between cementum and alveolar bone. Several types of cells reside in the periodontal ligament. It has been reported that cells isolated from human periodontal ligament tissues expressed several embryogenic stem cell markers, mesenchymal stem cell markers and neural crest markers.[11] The isolated stem cells also have colony-forming unit ability and can differentiate into osteogenic/cementogenic, adipogenic, and fibroblastic lineages.[12] However, evidence of Notch signaling in control of human periodontal ligament cell function is still very limited. The aim of this study was to evaluate the ability to control differentiation of human periodontal ligament-derived stem cells (HPDLs) using modified surfaces containing affinity immobilized notch ligands, Jagged-1.

## MATERIALS AND METHODS

### Reagents

Dulbecco's modified Eagle's medium (DMEM), neurobasal medium, B-27, fetal bovine serum (FBS), L-glutamine, and penicillin–streptomycin–amphotericin B solution were purchased from Gibco, USA. Bovine serum albumin (BSA), 2-amino-2methyl-1-propanol, Alizarin Red S, cetylpyridinium chloride monohydrate, β-glycerophosphate, Strep-FITC, Oil Red O, dexamethasone (Dex), IBMX, ascorbic acid, indomethasin, and recombinant epidermal growth factor (EGF) were purchased from Sigma, USA. Recombinant basic fibroblast growth factor (bFGF), *p*-nitrophenol phosphate and Tag polymerase were purchased from Invitrogen, USA. Mouse anti-human Jagged-1, anti-human Rex-1 antibody and recombinant human Jagged-1/Fc (Jagged-1/Fragment, crystallizable region of antibody; Jagged-1) were purchased from R&D systems, USA. Mouse anti-β3-Tubulin and reverse transcriptase enzyme were purchased from Promega, USA. Biotinylated rabbit anti-mouse antibody, biotinylated rat anti-mouse antibody and recombinant protein G were purchased from Zymed, USA. Mouse anti-human Stro-1 monoclonal antibody was purchased from Chemicon, USA. BCA assay kit was purchased from Thermo Scientific, USA. Anti-human CD44 antibody, anti-human CD73 antibody and anti-human CD90 were purchased from Abcam, USA. Human IgG Fc fragment was purchased from Jackson Immunoresearch Laboratory, USA.

### Isolation and culture of HPDLs

The protocol for the isolation of HPDLs was approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Healthy adult subjects undergoing surgical treatment for tooth removal due to third molar impaction were recruited for the isolation of HPDLs using methods previously described.[13] Briefly, periodontal tissues were scraped from the middle of the root surface and cultured in DMEM containing 10% FBS, 2 m*M* L-glutamine, 100 U $mL^{-1}$ penicillin, 100 μg $mL^{-1}$ streptomycin, and 5 μg $mL^{-1}$ amphotericin B in 100% humidity, 37°C and 5% carbon dioxide. Medium was changed every 48 h. After reaching confluence, the cells were subcultured at a 1:3 ratio. Colony forming unit and doubling time were determined according to Osathanon et al.[14] and Singhatanadgit et al.,[15] respectively. In some experiments, γ-secretase inhibitor (DAPT) was added to the culture medium at a concentration of 25 μ*M* to inhibit the cleavage of Notch intracellular domain.

### Flow cytometry analysis

Single-cell suspensions were incubated with fluorescent-conjugated anti-human CD90 or purified anti-STRO-1 antibody. Cells were then incubated with biotinylated secondary antibody anti-mouse IgM. Stained cells were analyzed using the Cytomic FC500 series (Beckman Coulter). Mouse IgM was used as an isotype control. Values are the mean fluorescence intensity (MFI). Data are representative of two independent experiments.

### HPDLs differentiation assay

To examine osteogenic differentiation of HPDLs, cells were seeded at a density of 25,000 cells per wells in a 24-well plate and maintained in an osteogenic medium [growth medium supplemented with ascorbic acid (50 μg $mL^{-1}$), Dex (100 n*M*), and β-glyceraphosphate (10 m*M*)]. The medium was changed every 48 h. Alkaline phosphatase activity, osteoblast marker gene expression and mineral deposition were investigated using the methods described below.

For adipogenic differentiation, cells were plated in 24-well plate and maintained in DMEM containing insulin (0.1 mg $mL^{-1}$), Dex (1 μ*M*), IBMX (1 m*M*), and indomethacin (0.2 m*M*) for 14 days. The adipogenic marker gene mRNA expression and lipid droplet accumulation were respectively examined using reverse-transcriptase polymerase chain reaction (RT-PCR) and Oil Red O staining as described below.

Neurogenic differentiation of mesenchymal stem cells was performed according to protocol previously published by our group.[14] Briefly, the cells were seeded in 60-mm Petri dishes ($5 \times 10^5$ cells per plate) and maintained in neurobasal medium containing B27 (2%), L-glutamine (2 m*M*), penicillin (100 U $mL^{-1}$), streptomycin (100 μg $mL^{-1}$), and amphotericin B (5 μg $mL^{-1}$), basic FGF (20 ng $mL^{-1}$), and EGF (20 ng $mL^{-1}$) for 7 days. Subsequently, the

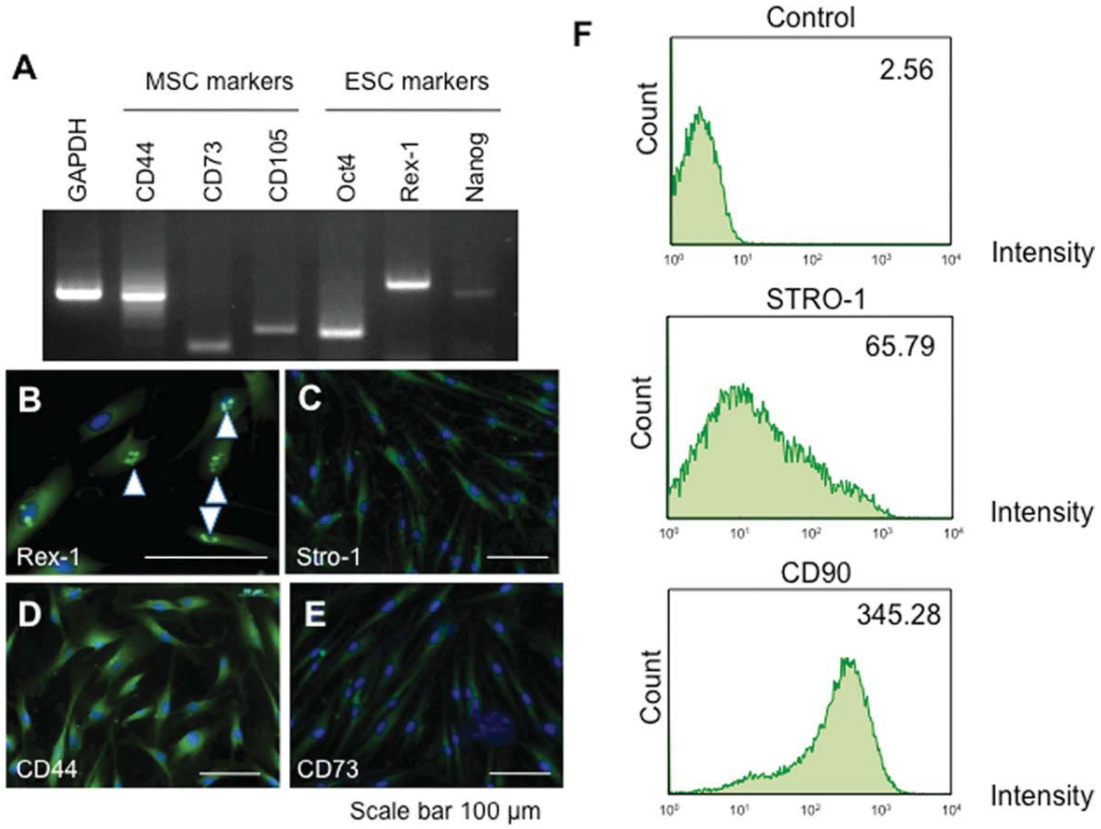


**FIGURE 1.** Characterization and multipotency of human periodontal ligament stem cells. Cells isolated from human periodontal ligament tissues expressed stem cell markers. The mRNA expression of mesenchymal stem cell markers (CD44, CD73, and CD105) and embryonic stem cell markers (Oct4, Rex-1, and Nanog) were noted (A). Correspondingly, the cells exhibited the protein expression of Rex-1 (B), Stro-1 (C and F), CD44 (D), CD73 (E), and CD90 (F) as determined using immunocytochemical staining or flow cytometry analysis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

neurospheres were seeded on collagen IV coated dishes and maintained in the neurogenic medium for 7 days. The mRNA expression of neurogenic markers was evaluated using RT-PCR. The expression human β3-tubulin was also evaluated by immunocytochemical staining.

### Fabrication of surface-bounded Jagged-1 protein on surface

Surface-bound Notch ligand, Jagged-1 was fabricated by an indirect affinity immobilization method previously reported by Beckstead et al.[9,10] Briefly, recombinant protein G was incubated with tissue culture plate surface at concentration 50 μg $mL^{-1}$ for 16 h. After three washes with sterile PBS, the surface was incubated with 10 mg $mL^{-1}$ BSA for 2 h. Subsequently, the surface was washed three times with sterile PBS and further incubated with Jagged-1 for 2 h. Equal amount of human IgG, Fc fragment was employed as a control. Prior to seed the cells, the surfaces were washed one time with culture media.

The presence of Jagged-1 protein on surface was confirmed using enzyme-linked immunosorbent assay. Following Jagged-1 immobilization, the surfaces were incubated with mouse anti-human Jagged-1 for 1 h. Subsequently, the biotinylated rat anti-mouse antibody was added for 1 h, followed with streptavidin/HRP solution for 30 min. The substrate was then added to develop the color. The reaction was stopped using 4.5*M* sulfuric acid and absorbance at 450 nm was measured.

### Alkaline phosphatase activity assay

The cells were lysed in alkaline lysis buffer. Aliquots were incubated at 37°C in a solution containing 2 mg $mL^{-1}$ *p*-nitrophenol phosphate, 0.1*M* 2-amino-2-methyl-1-propanol and 2 m*M* $MgCl_2$. After 15 min, 50 m*M* 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 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 m*M* sodium phosphate at room temperature for 15 min. The absorbance was measured at 570 nm.

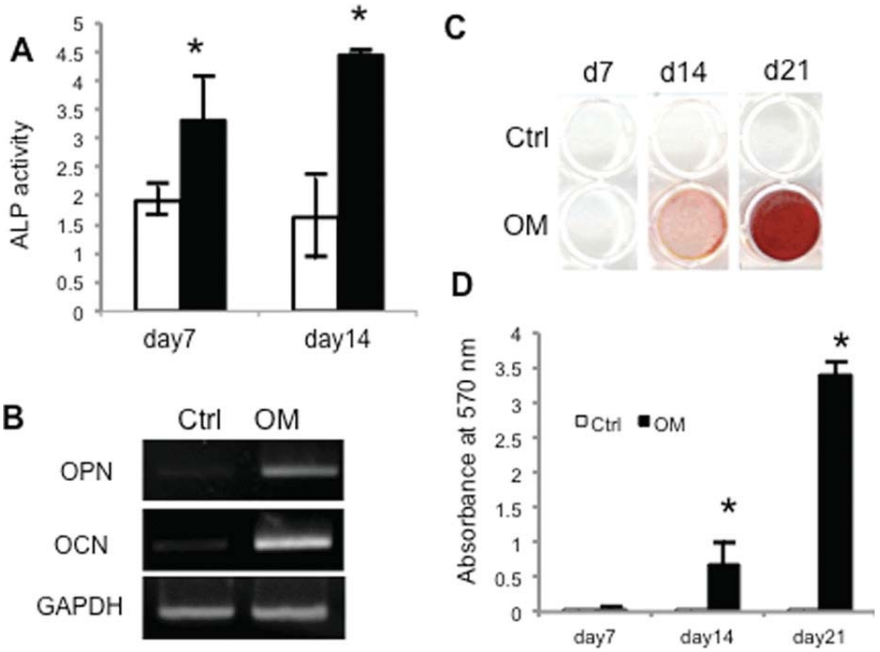


**FIGURE 2.** Characterization and multipotency of human periodontal ligament stem cells. Cells isolated from human periodontal ligament tissues were able to differentiate into osteogenic lineage. An increase of alkaline phosphatase (ALP) enzymatic activity (at 7 and 14 days) (A), osteoblast marker genes mRNA expression levels (at 14 days) (B) and mineralization (at 7, 14 and 21 days) (C,D) were noted using ALP activity assay, RT-PCR and Alizarin Red S staining, respectively. Asterisk represented the statistical significance ($p < 0.05$) compared to the control. (Ctrl; control and OM; osteogenic medium). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### Oil red O staining

Cells were fixed with 10% buffered formalin for 30 min. After washing with PBS, cells were rinsed briefly with 60% isopropanol. Subsequently, 0.2% Oil Red O solution was added and incubated for 15 min. Cells were then rinsed with distilled water and further examined under light microscope.

### RT-PCR and real-time quantitative PCR

Total cellular RNA was extracted with Trizol reagent. RNA samples (1 μg) were converted to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase. A semiquantitative polymerase-chain reaction (PCR) was performed using Tag polymerase. The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining. The band density was determined using ImageJ software. For real-time quantitative PCR experiment, PCR was performed in a MiniOpticon system (Bio-rad) 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 was quantified with GAPDH as the reference gene. The oligonucleotide sequences of the primers were shown in Supporting Information Table I.

