RESEARCH REPORTS
Biological

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Inhibition of Human Dental Pulp Stem Cell Differentiation by Notch Signaling

ABSTRACT

Notch signaling plays a critical role in development and cell fate specification. Notch receptors and ligands have been found to be expressed in dental epithelium or mesenchyme in the developing tooth, suggesting that Notch signaling may regulate odontogenesis. Post-natal human dental pulp stem cells (DPSCs) isolated from the dental pulp have characteristics of mesenchymal stem cells and can differentiate into odontoblasts. In this study, we examined whether Notch signaling regulated the odontoblastic differentiation of DPSCs. We found that over-expression of the Notch ligand, Jagged-1, activated the Notch signaling pathway in DPSCs. Jagged-1 inhibited the odontoblastic differentiation of DPSCs *in vitro*. Jagged-1-expressing DPSCs could not form mineralized tissues *in vivo*. Moreover, over-expression of the constitutively activated Notch1 intracellular domain (Notch-ICD) also inhibited odontoblastic differentiation of DPSCs. Taken together, our results demonstrate that Notch signaling can inhibit the odontoblastic differentiation of DPSCs.

KEY WORDS: Notch, dental pulp stem cells, differentiation, tooth development, odontoblasts.

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INTRODUCTION

The Notch signaling pathway is evolutionarily conserved in vertebrates and invertebrates and plays a critical role in development and cell fate specification (Shawber and Kitajewski, 2004; Rehman and Wang, 2006). There are 4 mammalian Notch receptor genes (Notch1-Notch4) and 5 ligands (Jagged1, Jagged2, Delta-like [DLL] 1, DLL3, and DLL4). They contain single-pass transmembrane domains and are expressed on the cell surface. The Notch receptor is activated through direct interaction with membrane-bound ligands, and Notch signaling is important in mediating communication between adjacent cells expressing either the receptors or ligands (Shawber and Kitajewski, 2004). The Notch receptor contains extracellular and intracellular domains that are synthesized as a single polypeptide. Upon receptor-ligand binding, the intracellular domain of the Notch receptor (NICD) is proteolytically cleaved by γ-secretase and translocates to the nucleus, where it binds to transcription factor CSL [**C**-promoter binding factor-1 (CBF-1), **S**uppressor of Hairless (Su[H]), **L**AG-1], also known as mammalian recombination signal-binding protein-Jκ (RBP-Jκ). Subsequently, the Notch target genes are induced (Kato *et al*., 1997; Zeng *et al.*, 2005).

The Notch receptors and ligands have been found to be expressed during tooth development. Jagged-1 has been found to be expressed at sites of epithelial-mesenchymal interactions in the developing teeth (Mitsiadis *et al.*, 1997). Epithelium induced Jagged-1 expression in mesenchyme, while mesenchyme-derived signals inhibited Jagged-1 expression in epithelial cells. Interestingly, fibroblast growth factor (FGF)-4, but not bone morphogenetic proteins (BMPs), induced Jagged-1 in dental mesenchyme. Also, it has been found that Delta-1 was expressed in the epithelium-derived ameloblasts and the mesenchyme-derived odontoblasts. Notch1, Notch2, and Notch3 were detected in dental epithelial and mesenchymal cells (Mitsiadis *et al.*, 1998). Stem cells isolated from mouse incisors have been found to express Notch1 (Harada *et al.*, 1999). FGF signaling from the mesenchyme could regulate the Notch signaling pathway in dental epithelial stem cells *via* stimulation of lunatic fringe expression. It has been demonstrated that precursor cells from human dental follicle expressed Notch1 (Morsczeck *et al.*, 2005). These precursor cells contained stem cells, which can differentiate into cementoblasts, periodontal ligament cells, and osteoblasts. It has been reported that Notch2 was induced in odontoblasts and subodontoblasts during dentin repair (Mitsiadis *et al.*, 2003). Transforming growth factor beta-1, which stimulated odontoblast differentiation and hard tissue formation after dental injury, inhibited Notch2 expression in cultured human dental slices. Interestingly, studies have found that Notch signaling was activated in response to injury and associated with the differentiation of pulp cells into perivascular cells and odontoblasts (Lovschall *et al.*, 2005). These investigators observed that the expression of Notch1, Notch2, and Notch3 was induced in pulp tissues after

the pulp capping of adult first upper rat molars. Taken together, these observations suggest that Notch signaling is an important element in dental physiological and pathogenic conditions. However, the functional role of Notch signaling in differentiation of dental epithelial and mesenchymal cells is not clear.

DPSCs are unique mesenchymal stem cells, which were isolated from human dental pulp tissues (Gronthos *et al.*, 2000). DPSCs have the potential to differentiate into odontoblasts, adipocytes, and neural-like cells *in vitro*. DPSCs differentiated into odontoblasts and formed calcified nodules *in vitro* under mineralization-inducing conditions. *In vivo* transplantation studies have found that DPSCs were capable of forming dentin-pulp-like tissues (Gronthos *et al.*, 2000, 2002; Shi and Gronthos, 2003). Since the Notch receptors and/or ligands have been found to be expressed in the dental mesenchyme and to be induced during dental pulp injury, in this study, we examined whether Notch signaling regulated the odontoblastic differentiation of DPSCs.

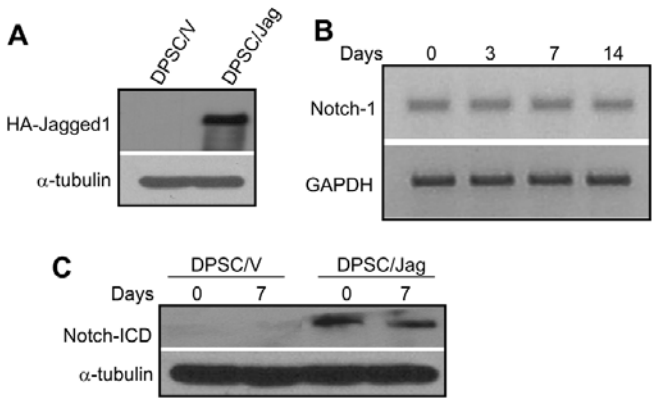

Figure 1. Generation of Jagged-1-expressing DPSCs. **(A)** DPSCs were stably infected with retroviruses expressing HA-Jagged-1 or control vector. Fifty-μg aliquots of protein extracts isolated from both DPSC/V and DPSC/Jag cells were probed with monoclonal antibodies against HA. **(B)** DPSCs expressed Notch1. DPSCs were induced to differentiate for 0, 3, 7, and 14 days, and total RNA was isolated. Notch mRNA was determined by RT-PCR. GAPDH was used as internal control. **(C)** Both DPSC/V and DPSC/Jag cells were grown in differentiation-inducing media for 0 and 7 days. Fifty-μg aliquots of protein extracts from these cells were probed with monoclonal antibodies against Notch1-ICD.

MATERIALS & METHODS

Cell Cultures and Retroviral Infection

DPSCs were isolated and characterized as described previously (Gronthos *et al.*, 2000). Tissues were obtained at the Dental Clinic of the National Institute of Dental and Craniofacial Research (Bethesda, MD, USA) under approved guidelines set by the US National Institutes of Health Office of Human Subjects Research and the University of Southern California IRB, with informed donor consent. Cells were grown in a humidified 5% CO_2 incubator at 37°C in alpha modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Invitrogen). To prepare retroviruses for cell infection, we co-transfected 293T cells with the retroviral vector pHytc-HA-Jagged-1, pQNCII-HA-Notch1-ICD, or control vector and the packaging vectors, using the calcium phosphate method. Forty-eight hrs after transfection, supernatants were harvested and filtered with 0.45-μM filters for the removal of cell debris. For viral infection, DPSCs were plated overnight and then infected with retroviruses in the presence of polybrene (6 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 6 hrs. Thirty-six hrs after infection, cells were selected with hygromycin (500 μg/mL) for 1 wk. The resistant clones were pooled and subjected to Western blot analysis.