### Immunocytochemistry staining

The cells were fixed in 10% buffered formalin at room temperature for 30 min, permeabilized with 0.15% Triton®-X100 in PBS and 10% horse serum for 1 h at room temperature. The cells were then incubated with primary antibody at a 1:200 dilution for 18 h at room temperature. After washing with PBS, the cells were incubated with secondary antibody for 30 min at 1:500 dilutions. Subsequently, the antibody was detected using Strep-FITC and the nuclei were counterstained with DAPI (0.1 μg $mL^{-1}$). The cells were analyzed with a fluorescent microscope.

### Statistical analyses

Data are 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

### Cells isolated from human periodontal ligament demonstrated stem cell-like characteristics

Cells isolated from human periodontal ligament exhibited fibroblast-like morphology. Cells were able to proliferate *in vitro* with a doubling time of 38 ± 2.02 h (data not shown). Colony forming unit were observed as early as 7 days in culture and the average colony was 39 ± 14.78 at 14 days in culture (data not shown). These cells expressed mRNA markers for embryonic stem cells (Oct4, Rex-1 and Nanog) and mesenchymal stem cells (CD44, CD73, and CD105) [Fig. 1(A)]. In addition, the protein expression of stem cell markers (Rex-1, Stro-1, CD44, CD73, and CD90) was confirmed using immunocytochemistry staining and

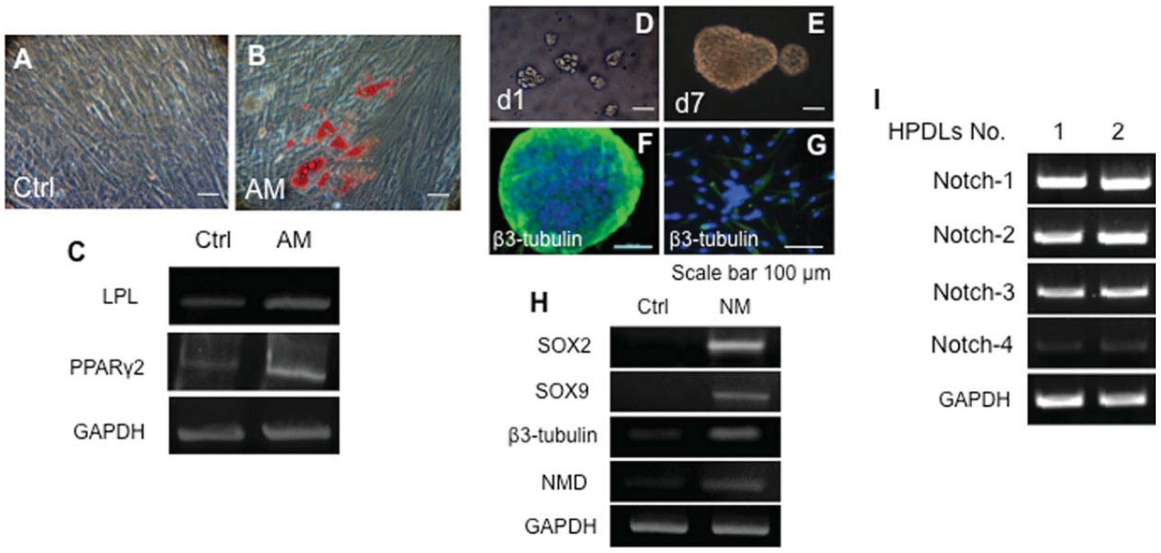


**FIGURE 3.** Characterization and multipotency of human periodontal ligament stem cells. Cells isolated from human periodontal ligament tissues were able to differentiate into adipogenic and neurogenic lineage. Lipid droplets were observed in the cells cultured in adipogenic medium (B) but not in the control medium (A), corresponding with the upregulation of adipogenic marker genes, lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor gamma (PPARγ) (C). For neurogenic differentiation, neurospheres were formed (D,E). The cells were positively stained with β3-tubulin in both sphere and attached cells (F,G). Increase neurogenic mRNA markers were observed (H). The mRNA expression of Notch receptors in two line of cells isolated from human periodontal ligament was illustrated (I). (Ctrl; control, AM; adipogenic medium and NM; neurogenic medium). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

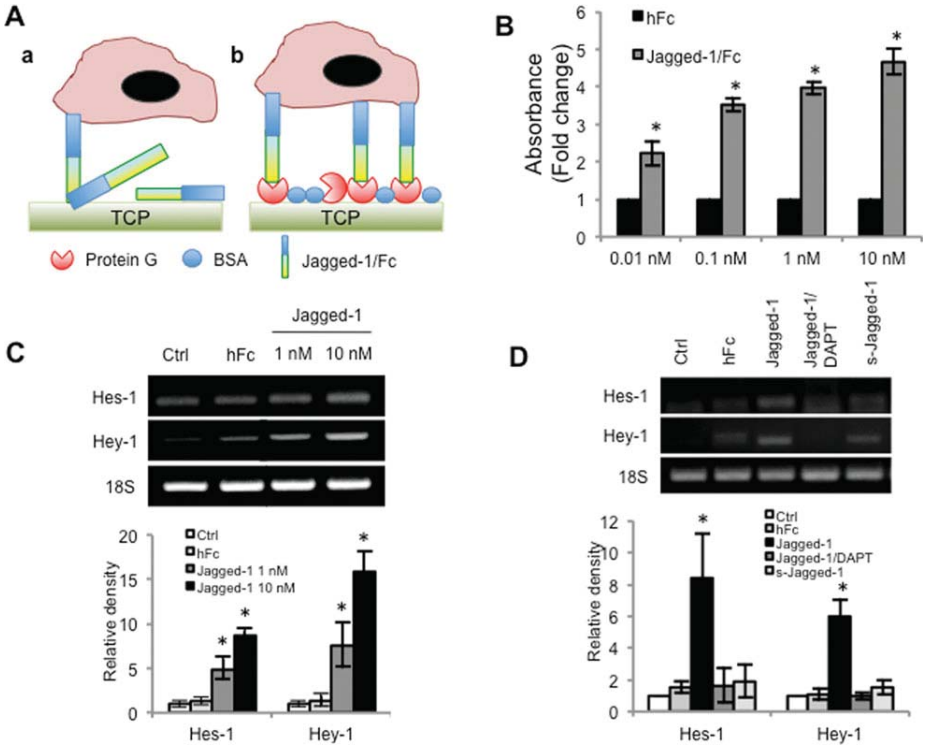


**FIGURE 4.** Jagged-1 was successfully immobilized on the tissue culture surfaces. The picture illustrates an orientated Jagged-1 obtained by an indirect affinity immobilization technique (Ab) compared to random absorbed protein (Aa) by normal coating technique. The presence of Jagged-1 was determined using ELISA technique (B). Human periodontal ligament derived mesenchymal stem cells were seeded on the modified surface for 48 h. The hFc protein was used as the control. Jagged-1 modified surface induced the expression of Notch target genes in dose dependent manner (C). This activity inhibited by γ-secretase inhibitor; DAPT (D). Asterisk represented the statistical significance ($p < 0.05$) compared to the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

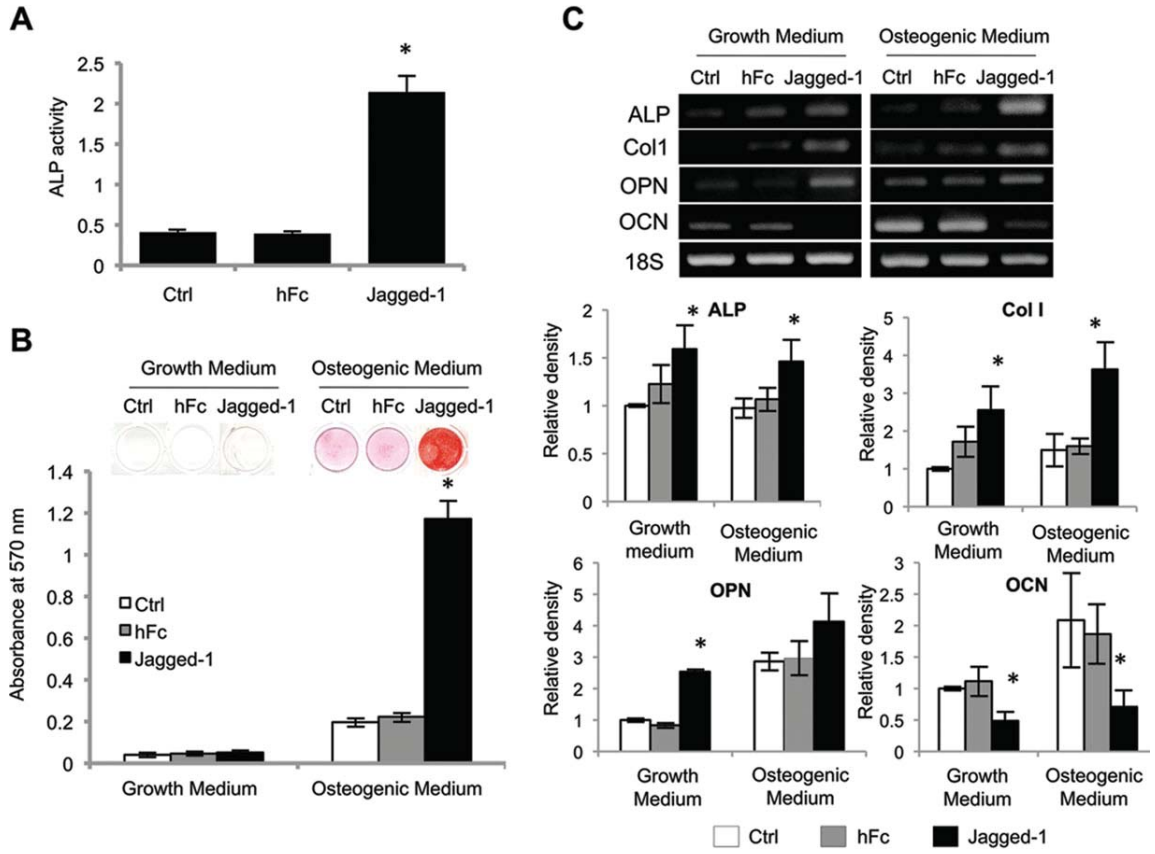


**FIGURE 5.** Jagged-1 modified surface enhanced osteogenic differentiation of human periodontal ligament-derived mesenchymal stem cells. At 7 days, cells on Jagged-1 bound surface had higher alkaline phosphatase enzymatic activity (A), mineralization (B) and mRNA expression of osteogenic marker genes (C). However, a decrease in OCN mRNA expression was noted. Asterisk represented the statistical significance ($p < 0.05$) compared to the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

flow cytometry analysis [Fig. 1(B–F)]. Further, the multipotential differentiation ability of isolated cells was investigated. The cells were able to differentiate into osteogenic, adipogenic, and neurogenic lineage. Upon culturing cells in osteogenic medium, an increase of alkaline phosphatase enzymatic activity was observed at 7 and 14 days [Fig. 2(A)]. In addition, the expression of osteogenic mRNA markers; OPN and OCN, was upregulated at 14 days [Fig. 2(B)]. Mineral deposition was dramatically noted at 14 and 21 days [Fig. 2(C,D)]. For adipogenic differentiation, intracellular lipid accumulation was noted in those cells maintained in adipogenic medium as demonstrated by Oil red O staining [Fig. 3(A,B)]. Correspondingly, adipogenic differentiation mRNA marker genes (LPL and PPAR$\gamma$2) were markedly increased [Fig. 3(C)]. Further, neurogenic potential of HPDLs was examined using neurosphere formation assay.[14] Sphere formation was observed in neurogenic medium as early as 1 day in culture [Fig. 3(D)]. At 7 days, the spheres were greater in size compared to day 1, suggesting cell proliferation in the spheres [Fig. 3(E)]. β3-tubulin was expressed in floating spheres and adhering cells [Fig. 3(F,G)]. These spheres expressed higher Sox2, Sox9, β3-tubulin, and NMD mRNA levels compared to the control, indicating neurogenic differentiation [Fig. 3(H)]. Together, these results suggest stem cell-like properties of the cells isolated from human periodontal ligament tissues.

### Oriented surface-bound notch ligands stimulated notch target genes

First, the expression of notch receptors was examined using RT-PCR methods. HPDLs expressed Notch-1, -2, -3, and -4, implying that these cells may able to response to the immobilized ligands [Fig. 3(I)].

The effect of Notch signaling activation using an indirect affinity immobilization of the Notch ligand, Jagged-1, on the surface was investigated in HPDLs. Recombinant protein G was used to immobilize and orient Jagged-1/Fc fusion protein on the surface [Fig. 4(A)]. An antibody-sandwich ELISA was used to confirm the presence of Jagged-1 on the immobilized surface. The results demonstrated dose dependent immobilization of Jagged-1 on the surface [Fig. 4(B)]. Immobilized hFc in equal amounts was used as control.

The effectiveness of the bound Jagged-1 surface was determined by examining the expression of Notch target genes; Hes-1 and Hey-1. HPDLs seeded on bound Jagged-1 exhibited higher mRNA expression of Hes-1 and Hey-1 compared to untreated surfaces or those containing bound hFc at 48 h after seeding. The expression of these target genes

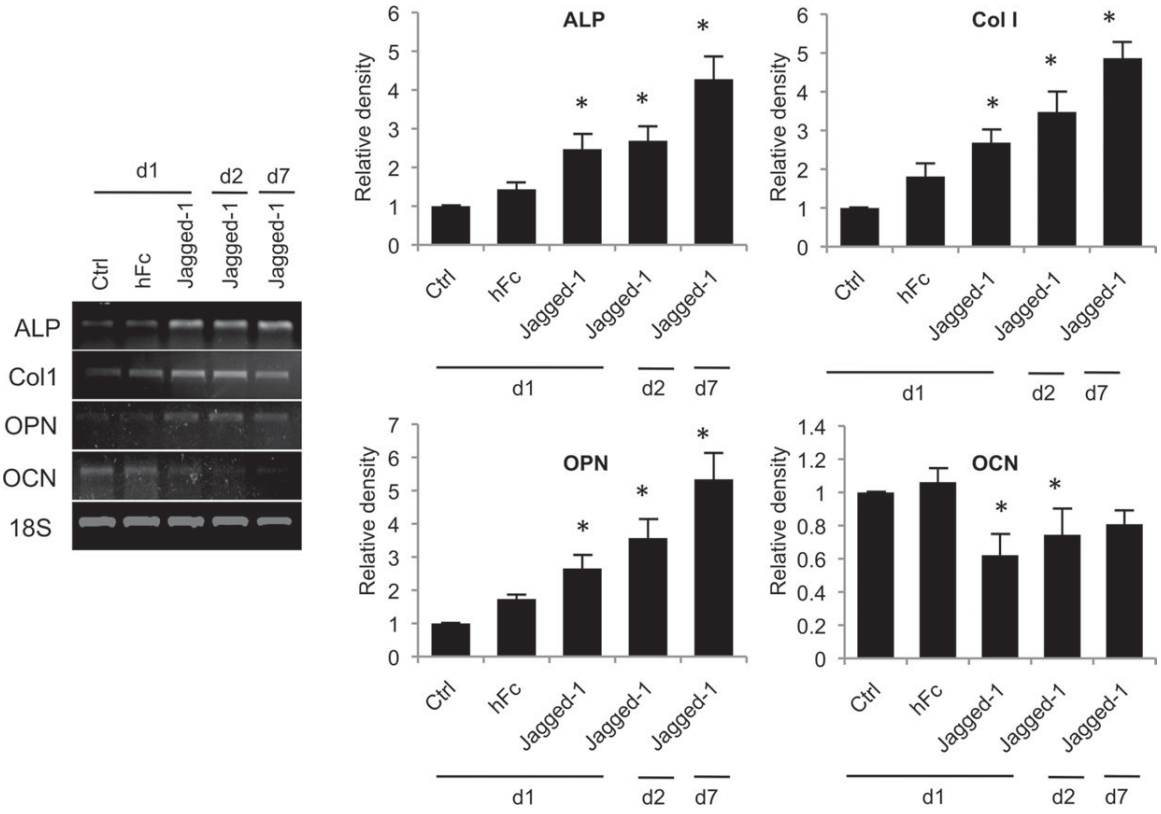


**FIGURE 6.** Jagged-1 modified surface enhanced osteogenic differentiation of human periodontal ligament-derived mesenchymal stem cells. The increase mRNA expression of ALP, Col I, and OPN as well as the decrease of OCN mRNA expression were noted in time dependent manner. Asterisk represented the statistical significance ($p < 0.05$) compared to the control.

was dependent on the coating concentration of Jagged-1 [Fig. 4(C)], and was attenuated after treatment with γ-secretase inhibitor, DAPT. In addition, the levels of Hes-1 and Hey-1 mRNA expression was lower upon exposure of HPDLs to soluble Jagged-1 compared to surface-bound Jagged-1 [Fig. 4(D)]. Together, these results suggest that surface-bound Jagged-1 is capable of potently activating the notch signaling pathway in HPDLs.

### Oriented surface-bound notch ligands enhanced osteogenic differentiation of HPDLs

HPDLs were seeded on surfaced-bound Jagged-1 at concentration 10 n*M* and further maintained in medium for 7 days. In osteogenic medium, HPDLs on the Jagged-1 immobilized surface had significantly higher alkaline phosphatase enzymatic activity and mineralization than those on tissue culture or the hFc immobilized control [Fig. 5(A,B)]. Correspondingly, the Jagged-1 immobilized surface significantly enhanced mRNA expression of osteogenic marker genes; ALP, Col 1 and OPN in both growth medium and osteogenic medium [Fig. 5(C)]. Interestingly, OCN mRNA expression was significantly downregulated in those cells cultured on Jagged-1 immobilized surface. A significant upregulation of ALP, Col 1 and OPN mRNA expression by HPDLs seeded on Jagged-1 bound surface was observed as early as 1 day and increased with time. On the contrary, mRNA expression of OCN was decreased significantly at day 1 and the downregulation of OCN mRNA expression was still observed at 7 days after culture (Fig. 6).

To further confirm an involvement of Notch signaling in the ability of surface-bound Jagged-1 to induce osteogenic differentiation, HPDLs were cultured in osteogenic medium and DAPT was added to inhibit γ-secretase activity. The results demonstrated that DAPT was able to inhibit the increase of alkaline phosphatase activity and mineralization by HPDLs on the Jagged-1 bound surface [Fig. 7(A,B)]. In addition, DAPT was able to attenuate the ALP, Col 1, and OPN mRNA expression level of HPDLs on the Jagged-1 bound surface. The mRNA expression of OCN, which was downregulated by the Jagged-1 bound surface, recovered to levels comparable to those of HPDLs on tissue culture and the hFc bound surface [Fig. 7(C)].

To determine potential mechanism(s) by which surface-bound Jagged-1 enhanced osteogenic differentiation of HPDLs, cells were exposed to the hFc or Jagged-1 immobilized surfaces and key osteogenic differentiation factors were examined. The negative regulators of osteogenic differentiation, TWIST1 and TWIST2, were downregulated. No statistical significance was noted for TWIST1 mRNA expression [Fig. 8(A)]. On the contrary, the TWIST2 mRNA expression significantly decreased compared to cells on hFc bound surface as early as 1 day in culture, suggesting a prospective control mechanism [Fig. 8(B)]. The expression of Cbfa-1 and

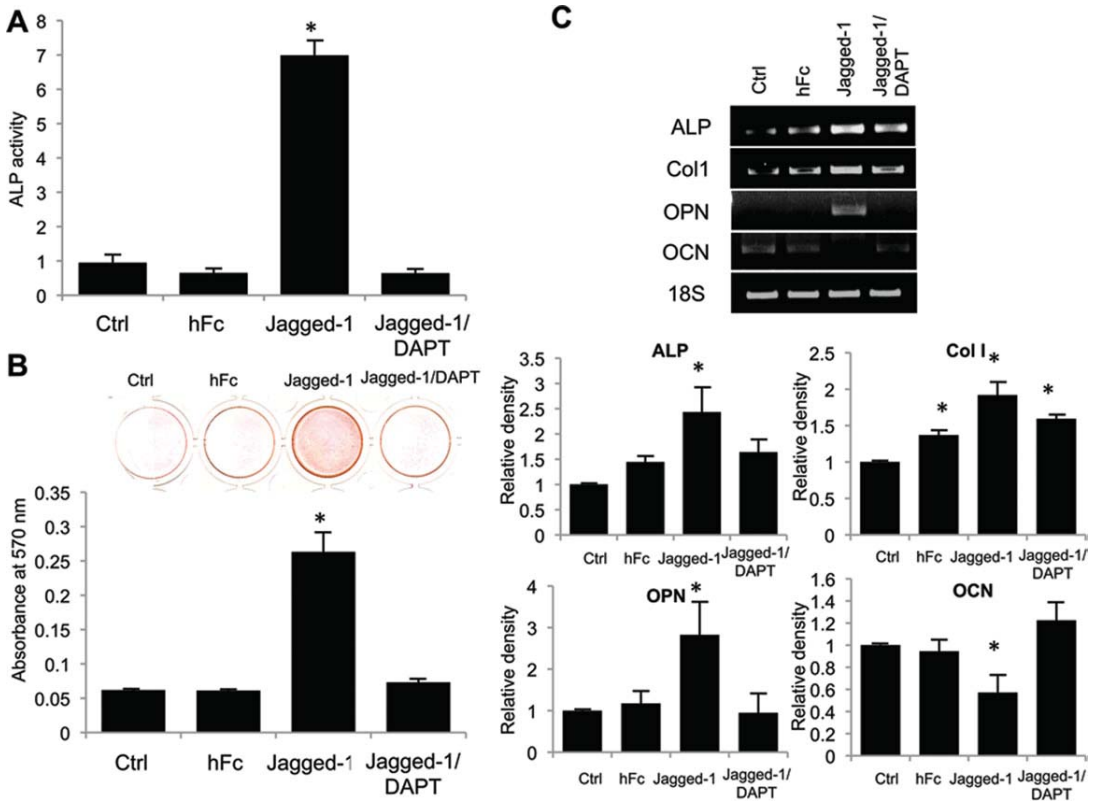


**FIGURE 7.** The effect of Jagged-1 bound surface on HPDLs osteogenic differentiation was attenuated by γ-secretase inhibitor; DAPT. After seeding cell for 7 days, DAPT treated cells on Jagged-1 modified surface had alkaline phosphatase enzymatic activity (A), mineralization (B) and mRNA expression of osteogenic marker genes (C) similar to those of the control surface, but significantly different from those of cells on the Jagged-1 bound surface. Asterisks represent statistical significance ($p < 0.05$) compared to the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Osterix, positive regulators of osteogenic differentiation, was slightly increased compared to those cells exposed to the hFc control surfaces, however, the mRNA expression levels were not significantly different [Fig. 8(C,D)].

## DISCUSSION

In the present study, the notch ligand, Jagged-1, was successfully immobilized on a material surface using an indirect affinity technique, and shown to retain its ability to initiate notch signaling *in vitro*. In addition, surface-bounded Jagged-1 promoted osteogenic differentiation of HPDLs. In this regard, alkaline phosphatase enzymatic activity and mineralization as well as mRNA expression of ALP, Col 1 and OPN were significantly increased compared to those of control surfaces. In contrast, OCN mRNA expression levels were decreased when cells were exposed to Jagged-1. Further, Jagged-1 downregulated TWIST1 and TWIST2 gene expression, but did not alter Cbfa-1 mRNA levels, suggesting that notch signaling might stimulate osteogenic differentiation in HPDLs through a TWIST-dependent mechanism.

In this study, the bioactive surface-bound Jagged-1 was successfully fabricated. The bound ligand was able to significantly activate Notch signaling compared to soluble ligand, as indicated by an increase in Notch target gene, Hes-1 and Hey-1, expression. Consistent with this study, Beckstead et al. previously reported that an immobilized notch ligand on a material surface could induce notch signaling to a greater extent than solubilized notch ligand.[9] Dose-dependent activation of CBF-1 luciferase reporter was observed on Jagged-1 immobilized surface. Using an indirect affinity immobilization, the activity of CBF-1 luciferase reporter was significantly increased.[10] These data confirm that the form of the notch ligand, as well as the method to introduce notch ligands to cells influences biological activity and cell function. The concept of an indirect Jagged-1 immobilized surface and its potential mechanism on notch signaling initiation were formerly reported and discussed in our previous studies.[9,10]

Notch signaling has been proposed as one of the mechanisms involving in osteoblast differentiation and bone regeneration.[16–18] Using murine tibia fracture and calvarial defect models, the up-regulation of Notch ligands and receptors were noted in mesenchymal cells during the regeneration process.[17] Particularly, Jagged-1 and Notch-2 were highly expressed.[17] In addition, it has been reported that intrinsic expression of Jagged-1 in bone marrow stromal cells was related to cellular osteogenic differentiation capacity.[6,7] Jagged-1 also enhanced BMP-2-induced osteoblast differentiation of murine calvarial osteoblast cell line.[19] Consistent with these studies, surface-bound, oriented Jagged-1 promoted osteogenic differentiation in HPDLs. Upregulation of

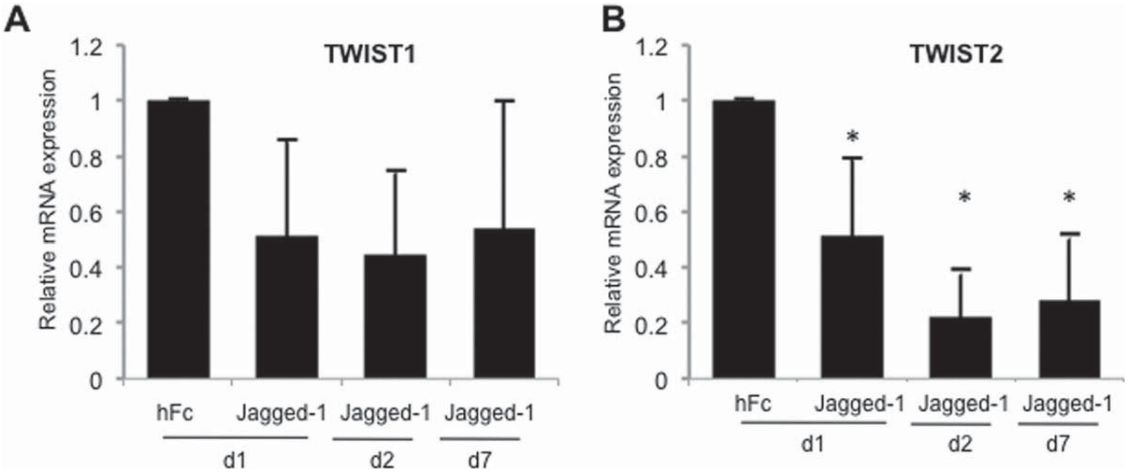


**FIGURE 8.** The mRNA expression of transcriptional factors that positively or negatively regulated osteogenic differentiation of human periodontal ligament-derived mesenchymal stem cell at 1, 2, and 7 days after seeded on the Jagged-1 bound surface. Asterisk represented the statistical significance ($p < 0.05$) compared to those cells exposed to the hFc control surfaces. (TWIST1 and TWIST2; negative regulators of osteogenic differentiation).

alkaline phosphatase enzymatic activity, mineralization and osteoblast-related genes were significantly noted in HPDLs on the Jagged-1 surface. Together, Jagged-1 enhanced osteogenic differentiation of mesenchymal cells and may be a candidate molecule used for promoting periodontal tissue regeneration. However, over-expression of Jagged-1 in dental pulp stem cells resulted in inhibition of odontoblast differentiation *in vitro* and mineralization *in vivo*.[20] The discrepancy may be due to intrinsic factors in different cell types. One intrinsic factor, which may involve in this discrepancy, is a cell origin. Mesenchymal cells in craniofacial region including dental pulp stem cells are derived from ectodermal origin known as neural crest cells,[21] unlike other part of the body which mesenchymal cells are derived from mesodermal origin.[22] In addition, the different gene expression was noted between human dental pulp stem cells and bone marrow stem cells as evaluated by cDNA microarray analysis.[23] Various publications have reported the different cell behaviors derived from these two sources.[24,25] Thus, the evidences may imply that an influence of Notch signaling in various cell types could be different due to the cell intrinsic factors.