Western Blot Analysis

Cells underwent lysis in RIPA buffer (10 mM Tris-HCL, 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1:100 proteinase inhibitor cocktail, 50 mM β-glycerophosphate, 50 mM sodium fluoride). The samples were separated on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane by a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were treated with 5% milk for 2 hrs and then incubated with primary antibodies overnight. The immunocomplexes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, Madison, WI, USA) and visualized with SuperSignal reagents (Pierce, Rockford, IL, USA). Primary antibodies were purchased from the following commercial sources: monoclonal antibodies against Notch1-ICD (1:500; University of Iowa, Iowa City, USA); monoclonal antibodies against HA epitope (1:1000; Covance, Princeton, NJ, USA); polyclonal antibodies against ALP and dentin sialoprotein (DSP) (NIDCR/NIH, Bethesda, MD, USA); and monoclonal antibodies against α-tubulin (1:7500; Sigma-Aldrich, St. Louis, MO, USA).

Alkaline Phosphatase and Alizarin Red Staining and Reverse-Transcriptase/Polymerase Chainreaction (RT-PCR)

DPSCs were grown in mineralization-inducing media containing 100 μM/mL ascorbic acid, 2 mM β-glycerophosphate, and 10 nM dexamethasone. For alkaline phosphatase (ALP) staining, 1 or 2 wks after induction, cells were fixed with 4% paraformaldehyde and incubated with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue BB dissolved in 0.1 M Tris buffer (pH 9.3). ALP activity assay was performed with an ALP kit according to the manufacturer's protocol (Sigma-Aldrich), and ALP activities were normalized based on protein concentrations. For the detection of mineralization, cells were induced for 2 to 3 wks, fixed with 4% paraformaldehyde, and stained with 2% Alizarin red (Sigma-Aldrich). For quantitative determination of calcium mineral, Alizarin red was de-stained with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 30 min at room temperature. The concentration was determined by absorbance measurement at 562 nm on a multiplate reader, with a standard calcium curve in the same solution. The final calcium level in each group was normalized with the total protein concentrations prepared from a duplicate plate (Stanford *et al.*, 1995). Total RNA were isolated from DPSCs with Trizol reagents (Invitrogen, Carlsbad, CA, USA). The primers for Notch1 are: forward, 5′-GGACCTCAT CAACTCACACG-3′; reverse, 5′-TTCTTCAGGAGCACAA CTGC-3′. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are: forward, 5′- GATCATCAGCAATGCC TCCT-3′; reverse, 5′-ACCTGGTGCTCAGTGTAGCC-3′.

Transplantation in Nude Mice

Approximately 4.0 x 10^6 of DPSC/Jag or DPSC/V cells were mixed with 40 mg of hydroxyapatite/tricalcium phosphate