Interestingly, we observed that the mRNA expression of OCN was significantly decreased when HPDLs were seeded on Jagged-1 bounded surface, contrasting with other osteogenic marker genes. The conflicting results on the influence of surface-bound Jagged-1 on the mRNA expression of osteogenic markers led us to examine potential mechanisms of Jagged-1 induced osteogenic differentiation. We hypothesized that the Jagged-1 bound surface might activate Notch signaling and in turn, expression of downstream osteogenic regulators. In this study, while Cbfa-1 levels were unchanged, TWIST2 mRNA levels were significantly decreased in HPDLs on the Jagged-1 bound surface. TWIST was previously described as a negative regulator of osteogenic differentiation.[26] In murine calvarial cells, mutation of TWIST resulted in increased alkaline phosphatase and collagen synthesis, but decreased osteocalcin expression in both basal condition and during osteogenesis.[26] Moreover, over-expression of TWIST in human mesenchymal stem cells reduced the expression of osteogenic marker genes.[27] TWIST knockdown in human periodontal ligament cells led to higher ALP, OPN, and BSP expression.[28] However, Cbfa-1 and OCN expression were not different in that study.[28] Altogether, the data imply that surface-bound Jagged-1 might promote osteogenic differentiation of HPDLs through the regulation of TWIST expression. The detail mechanism of Jagged-1 regulated TWIST function in HPDLs is currently under investigation in our laboratory.

In summary, this study illustrated that surface-bound Jagged-1 enhanced HPDLs differentiation toward the osteoblast lineage under osteogenic induction condition. In addition, this process required Notch signaling and correlated with downregulation TWIST gene expression. In previous studies by Beckstead et al.,[9,10] Jagged-1 modified biomaterials induced decreased proliferation and increased differentiation of epithelial cells as well as inhibited epithelium migration down along the dermis. Ideally, guide tissue regeneration membrane used in periodontal tissue regeneration should prevent epithelium downgrowth in the defect as well as promote alveolar bone formation. Thus, Jagged-1 could be a candidate molecule to guide tissue regeneration of periodontal tissues as it promotes epithelial differentiation and attenuates downward migration as well as enhances osteogenic differentiation in periodontal ligament stem cells.

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# Notch Signaling Is Involved in Neurogenic Commitment of Human Periodontal Ligament-Derived Mesenchymal Stem Cells

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Notch signaling plays critical roles in stem cells by regulating cell fate determination and differentiation. The aim of this study was to evaluate the participation of Notch signaling in neurogenic commitment of human periodontal ligament-derived mesenchymal stem cells (hPDLSCs) and to examine the ability to control differentiation of these cells using modified surfaces containing affinity immobilized Notch ligands. Neurogenic induction of hPDLSCs was performed via neurosphere formation. Cells were aggregated and form spheres as early 1 day in culture. In addition, the induced cells exhibited increased mRNA and protein expression of neuronal markers that is, β3-tubulin and neurofilament. During neuronal differentiation, a significant increase of *Hes1* and *Hey1* mRNA expression was noted. Using pharmacological inhibition (γ-secretase inhibitor) or genetic manipulation (overexpression of dominant negative mastermind-like transcription co-activators), neurosphere formation was attenuated and a marked decrease in neurogenic mRNA expression was observed. To confirm the role of Notch signaling in neuronal differentiation of hPDLSCs, the Notch ligand, Jagged-1, is bound to the surface using an affinity immobilization technique. The hPDLSC cultured on a Jagged-1-modified surface had increased expression of Notch signaling target genes, *Hes-1* and *Hey-1*, confirming the activity and potency of surface-bound Jagged-1. Further, hPDLSC on surface-bound Jagged-1 under serum-free conditions showed multiple long and thin neurite-like extensions, and an increase in the expression of neurogenic mRNA markers was observed. Pretreatment of the cells with γ-secretase inhibitor, DAPT, before seeding on the Jagged-1-modified surface blocked development of the neurite-like morphology. Together, the results in this study suggest the involvement of Notch signaling in neurogenic commitment of hPDLSCs.

## Introduction

Periodontal ligament is located between cementum and alveolar bone. It assists the anchoring of teeth to alveolar bone [1]. The periodontal ligament mainly consists of type I collagen fibers and several types of cells, for example, periodontal ligament fibroblast cells, vascular endothelial cells, and smooth muscle cells [1]. Moreover, several studies have shown that mesenchymal stem cells can be isolated from periodontal ligament tissues of both primary and permanent teeth [2–4]. Our previous study showed that cells isolated from human periodontal ligament tissues expressed embryonic and mesenchymal stem cell markers: Oct4, Rex-1, Nanog, CD44, CD73, and CD105 [5]. These cells were able to differentiate into osteogenic/cementogenic, adipogenic, and neurogenic lineage in vitro [5]. In addition, human periodontal ligament-derived mesenchymal stem cells (hPDLSCs) could also differentiate into other specific cell types such as chondrocytes, cardiomyocytes, and vascular-like cells [6,7]. Upon subcutaneous implantation, hPDLSCs had the potential to generate cementum-periodontal ligament like tissues [1–3]. Seo et al. described the hPDLSCs'properties as clonogenic, high proliferative, and capable to regenerate cementum/periodontal ligament-like tissues [2]. Together, these results suggest stem cell-like properties of the cells isolated from human periodontal ligament tissues.

Regarding neuronal differentiation, our group and other investigators have illustrated that hPDLSCs were able to differentiate toward neurogenic lineage under specific conditions [4–7]. Neurite-like, long cellular processes and upregulation of neurogenic markers [β3-tubulin, nestin, and neurofilament (NF)] were noted after neurogenic induction

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[4,8]. Moreover, it has been demonstrated that hPDLSCs were able to integrate into brain tissues in vitro, evaluated by hippocampal organ culture model [9]. In addition to direct differentiation into neurons, hPDLSCs promoted survival, neurite outgrowth, and migration of neuronal cell line by secreting nerve growth factor [10]. Thus, hPDLSCs have been proposed as a candidate cell source for neurological regenerative treatment as they are capable of direct differentiation into neuron and indirect support nervous tissue regeneration.

Notch signaling has a critical role in maintaining stemness and directing differentiation in several types of stem cells [11–13]. Notch signaling is activated upon the binding of Notch to its ligands on adjacent cells [14]. Subsequently, the cleavage of Notch protein occurs intracellularly, resulting in the translocation of Notch intracellular domain (NICD) into the nucleus and further regulating Notch target gene expression [14]. In periodontal ligament cells, Notch signaling was shown to participate in osteogenic differentiation process and induction of osteoclastogenesis [4,15,16]. However, evidence of Notch signaling in hPDLSCs differentiation toward neuronal lineage is yet unknown. Therefore, the aim of this study was to evaluate the role of Notch signaling during neurogenic differentiation of hPDLSCs in vitro. Here, we reported that attenuation of Notch signaling resulted in the decrease expression of neurogenic marker, while the activation of Notch signaling under serum-free condition promoted neurogenic phenotypes.

## Materials and Methods

### Culture and neurogenic differentiation hPDLSCs

hPDLSCs were isolated and cultured according to formerly published procedure [17]. The protocol for the isolation of hPDLSCs was previously approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. hPDLSCs were maintained in Dulbecco's modified Eagle medium (Gibco) containing 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 5 μg/mL amphotericin B (Gibco) in 100% humidity, 37°C and 5% carbon dioxide. Medium was changed every 48 h.

Neurogenic differentiation of hPDLSCs was performed using the sphere formation technique [18]. Briefly, hPDLSCs were cultured in neurobasal medium (Gibco) containing B27 (2%), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (5 μg/mL), basic fibroblast growth factor (20 ng/mL; Invitrogen), and epidermal growth factor (20 ng/mL; Sigma) in 60-mm-petri dishes. In some experiments, γ-secretase inhibitor (DAPT; Sigma) was added to the culture medium at a concentration of 25 μM to inhibit the cleavage of NICD. Dimethyl sulfoxide was used as vehicle control. Subsequently, the spheres were seeded on collagen IV (Sigma) coated dishes and maintained in the neurogenic medium supplemented with retinoic acid for 7 days. The mRNA expression of neurogenic markers was evaluated using reverse transcriptase–polymerase chain reaction (RT-PCR). The expression of human β3-tubulin and NF was also evaluated by immunocytochemical staining. Six pictures of spheres were randomly captured on each plate using phase-contrast microscopy. Size of spheres was analyzed using ImageJ software.

The percentage of neurospheres with given diameters was calculated as follows: group 1: spheres with a diameter < 50 μm, group 2: spheres with a diameter 50–100 μm, and group 3: spheres with a diameter > 100 μm.

To determine sphere formation efficiency, limited dilution assay was employed. Cells were seeded in 60 mm low attachment Petri dishes at various density (from 10,000–100,000 cells/plates) and maintained in neurogenic induction medium for 7 days. The number of sphere formation was evaluated under phase-contrast microscope.

For calcium imaging, cells were loaded with Fluo-4 AM dye (Invitrogen) for 30 min at room temperature according to manufacturing protocol. Cell were washed twice and maintained in calcium-free culture medium. Cells were stimulated with *N*-methyl-D-aspartic acid (NMDA, 20 μM; Sigma) or dopamine hydrochloride (DPH, 10 mM; Sigma). Intracellular calcium change was imaged using inverted fluorescence microscopy at 20× magnification objective lens at 3 different time points: baseline before treatment, immediate after treatment, and 30 s after treatment.

### Cell viability assay

Live/dead fluorescence staining and MTT assay were employed to evaluated cell viability. For live/dead fluorescence staining, calcein acetoxymethyl ester, and ethidium homodimer-1 were added into the culture and incubated at 37°C for 20 min and subsequently observed under a fluorescent microscope using Apotome.2 apparatus (Carl Zeiss). Live cells generated green fluorescence and dead cells exhibited red fluorescence. For flow cytometry analysis, a single-cell suspension was obtained from spheres using trypsin dissociation. Cell suspension was allowed to recover in culture medium for 30 min before staining with live/dead fluorescence. MTT assay was performed according our previous publication [19].

### Transfection and retroviral transduction

The retroviral plasmid for expression of dominant negative mastermind-like transcriptional factor (DN-MAML; MSCV-Mam(12–74)-EGFP), a kind gift from Dr. Warren Pear (University of Pennsylvania) or the control vector (MSCV-IRES-GFP, Addgene plasmid 20672), was co-transfected with packaging construct pCL-Ampho (Imagenex) into HEK293 cells using the FuGene® HD transfection reagent (Roche) according to the manufacturer's instructions. Culture supernatants containing retroviruses were harvested twice at 48 and 72 h after transfection and were used to transduce hPDLSCs. Transduction efficiency was confirmed by fluorescent microscopy.

### Immobilization of Jagged-1

Surface-bound Notch ligand, Jagged-1, was fabricated by an indirect affinity immobilization method previously reported by our group [4,20,21]. Briefly, recombinant protein G (50 μg/mL; Zymed) was incubated with the tissue culture plate surface for 16 h and subsequently, incubated with bovine serum albumin (10 mg/mL; Sigma) for 2 h. The surface was further incubated with recombinant human Jagged-1 (10 μM; R&D Systems) for 2 h. The surface was washed 3 times with sterile phosphate-buffered saline (PBS) between each step. An equal amount of human immunoglobulin G Fc fragment (Jackson Immunoresearch Laboratory) was employed as a control. Before seeding the cells, the surfaces were washed

once with culture media. The presence of immobilized Jagged-1 was confirmed using enzyme-linked immunosorbent assay (ELISA) according to previously publish protocol [4,20,21].

### Polymerase chain reaction

For RT-PCR, total cellular RNA was extracted with Trizol reagent. RNA samples (1 μg) were converted to cDNA by reverse transcriptase enzyme (Promega). RT-PCR was performed using Taq polymerase (Invitrogen). The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining. In some experiments, the band density was determined using ImageJ software. For real-time quantitative PCR experiment, PCR was performed in a LightCycler® Nano (Roche) with LightCycler480 SYBR Green I Master kit (Roche Diagnostic). Reaction product was quantified with GAPDH as the reference gene. The primer sequences were shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd).

### PCR array

Total RNA was extracted using RNeasy Mini kit (Qiagen). The contaminating DNA was digested by RNase-free DNase I (Qiagen). One microgram of total RNA was employed to synthesize first-strand cDNA using $RT^2$ First Strand kit (Qiagen). Human Notch Signaling Pathway PCR array (PAHS-059; Qiagen) was performed with real-time PCR using $RT^2$qPCR Master Mixes (Qiagen) in LightCycler480 (Roche). The data were analyzed using $RT^2$ Profiler™ PCR array data analysis software.

### Immunocytochemistry

The cells were fixed in 10% buffered formalin at room temperature for 30 min, permeabilized with 0.15% Triton®-X100 in PBS and 10% horse serum for 1 h at room temperature. The cells were then incubated with mouse anti-β3-tubulin (Promega) or mouse anti-NF, medium chain (NF) (Invitrogen) at a 1:200 dilution for 18 h at room temperature. After washing with PBS, the cells were incubated with biotinylated rabbit anti-mouse antibody (Zymed) for 30 min at 1:500 dilution. Subsequently, the antibody was detected using Strep-Fluorescein isothiocyanate (Sigma) or Rhodamine Red™-X (Invitrogen) and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (0.1 μg/mL). The cells were analyzed with a fluorescent microscope using Apotome.2 apparatus (Carl Zeiss).