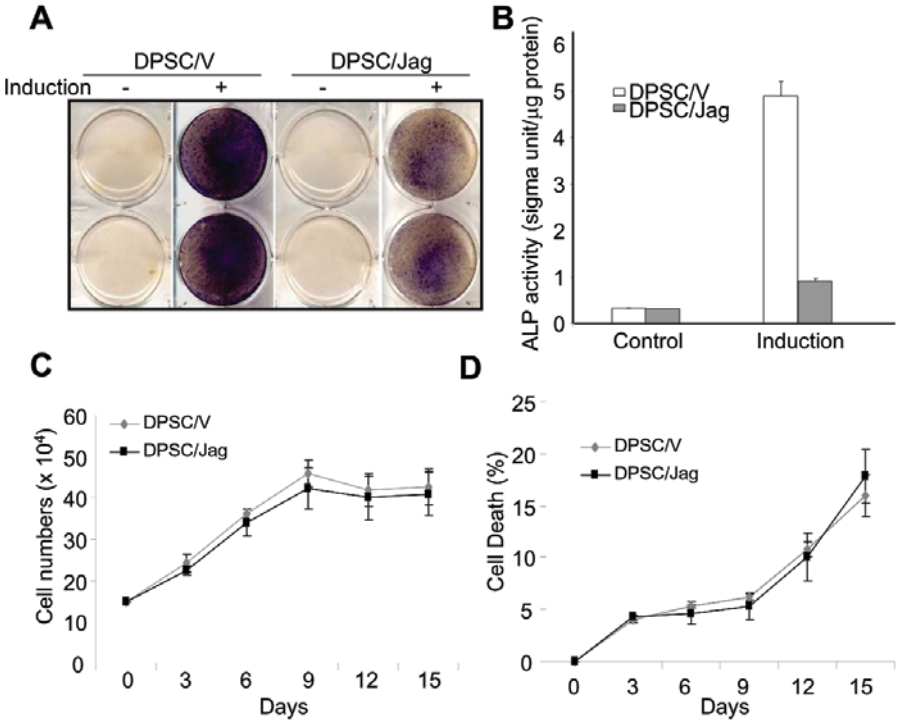

Figure 2. The activation of Notch signaling by Jagged-1 inhibits ALP activity in DPSCs. **(A)** Both DPSC/V and DPSC/Jag cells were treated with differentiation-inducing media for 2 wks. ALP activity was stained with 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue BB. **(B)** ALP activity assay was determined with an ALP assay kit according to the manufacturer's instructions. **(C)** Over-expression of Jagged-1 did not affect cell proliferation. Both DPSC/V and DPSC/Jag cells were treated with differentiation-inducing media for the indicated times. Cell numbers were counted. **(D)** Over-expression of Jagged-1 did not affect cell survival. Cell viability was determined with the Trypan blue exclusion assay.

(HA/TCP) ceramic particles (Zimmer Inc., Warsaw, IN, USA) and then transplanted subcutaneously into the dorsal surfaces of 10-week-old immunocompromised beige mice (bg-nu/nu-xid, Harlan Sprague-Dawley, Indianapolis, IN, USA) as previously described (Gronthos *et al.*, 2000). These procedures were performed in accordance with specifications of an approved animal protocol. Eight wks after transplantation, the transplants were harvested, fixed with 10% formalin, decalcified with buffered 10% EDTA (pH 8.0), and then embedded in paraffin. Sections were deparaffinized, hydrated, and stained with H&E.

RESULTS

To investigate whether the Notch signaling pathway modulated the odontoblastic differentiation of DPSCs, we transduced DPSCs with retroviruses expressing the Notch ligand Jagged-1 or control vector. To avoid clonal variation, we pooled resistant clones upon antibiotic selection for 10 days. DPSCs expressing HA-tagged Jagged-1 proteins (DPSC/Jag) were generated as determined by Western blot analysis (Fig. 1A). Jagged-1-expressing cells can bind to the Notch receptor of neighboring cells to activate Notch signaling. Since DPSCs expressed the Notch1 receptor (Fig. 1B), the over-expression of Jagged-1 in DPSCs should be able to activate the Notch signaling pathway *via* cell-to-cell contact. Both DPSC/Jag and DPSC/V cells were grown to over 80% confluence, allowing Jagged-1 to bind to the endogenous Notch1 receptor on adjacent cells. Upon receptor-ligand binding, the full-length endogenous Notch1 receptor was cleaved and liberated NICD to the nucleus to activate transcription. Western blot analysis revealed that the cleaved NICD was detected in DPSC/Jag cells, but not in control cells DPSC/V (Fig. 1C), suggesting that the over-expression of Jagged-1 activated Notch signaling in DPSCs.

Previous studies demonstrated that DPSCs could differentiate into odontoblast-like cells and form mineralized nodules *in vitro* when they were grown in differentiation-inducing medium containing ascorbic acid, β-glycerophosphate, and dexamethasone (Gronthos *et al.*, 2000). To examine whether the Notch signaling pathway modulated the odontoblastic differentiation of DPSCs, we treated both DPSC/Jag and DPSC/V cells with differentiation-inducing medium. One or 2 wks after induction, we examined the early differentiation marker ALP activity in these cells. Compared with control DPSC/V cells, ALP activity was significantly inhibited in DPSC/Jag cells, as determined by ALP staining or ALP activity assay (Figs. 2A, 2B). Importantly, the inhibition of ALP was unlikely due to the induction of cell death or the inhibition of cell proliferation by Jagged-1 in DPSCs (Figs. 2C, 2D).

Subsequently, we also determined whether Notch signaling inhibited DPSC mineralization *in vitro*. Calcified nodule formation in DPSC/Jag cells was significantly suppressed compared with that in DPSC/V cells after 3 wks of induction, as determined by Alizarin red staining (Fig. 3A). We also quantified calcium mineral and found that its level was significantly lower in DPSC/Jag cells than in DPSC/V cells (Fig. 3B). In addition to mineralization, we also examined whether the activation of Notch signaling by Jagged-1 inhibited the matrix protein formation during the late stage of odontoblast differentiation. DPSCs expressed dentin sialoprotein (DSP), which plays a critical role in dentin formation and tooth development (Gronthos *et al.*, 2000; Shi and Gronthos, 2003). Interestingly, we also observed that the basal level of DSP was significantly inhibited in DPSC/Jag cells. While DSP was modestly induced upon the induction of differentiation in DPSC/V cells, DSP expression was significantly inhibited in DPSC/Jag cells (Fig. 3C). We also transplanted both DPSC/V and DPSC/Jag cells into nude mice. Two mos after transplantation, the transplants were harvested and subjected to histological analysis. DPSC/V cells formed mineralized tissues, similar to previous results (Fig. 3D; Gronthos *et al.*, 2000). In contrast, DPSC/Jag cells did not generate any mineralized tissues *in vivo*.

Since Notch signaling could be bidirectional (LaVoie and Selkoe, 2003; Hiratochi *et al.*, 2007), to further confirm our

results, we also examined whether over-expression of the constitutively activated Notch-ICD could inhibit DPSC differentiation. Western blot analysis showed that we were able to stably express Notch-ICD in DPSCs (Fig. 4A). Compared with control DPSC/V cells, ALP activity was significantly inhibited in DPSCs expressing Notch-ICD (DPSC/ICD), as determined by ALP staining or ALP activity assay upon the induction of differentiation (Figs. 4B, 4C). Moreover, Notch-ICD also inhibited calcified nodule formation (Figs. 4D, 4E). Importantly, over-expression of Notch-ICD did not affect cell death or proliferation of DPSCs during the induction of differentiation (data not shown).