### Statistical analyses

Data are reported as mean ± standard deviation. Statistical analyses were performed using 2-independent Student *t* test for 2-group comparison. A one-way analysis of variance followed by Dunnett test was employed to compare in experiments containing 3 or more groups. Differences at $P < 0.05$ were considered to be statistically significant.

## Results

### hPDLSCs differentiated into neuronal linage

Neurosphere formation assay was employed to evaluate neurogenic differentiation ability of hPDLSCs. Similar to our previous reports [4], neurosphere formation was noted at day 1 after culturing cells in neurogenic medium (Fig. 1A). Size and density of the spheres were increased at day 3 and 7 compared to day 1 (Fig. 1B–D). However, the efficiency of the sphere formation was 2–4 spheres per 10,000 cells at day 7. After 7 days, the spheres were transferred to collagen IV coated dishes and maintained in neurogenic medium supplemented with retinoic acid for another 7 days. Cells migrated out from the spheres and exhibited neurite-like cellular processes (data not shown). β3-tubulin and NF protein expression were observed in the floating and adhered spheres (Fig. 1E–H). More cells expressed β3-tubulin than expressed NF in both floating and adhered conditions. The NF expression was noted in cells located at the out border of the spheres. Cell viability in floating and adhered condition was evaluated using live/dead fluorescence (calcein acetoxymethyl ester and ethidium homodimer-1) staining. The results illustrated that most of the cells were positively stained with green fluorescence, indicating cell survival (Fig. 1I, J). In addition, neurogenic differentiation was confirmed by the mRNA expression of neurogenic markers. The mRNA expression of *Sox2* and *Sox9* was significantly upregulated in floating spheres (Fig. 1K–P). For adhered spheres, it was also noted that *Sox2* and *Sox9* mRNA levels were decreased, while *β3-tubulin* and *NF* was increased when compared to the floating spheres. Moreover, the upregulation of *GABA receptor β3* was noted in both sphere and adhered condition. Intracellular calcium imaging was performed comparing calcium at baseline and after treatment with NMDA or DPH (Fig. 1Q). The results showed that levels of intracellular calcium were increased immediately after cells were exposed with the stimuli, implying neuronal function. The intracellular calcium was decreased to baseline within 30 s after stimulation. Together, these results suggest that hPDLSCs were able to differentiate into the neurogenic lineage.

### Notch signaling involved in neuronal differentiation of hPDLSCs

To examine a possible role for Notch signaling in neuronal differentiation of hPDLSCs, PCR array of human Notch signaling was performed to compare mRNA expression between control hPDLSCs those undergoing neurogenic differentiation. Thirteen genes were upregulated (data not shown). Among those, 2 Notch signaling target genes, *Hes1* and *Hey1*, were noted. The upregulation of *Hes1* and *Hey1* mRNA expression was confirmed by RT-PCR and real-time quantitative PCR (Fig. 2A–C). *Hes1* and *Hey1* mRNA levels were higher in neurospheres than in controls. In time course experiments, *Hes1* and *Hey1* mRNA levels increased upon culturing cells in neurogenic medium as early as 1 day compared to the control. Both *Hes1* and *Hey1* mRNA expression tended to decrease at later time points, although the expression was still relatively higher than the control (Fig. 2D–F). In addition, the decrease of *Hes1* and *Hey1* mRNA levels was also noted in adhered neurosphere-derived neuronal cells compared to floating spheres (data not shown), suggesting the reduction of Notch signaling may required in neuronal maturation process. Together, the upregulation of Notch signaling target genes upon neurogenic differentiation

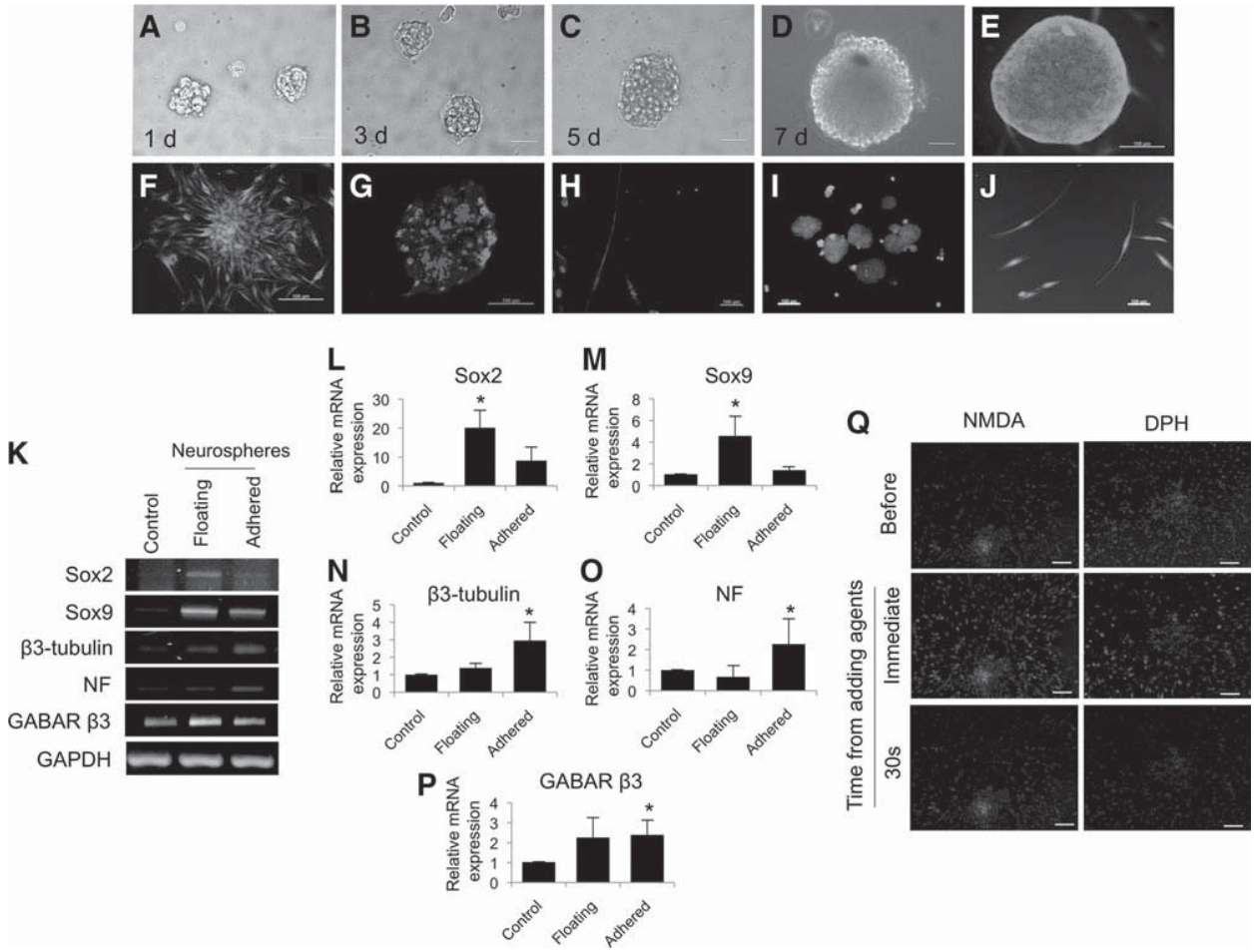


**FIG. 1.** Human periodontal ligament-derived mesenchymal stem cells (hPDLSCs) were able to differentiate into neurogenic lineage. Neurosphere formation was observed at day 1 **(A)**, 3 **(B)**, 5 **(C)**, and 7 **(D)** after exposing cells in the neurogenic medium. The protein expression of β3-tubulin and neurofilament (NF) was noted in floating [**(E, G)**, respectively] and adhering neurospheres [**(F, H)**, respectively]. Live/dead fluorescence staining in floating **(I)** and adhered spheres **(J)** was illustrated. The neurogenic mRNA expression was evaluated in both floating and adhering neurospheres compared to the control by conventional polymerase chain reaction (PCR) **(K)** and the results were further confirmed using real-time quantitative PCR **(L–P)**. Intracellular calcium of adhered cells before and after treatment with *N*-methyl-D-aspartic acid (NMDA) and dopamine hydrochloride (DPH) was observed using inverted phase contrast fluorescence microscopy **(Q)**. *Asterisks* indicate statistical significance compared to the control. (Scale bar = 100 μm).

suggest a role of Notch signaling in neurogenic commitment process in hPDLSCs.

To further confirm the involvement of Notch signaling in the cell fate decision toward the neurogenic lineage, a γ-secretase inhibitor, DAPT, was applied to inhibit Notch signaling initiation by impeding the cleavage of NICD. In the presence of DAPT, the spheres appeared smaller than the control (Fig. 3A–D). An average sphere diameter was decreased when cells were exposed to DAPT compared to control cells, but a statistical significant difference was not observed (Fig. 3E). A higher percentage of small-size neurospheres (< 100 μm) was observed with the addition of DAPT compared to the control at day 1 and 7 (Fig. 3F). Cell viability was not significantly different between the control and DAPT treated group as determined by flow cytometry and MTT analysis (Fig. 3G, H, respectively). At day 1, the DAPT-treated neurospheres had decreased *Hes1* and *Hey1* mRNA expression, confirming the inhibition of Notch signaling (Fig. 3I, J). Correspondingly, *Sox2* and *β3-tubulin* mRNA levels were decreased by treatment of the neurospheres with DAPT (Fig. 3I, J). In addition, overexpression of DN-MAML was employed. MAML is transcriptional co-activator and essential participant in Notch signaling. The DN-MAML overexpressed hPDLSCs resulted in a decrease of neurosphere's size at 1 day in neurogenic culture condition compared to the vector control transduced cells (Fig. 4A, B). Cell viability was not significantly different between the GFP vector control and DN-MAML group (Fig. 4C). In addition, a decrease in mRNA expression levels of Notch signaling target genes (*Hes-1* and *Hey-1*) as well as neurogenic marker genes (*Sox2* and *β3-tubulin*) was noted (Fig. 4D, E). Together, the data imply the involvement of Notch signaling in neuronal commitment of hPDLSCs.

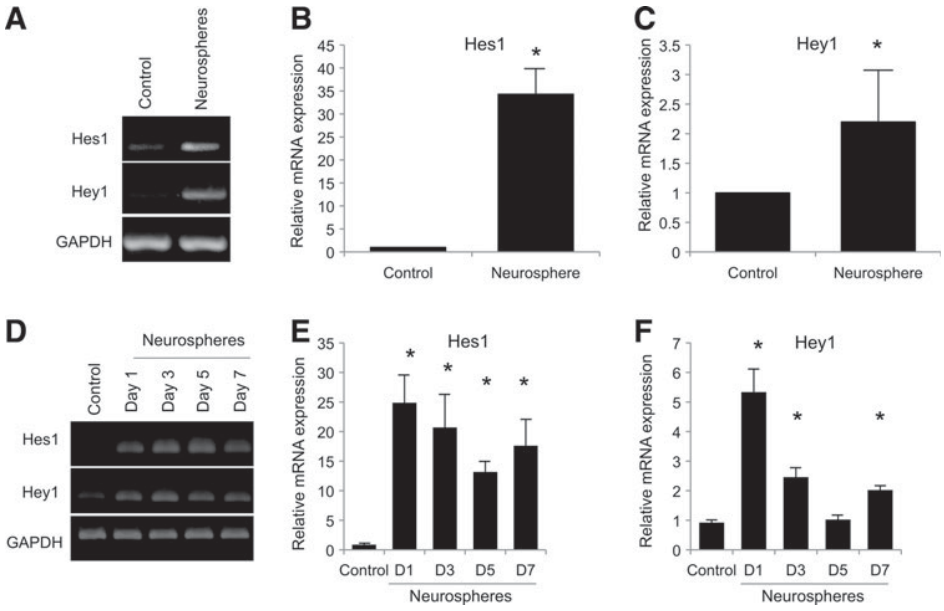


**FIG. 2.** hPDLSCs expressed Notch signaling target genes during neurogenic differentiation. The *Hes1* and *Hey1* mRNA levels were significantly upregulated after neurogenic induction for 7 days **(A–C)**. In time course study, *Hes1* and *Hey1* mRNA expression were significantly increased as early as 1 day after neurogenic induction, but a decrease expression in later time points was noted **(D–F)**. Real-time quantitative PCR was employed to confirm the upregulation of mRNA expression **(B, C, E, F)**. *Asterisks* indicate statistical significance compared to the control.

### *Surface-bound Jagged-1 induces neuronal differentiation in hPDLSCs.*

From PCR array analysis, Notch ligand, Jagged-1, was upregulated in neurospheres compared to the control (data not shown). The increased mRNA level of *Jagged-1* was confirmed by real-time quantitative PCR (Fig. 5A). This result suggests a role of Jagged-1 in neurogenic commitment of hPDLSCs. We, therefore, utilized a Jagged-1-immobilized surface to further elucidate the role of Notch signaling in neurogenic commitment by hPDLSCs. The presence of bound Jagged-1 on the surface was confirmed by ELISA assay (Fig. 5B). The hPDLSCs seeded on the Jagged-1-immobilized surface exhibited a dramatic increase of Notch target gene (*Hes-1* and *Hey-1*) expression compared to those exposed to normal culture surface and hFc bound surface (Fig. 5C, D). Moreover, the induction of Notch signaling by the Jagged-1-immobilized surface was inhibited by the addition of DAPT in culture medium, confirming the Notch signaling ability of the Jagged-1-bound surface.

To examine the role of Jagged-1 on neurogenic differentiation of hPDLSCs, the cells were seeded on a Jagged-1-modified surface in serum-free condition for 72 h. These cells exhibited multiple long, thin neurite-like extensions in both 1 and 10 nM concentration of Jagged-1 (Fig. 6C, D). On the contrary, hPDLSCs on untreated and bound Fc control surfaces had a fibroblast-like morphology (Fig. 6A, B). By immunocytochemical analyses, cells seeded on Jagged-1-bound surface were also positively stained with an antibody to β3-tubulin (Fig. 6G, H). However, β3-tubulin positive cells were not observed on either of the control surfaces (Fig. 6E, F). Moreover, an increase in the expression of neurogenic mRNA markers, *Sox2* and *β3-tubulin*, was observed in cells seeded on surface-bound Jagged-1 in a dose-dependent manner (Fig. 6I, J). These data indicate that Jagged-1 regulates neuronal differentiation of hPDLSCs. In addition, pretreatment of the cells with DAPT before seeding on the Jagged-1-modified surface blocked development of the neurite-like morphology and β3-tubulin protein expression (Fig. 7C, G, respectively). Moreover, the cells treated with soluble Jagged-1 displayed similar morphology and β3-tubulin protein expression to those on untreated surface and bound Fc surface (Fig. 7D, H, respectively). *Sox2* and *β3-tubulin* mRNA expression were significant lower on DAPT pretreated cells on surface-bound Jagged-1- and soluble Jagged-1-treated cells compared to those on surface-bound Jagged-1 surface (Fig. 7I, J).

## Discussion

In the present study, we identified a role for Notch signaling on neurogenic commitment of hPDLSCs. The results showed that *Hes-1* and *Hey-1* were upregulated in hPDLSC-derived neurospheres. The inhibition of Notch signaling by either pharmacological or genetic approaches was able to attenuate neurosphere formation and neurogenic mRNA expression by hPDLSCs. Further, a transcriptional activity of the Notch/CSL complex was required during neurogenic commitment of hPDLSCs as the overexpression of DN-MAML attenuated this process. In addition, hPDLSCs exposed to a Jagged-1-immobilized surface exhibited neuronal-like morphology with an increase in neurogenic mRNA and protein expression. Together, these results suggest the involvement of Notch signaling in neuronal differentiation of hPDLSCs.

Upon induction, hPDLSCs were able to differentiate into neuronal-like cells. These induced cells exhibited neurite formation and expressed neurogenic markers in both mRNA and protein levels. In addition, calcium ions initiate intracellular signaling that correspond to neuronal function [22]. NMDA receptor and DPH receptor are expressed in neuronal cells. Thus, the change of intracellular calcium resulted from NMDA or DPH treatment in the present study, implying the expression of particular receptors and their function. Similar to the present study, neuronal differentiation of hPDLSCs has previously been reported [5,23].

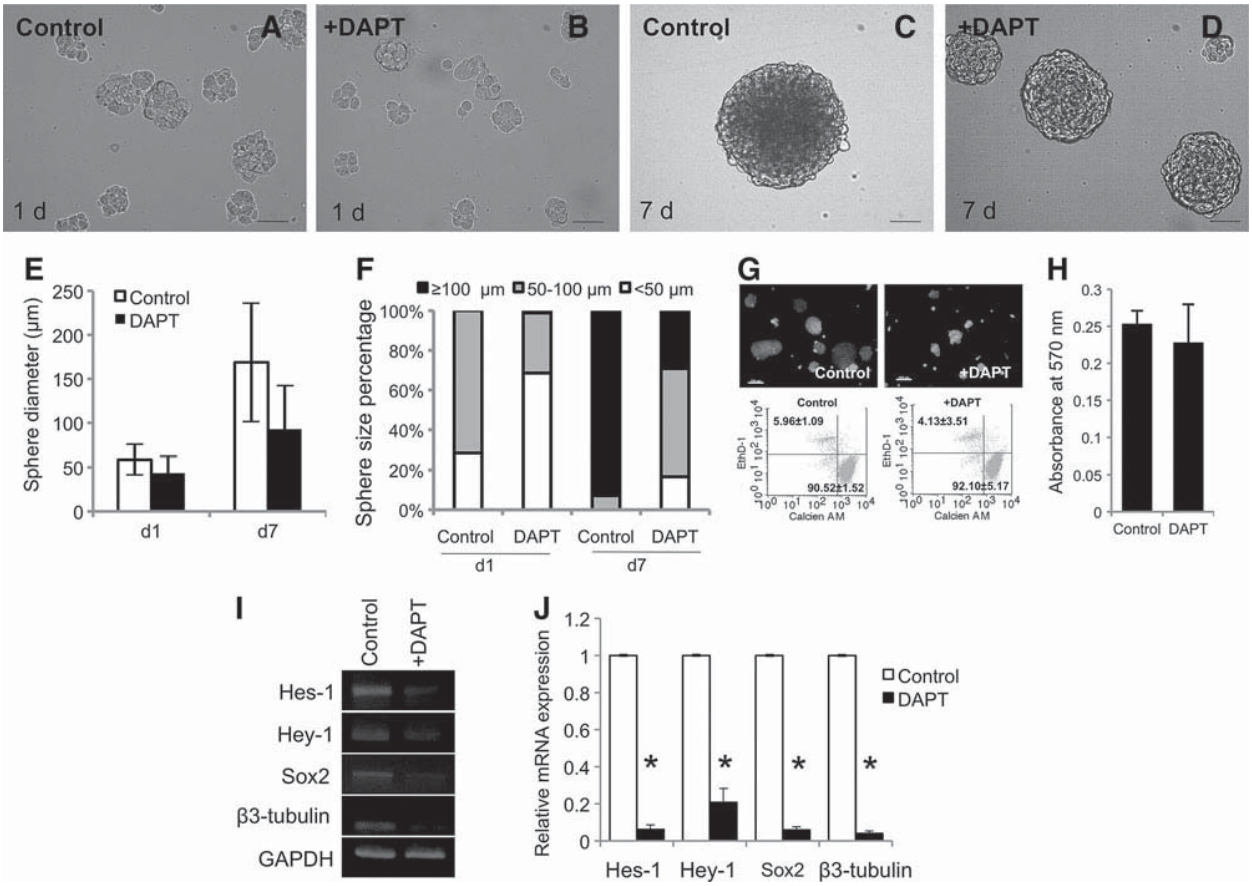


**FIG. 3.** Pharmacological inhibition of Notch signaling attenuated neurosphere formation by hPDLSCs. A γ-secretase inhibitor (DAPT) was employed to inhibit the initiation of Notch signaling. Cells treated with DAPT resulted in small-sized neurospheres as noted at 1 **(B)** and 7 **(D)** days upon neurogenic induction compared to the control at similar time points **(A, C)**. The average diameter of neurospheres was decreased compared to the control in both days 1 and 7; however, no statistical significance was observed **(E)**. By categorizing the neurosphere size into 3 groups, DAPT-treated cells exhibited more small-size neurospheres compared to the control **(F)**. Cell viability in spheres was evaluated using live/dead fluorescence staining **(G)** and MTT assay **(H)**. In addition, downregulation of *Hes1* and *Hey1* was noted, confirming the inhibition of Notch signaling in DAPT-treated groups. Correspondingly, a decrease of neurogenic mRNA levels was observed in DAPT-treated cells as determined by conventional and real-time quantitative PCR **[(I, J)**, respectively). *Asterisks* indicate statistical significance compared to the control. (Scale bar = 100 μm).

Undifferentiated hPDLSCs expressed baseline mRNA levels of neurogenic markers (ie, *β3-tubulin*), but the protein expression was not observed [24]. Keeve et al. hypothesized that this phenomenon may be due to the translation repression of *β3-tubulin* mRNA by miRNA [24]. Agreeing with our observation, the *β3-tubulin* mRNA expression was noted in the control and hFc control surface. However, the protein expression was not observed by immunocytochemistry staining. In addition, it has been identified that periodontal ligament tissues contained a subpopulation that is derived from neural crest cells [10,25,26]. Thus, these cells may contain the intrinsic characteristics of neuroectodermal lineage. Together, hPDLSCs may be an alternative source for cell therapy in regenerative neuronal disease.

Notch signaling has been shown to regulate neural stem cell maintenance [27–29]. Inhibition of Notch signaling resulted in an abundance of self-renewal stage and entry to neurogenic differentiation of neuronal stem cells [28]. However, an overexpression of Notch target gene, *HeyL*, promoted neuronal differentiation of neural progenitor cells both in vitro and in vivo [30]. Together, these data underscore the complex regulation of Notch signaling in neural stem cell maintenance and differentiation. In regard to mesenchymal stem cells, mRNA levels of Notch signaling pathway components were decreased in human adipose-derived mesenchymal stem cells upon neurogenic differentiation [31]. The inhibition of Notch1 expression enhanced neurogenic differentiation in murine bone marrow-derived mesenchymal stem cells [32]. On the contrary, some investigators reported that an activation of Notch signaling in mesenchymal stem cells promoted neuronal commitment. In this respect, overexpressing NICD in bone marrow stromal cells led to an increase in neurogenic markers, and these cells demonstrated action potentials as recorded by whole-cell patch clamp [33]. In addition, these Notch-induced mesenchymal-derived neuronal cells significantly improved

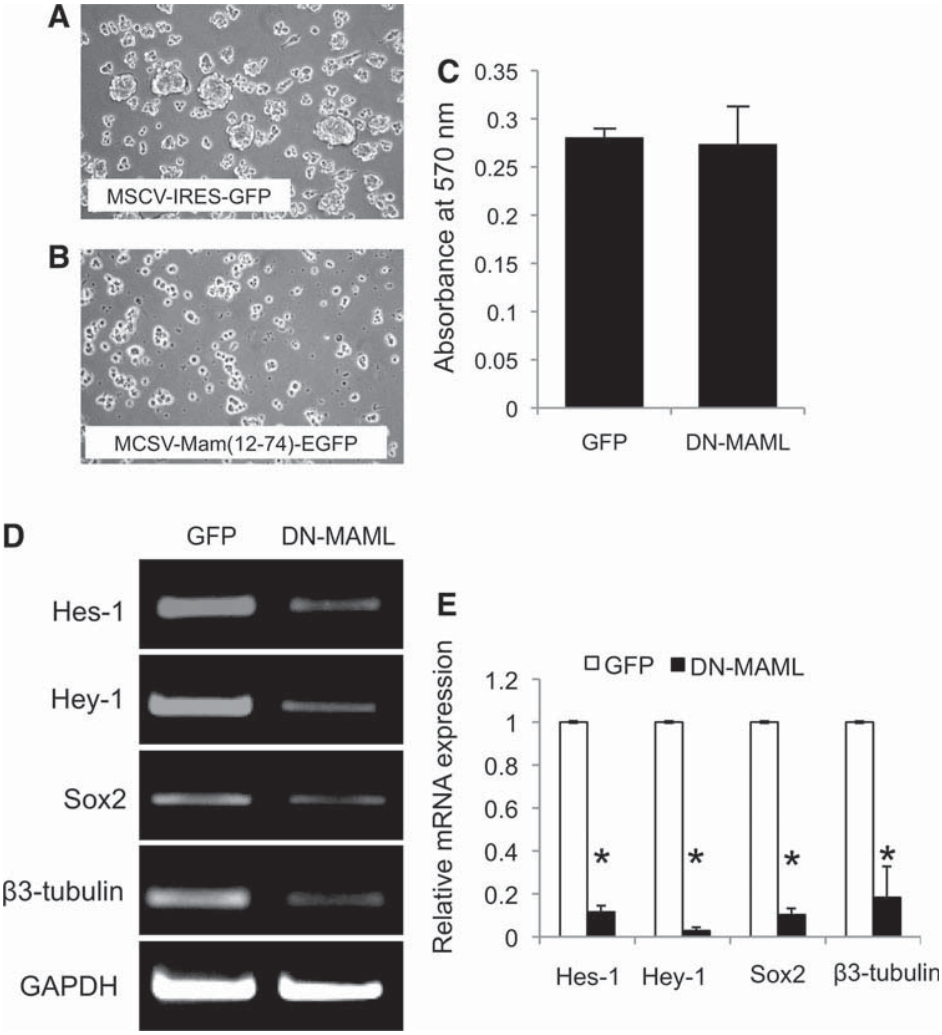


**FIG. 4.** The suppression of Notch signaling by genetic manipulation impaired neurosphere formation by hPDLSCs. Overexpression of dominant negative mastermind-like transcriptional factor (DN-MAML) in hPDLSCs resulted in the reduction of neurosphere formation compared to those transduced with the control vector **(A, B)**. Cell viability in spheres was evaluated using MTT assay **(C)**. The downregulation of neurogenic mRNA levels was noted in correspondence with the decrease of *Hes1* and *Hey1* mRNA expression as determined by conventional and real-time quantitative polymerase reaction [**(D, E)**, respectively]. *Asterisks* indicate statistical significance compared to the control.

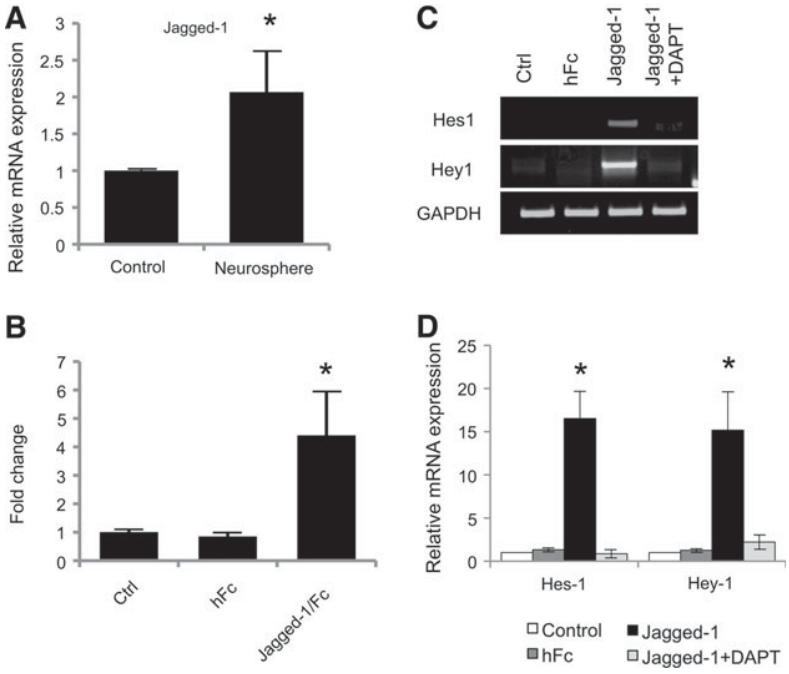


**FIG. 5.** Surfaced-bound Jagged-1 initiated Notch signaling in hPDLSCs. The *Jagged-1* mRNA expression was significantly increased after culturing cells in neurogenic medium for 7 days, as evaluated by real-time PCR **(A)**. The presence of immobilized Jagged-1 on tissue culture surface was confirmed by enzyme-linked immunosorbent assay **(B)**. The hPSLDCs expressed significantly higher *Hes1* and *Hey1* mRNA levels after seeding on a Jagged-1-modified surface. The upregulation of Notch target gene expression was inhibited by pretreatment cells with DAPT as determined by conventional and real-time quantitative PCR [**(C, D)**, respectively]. *Asterisks* indicate statistical significance compared to the control.

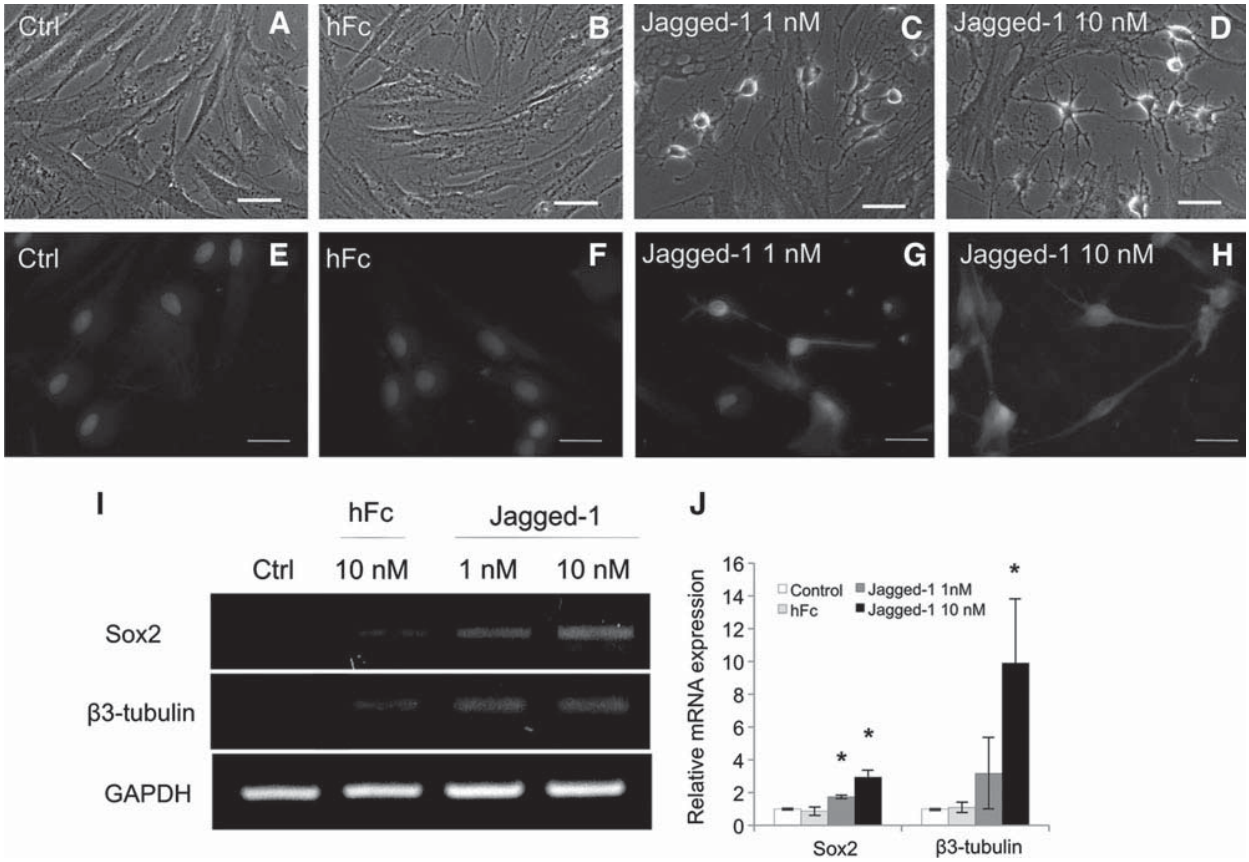


**FIG. 6.** Surface-bound Jagged-1 initiated neuronal differentiation by hPDLSCs in serum-free condition. At 72 h, the hPDLSCs on untreated surface and bound Fc surface appeared fibroblast-like morphology **(A, B)**. On the contrary, the cells exhibited multiple long and thin neurite-like extensions on surface-bound Jagged-1 groups **(C, D)**. The β3-tubulin protein expression was not observed in those cells seeded on the control and hFc bound surface **(E, F)** but markedly expression was noted in those cells exposed to a Jagged-1-modified surface **(G, H)**. An increase in the expression of neurogenic mRNA markers, *Sox2* and *β3-tubulin*, was observed in cells seeded on surface-bound Jagged-1 as determined by conventional and real-time quantitative PCR **[(I, J)**, respectively]. *Asterisks* indicate statistical significance compared to the control. (Scale bar = 100 μm).

behavioral responses upon implantation in a rat stoke model [34,35]. For embryonic stem (ES) cells, overexpression of NICD resulted in accelerated differentiation toward neuronal lineages [36]. NICD overexpression in ES cells resulted in the inhibition of non-neurogenic differentiation [36]. In addition, it was demonstrated that the co-culture of ES cells with stromal cells overexpressed Delta-like1 were able to promote the expression of neurogenic markers [36].

The discrepancies in observations regarding the role of Notch signaling in neurogenic differentiation may have several explanations. First, it has been shown that function of Notch signaling is cell type and developmental stage specific [37,38]. In this regard, a differential expression of Notch receptors was noted at different stages of development, which may influence intracellular signaling and cell fate decision [39,40]. Second, differences in neuronal induction protocols are likely to influence differentiation mechanism and efficiency [41]. Various reports employed β-mercaptoethanol as a component in neuronal induction medium [42]. Although, the protein expression of neuronal markers was upregulated, the neurogenic mRNA levels were not changed. Some studies showed that these β-mercaptoethanol-induced neurons did not exhibit neuron-like electrophysiological characteristics, suggesting pseudo-neuronal differentiation [43,44]. In the present study, the neurosphere formation method was employed. Neurosphere-derived neuronal cells exhibited similar characteristics to those of bonafide neurons in term of mRNA and protein expression as well as electrophysiological properties [45,46]. Thus, the evaluation of Notch signaling using neurosphere formation technique could give a better understanding the mechanisms controlling true neuronal differentiation.

In the present study, we described the upregulation of *Hes1* and *Hey1* during neurogenic differentiation by hPDLSCs. However, the reduction of *Hes1* and *Hey1* mRNA levels was noted at later stages. These data suggest that Notch signaling may be required in neurogenic commitment of hPDLSCs and but that Notch signaling should fade out to permit neuronal maturation processes. Corresponding with our study, it was demonstrated that *Hes1* mRNA levels were increased after exposing human bone marrow stem cells in neuroinduction medium [47]. However, it has to be noted that these genes have other potential functions, that is, cell proliferation and stemness maintenance, which should be

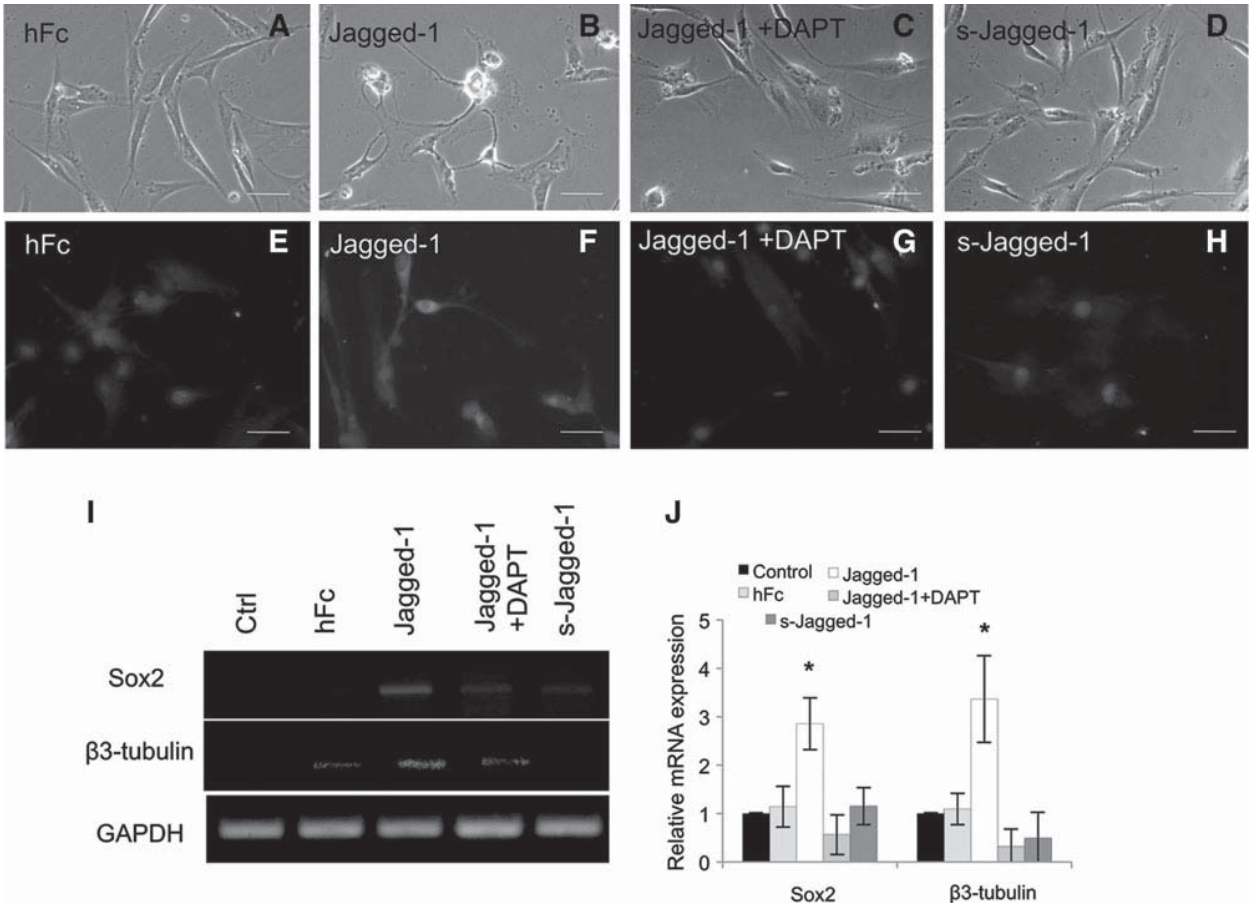


**FIG. 7.** Surface-bound Jagged-1 initiated neuronal differentiation by hPDLSCs in serum-free condition. The hPDLSCs on the control bound Fc surface had a fibroblast-like morphology and were not stained with anti β3-tubulin antibody **(A, E)**. On the contrary, the cells exhibited neuronal-like morphology upon exposed to Jagged-1-modified surface **(B)**. Positive staining with anti β3-tubulin antibody was noted **(F)**. Pretreatment of the cells with DAPT before seeding on the Jagged-1-modified surface or treatment of the cells with soluble Jagged-1 resulted in similar results to those of the control **(C, D, G, H)**. *Sox2* and *β3-tubulin* mRNA expression were lower on DAPT pretreated cells on surface-bound Jagged-1- and soluble Jagged-1-treated cells compared to those on surface-bound Jagged-1 surface as determined by conventional and real-time quantitative PCR [**(I, J)**, respectively]. *Asterisks* indicate statistical significance compared to the control. (Scale bar = 100 μm).

considered [48]. In this regard, it has been reported that Hes1 prevented irreversible cell cycle exit in human fibroblast and rhabdomyosarcoma cells [49]. Thus, specific function of the upregulation of these genes in hPDLSCs should also be further investigated.

A role of Jagged-1 in cell fate decision control has been reported in several cell types. Jagged-1 promoted neuroectodermal commitment of ES cells [12]. Similar to that study, we illustrated that exposing hPDLSC to Jagged-1-modified surface promoted neurogenic commitment in serum-free culture medium as determined by the expression of neurogenic markers, *Sox2* and *β3-tubulin*. However, our previous publication observed that Jagged-1 enhanced osteogenic differentiation in hPDLSCs in normal growth medium as well as in osteogenic medium [4]. These contrary results may have occurred for several reasons. First, the activation of Notch signaling prompts hPDLSCs to differentiate. However, the specific lineage determination is regulated by other external stimuli in the culture environment. In this respect, it has been reported that the activation of Notch target genes was differently regulated by extrinsic factor. Different culture medium supplementation influenced other intracellular signaling, which further controlled and interacted with Notch signaling [50]. Moreover, a withdrawal of trophic factors resulted in the reversible change of Numb isoforms that further controlled the activation of Notch signaling target genes [51]. Together, these results suggest that culture environment has considerable influences on the different regulation of Notch signaling target gene expression and cell's behaviors. Corresponding to our observation, Jagged-1 promoted neurogenic commitment of hPDLSCs in serum-free condition, but in the present of 10% serum, osteogenic differentiation was enhanced, implying the role of culture condition of Notch signaling-influenced cell function. Second, the difference in cell fate determination upon exposure to Notch ligand might have occurred due to different Notch receptor interactions as well as downstream intracellular signaling cascade. In this regard, it has been illustrated

that an activation of canonical Notch signaling resulted differences in regulation and cell behavior compared to the noncanonical pathway [52]. In addition, it has been demonstrated that an alteration of Notch receptor expression may impact control of specific cell lineages as shown in olfactory epithelium [53]. In our laboratory, we observed the differential Notch receptor expression after hPDLSCs was induced to osteogenic, adipogenic, and neurogenic lineage (unpublished data). The mechanism for this requires further investigation.

In summary, we illustrated that the expression of Notch signaling target genes was changed during neuronal induction of hPDLSCs. Exposing cells to a Jagged-1-immobilized surface could promote morphological changes and expression of neurogenic markers. Together, these results suggest the potential role of Notch signaling in neuronal commitment of hPDLSCs. Further investigation on molecular mechanism is needed in order to elucidate a method to precisely control differentiation of these cells for clinical utilization.

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## Author Disclosure Statement

No benefit of any kind will be received either directly or indirectly by the author(s). No competing financial interests exist.

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# Directing Differentiation of Human-periodontal-ligament-derived Stem Cells using Notch-ligand Modified Surfaces


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## Introduction

Notch signaling is critical for maintaining stemness and inducing cell differentiation was reported. Over-expression of Delta-1, a Notch ligand, in dental pulp stem cells enhanced proliferation and differentiation into odontoblast-like cell (1). On the contrary, over-expression of another notch signaling ligand, Jagged-1, in dental pulp stem cells resulted in inhibition of odontoblast differentiation in vitro and mineralization in vivo (2). The function of Notch ligands, Delta-1 and Jagged-1, on cell behaviors was also reported in other cell types such as human hematopoietic progenitor cells (3). In periodontal tissues, up-regulation of Delta-1 ligands during osteogenic differentiation of human periodontal ligament cells was reported (4). However, evidence for different Notch ligands in control of periodontal ligament cell function was lacking. The **aim of this study was to evaluate the ability to control differentiation of human periodontal ligament-derived stem cells (HPDL-SC) using modified surfaces containing affinity immobilized Notch ligands.**

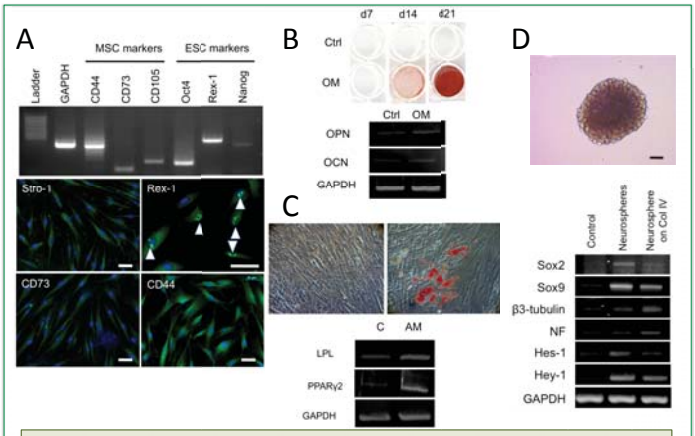


**Fig. 1 Cells isolated from human periodontal ligament demonstrated stem cell-like characteristics.**

Cells isolated from human periodontal ligament (HPDLs) exhibited fibroblast-like morphology. Cells were able to proliferate in vitro with doubling time at 38±2.02 h. Colony forming unit were observed as early as 7 days in culture and the average colony was 39±14.78 at 14 days in culture. These cells were expressed both embryonic and mesenchymal stem cell markers in both mRNA and protein levels **(A)**. HPDLs were able to differentiate into osteogenic, adipogenic and neurogenic lineage. Upon culture cells in osteogenic medium, mineralization deposition was dramatically noted at 14 and 21 days after culture. The increase of alkaline phosphatase enzymatic activity was also observed (data not shown). In addition, the expression of osteogenic mRNA markers; OPN and OCN, was upregulated **(B)**. For adipogenic differentiation, intracellular lipid accumulation was noted in those cells maintained in adipogenic medium as demonstrating by Oil red O staining. Correspondingly, adipogenic differentiation mRNA marker genes (LPL and PPAR$\gamma$2) were markedly increased **(C)**. Further, neurogenic potential of these cells was examined using neurosphere formation assay. These spheres were expressed higher Sox2, Sox9, $\beta$3-tubulin and NF mRNA levels compared to the control, implying the neurogenic differentiation of these cells **(D)**. *Together, these results suggest stem cell-like properties of HPDLs.*

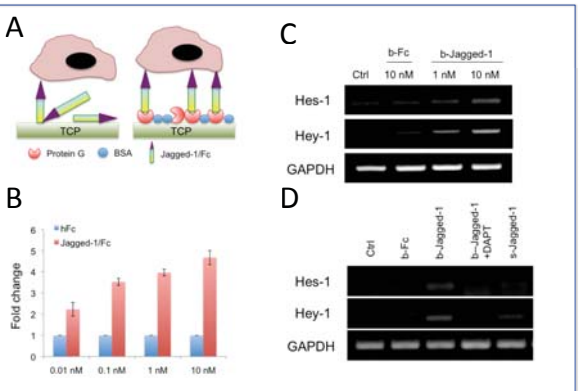


**Fig. 4 Oriented surface-bound Notch ligands stimulated Notch target genes.**

To further determine a participation of Notch signaling in neuronal differentiation of HPDLs, the effect of Notch signaling activation using an indirect affinity immobilization of Notch ligand, Jagged-1, on the surface was investigated in HPDLs. Recombinant protein G was used to immobilize Jagged-1/Fc fusion protein on the surface. This method obtained the oriented surface-bound Jagged-1 for the study **(A)**. An antibody-sandwich ELISA was used to confirmed the immobilized Jagged-1 on the surface. The results illustrated the dose dependent levels of bound Jagged-1 on the surface. The immobilized Fc in equal amount was used as the control **(B)**. The effectiveness of bound Jagged-1 surface was determined by the expression of Notch target genes; Hes-1 and Hey-1. HPDLs seeded on bound Jagged-1 surface exhibited higher mRNA expression of Hes-1 and Hey-1 compared to untreated surface and bound Fc surface. The expression of these target genes was dose dependent on the concentration of Jagged-1 **(C)**. Upregulation of these genes was attenuated after treatment with $\gamma$-secreatase inhibitor, DAPT. In addition, the levels of Hes-1 and Hey-1 mRNA expression was lower upon exposure of HPDLs to soluble Jagged-1 compared to surface-bound Jagged-1 **(D)**. *Together, these results suggest that the activity and potency of surface-bound Jagged-1 occur through Notch signaling pathway.*

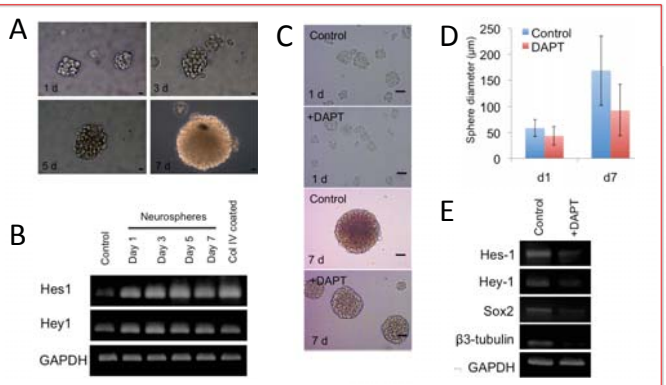


**Fig.2 Notch Signaling involved in neuronal differentiation of HPDLs.**

The sphere formation was observed as early as 1 day after culture cells in neurogenic medium. The size and cellular density of the spheres were increased at 3 and 7 days compared to 1 day in culture, suggesting the proliferation ability of the cells in spheres **(A)**. To determine a participation of Notch signaling in neuronal differentiation of HPDLs, mRNA expression of Notch target genes; Hes-1 and Hey-1, was investigated. Hes-1 and Hey-1 mRNA levels were higher in cells cultured in neurogenic medium both in floating neurosphere and adherent neurosphere-derived single cells on collagen type IV coated dishes **(B)**. The neurospheres treated with a $\gamma$-secretase inhibitor; DAPT, had relatively smaller in size but not significant compared to the control **(C and D)**. In addition, DAPT was able to decrease Hes-1 and Hey-1 mRNA expression, confirming the inhibition of Notch signaling. Correspondingly, Sox2 and $\beta$3-tubulin mRNA levels were decreased by treatment the neurospheres with DAPT **(E)**. *Together, the data imply the involvement of Notch signaling in neuronal differentiation of HPDLs.*

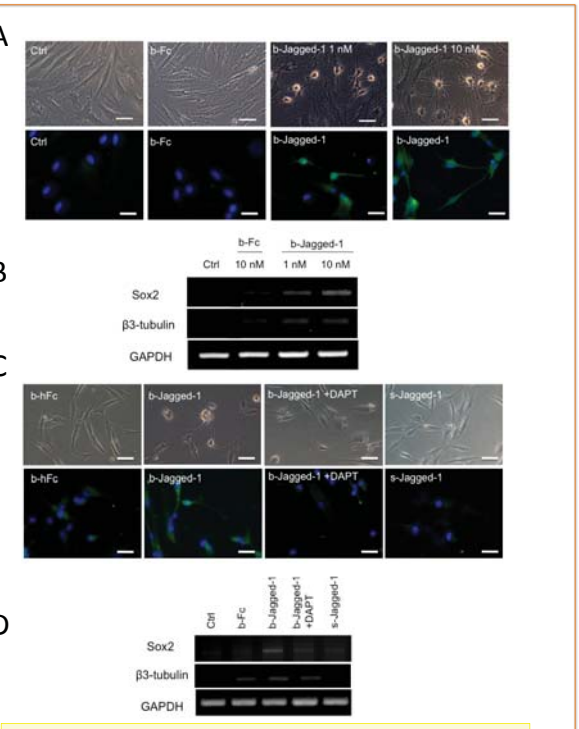


**Fig. 5 Surface-bound Jagged-1 initiated neuronal differentiation by HPDLs.**

Upon seeded HPDLs on surface-bound Jagged-1 surface, the cells exhibited multiple long and thin neurite-like extensions in both 1 nM and 10 nM concentration of Jagged-1. On the contrary, HPDLs on untreated surface and bound Fc surface appeared fibroblast-like morphology **(A)**. An increase in the expression of neurogenic mRNA markers; Sox2 and $\beta$3-tubulin, was observed in cells seeded on surface-bound Jagged-1 **(B)**. Pretreatment of the cells with DAPT prior to seeding on the Jagged-1 modified surface blocked development of the neurite like morphology. In addition, the cells treated with soluble Jagged-1 displayed similar morphology to those on untreated surface and bound Fc surface **(C)**. Sox2 and $\beta$3-tubulin mRNA expression were lower on DAPT pretreated cells on surface-bound Jagged-1 and soluble Jagged-1 treated cells compared to those on surface-bound Jagged-1 surface **(D)**.

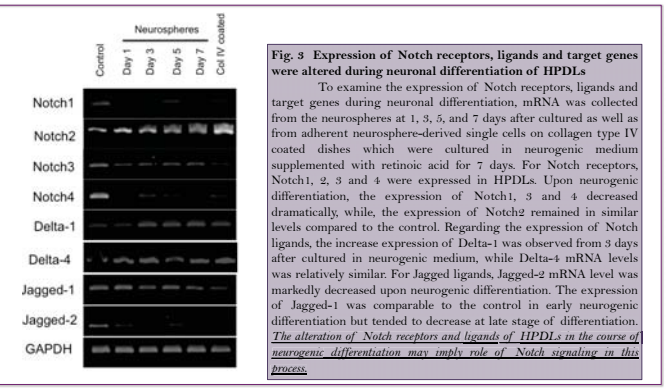


**Fig. 3 Expression of Notch receptors, ligands and target genes were altered during neuronal differentiation of HPDLs**

To examine the expression of Notch receptors, ligands and target genes during neuronal differentiation, mRNA was collected from the neurospheres at 1, 3, 5, and 7 days after cultured as well as from adherent neurosphere-derived single cells on collagen type IV coated dishes which were cultured in neurogenic medium supplemented with retinoic acid for 7 days. For Notch receptors, Notch1, 2, 3 and 4 were expressed in HPDLs. Upon neurogenic differentiation, the expression of Notch1, 3 and 4 decreased dramatically, while, the expression of Notch2 remained in similar levels compared to the control. Regarding the expression of Notch ligands, the increase expression of Delta-1 was observed from 3 days after cultured in neurogenic medium, while Delta-4 mRNA levels was relatively similar. For Jagged ligands, Jagged-2 mRNA level was markedly decreased upon neurogenic differentiation. The expression of Jagged-1 was comparable to the control in early neurogenic differentiation but tended to decrease at late stage of differentiation. *The alteration of Notch receptors and ligands of HPDLs in the course of neurogenic differentiation may imply role of Notch signaling in this process.*

## Conclusions

Indirect affinity immobilization of Jagged-1 on a material surface could direct HPDL-SC differentiation toward a neurogenic lineage. Thus, an alteration of biomaterial interface using Notch ligands illustrates a promising system to control stem cell differentiation.

**Methods**

Surface-bound Notch ligand, Jagged-1, was fabricated by an indirect affinity immobilization method. The present of Jagged-1 protein on surface was confirmed using enzyme-linked immunosorbent assay. The mRNA expression was analyzed using reverse transcriptase polymerase chain reaction. $\beta$3-tubulin protein expression was determined using immunohistochemical staining.


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