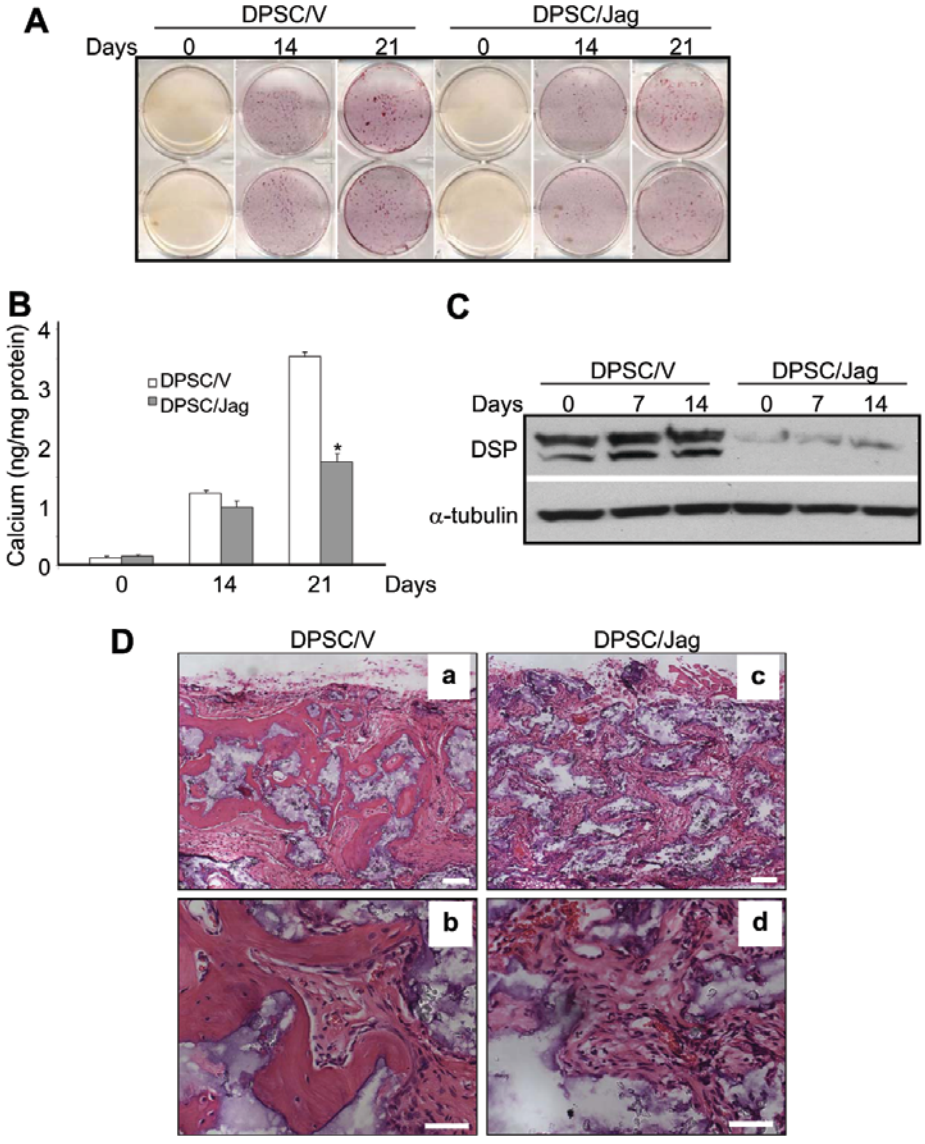

Figure 3. The activation of Notch signaling by Jagged-1 inhibits the odontoblastic differentiation of DPSCs *in vitro*. **(A)** Both DPSC/V and DPSC/Jag cells were treated with differentiation-inducing media for 0, 2, and 3 wks. Cells were fixed with 4% paraformaldehyde and stained with 2% Alizarin red. **(B)** Cells were de-stained with 10% cetylpyridinium chloride in sodium phosphate buffer. The concentration was determined by absorbance measurement at 562 nm on a multiplate reader. We performed Student's *t* test to determine statistical significance. *$p < 0.01$. **(C)** Both DPSC/V and DPSC/Jag cells were treated with differentiation-inducing media for 0, 1, and 2 wks. Aliquots (50 μg) of protein extracts were probed with polyclonal antibodies against DSP. For loading control, the membrane was stripped and re-probed with monoclonal antibodies against α-tubulin. **(D)** The activation of Notch signaling by Jagged-1 inhibits the formation of mineralized tissues by DPSCs *in vivo*. Both DPSC/V and DPSC/Jag cells were transplanted subcutaneously into the dorsal surfaces of 10-week-old immunocompromised beige mice. Eight wks after transplantation, the transplants were sectioned and stained with H&E. The transplants of DPSC/V (**a** and **b**), but not of DPSC/Jag (**c** and **d**), generated mineralized tissues in mice. Scale bar, 100 μm.

DISCUSSION

Our results presented here are the first demonstration that the activation of Notch signaling by Jagged-1 inhibited the odontoblastic differentiation of DPSCs *in vitro* and *in vivo*. It is known that Notch signaling plays a critical role in the maintenance of the hematopoietic stem cell niche (Shawber and Kitajewski, 2004). DPSCs were originally isolated from human dental pulp and expressed multiple mesenchymal stem cell markers. Currently, the stem cell properties of DPSCs have not been fully characterized. How the dental pulp microenvironment supports and sustains DPSCs *in vivo* is unknown. Although we cannot conclude that Notch signaling helps to maintain the stem cell niche of DPSCs, our results suggest that Notch signaling may play a critical role in keeping DPSCs in an undifferentiated state. According to our findings, the manipulation of Notch signaling may help us to expand these cells *in vitro* for tissue regeneration.

Recently, we have found that the bacterial by-product lipopoly-saccharide (LPS) could activate nuclear factor-kappa B in DPSCs, suggesting that DPSCs may play a role in immune responses (Chang *et al.*, 2005). Interestingly, the Notch ligand Jagged-1 has been found to be induced during dental pulp injury. According to our results, the induction of Jagged-1 expression may inhibit odontoblastic differentiation. The Notch receptors and ligands have been found to be expressed in osteoblasts and skeletal tissues. Consistent with our studies, early work found that the over-expression of NICD in mesenchymal C2C12 cells inhibited OCN expression (Nofziger *et al.*, 1999), suggesting that Notch signaling may inhibit osteoblast differentiation. Other investigators found that the over-expression of NICD in pre-osteoblastic MC3T3 cells and ST-2 mesenchymal cells inhibited osteoblast differentiation (Sciaudone *et al.*, 2003). They demonstrated that NICD inhibited the expression of type I

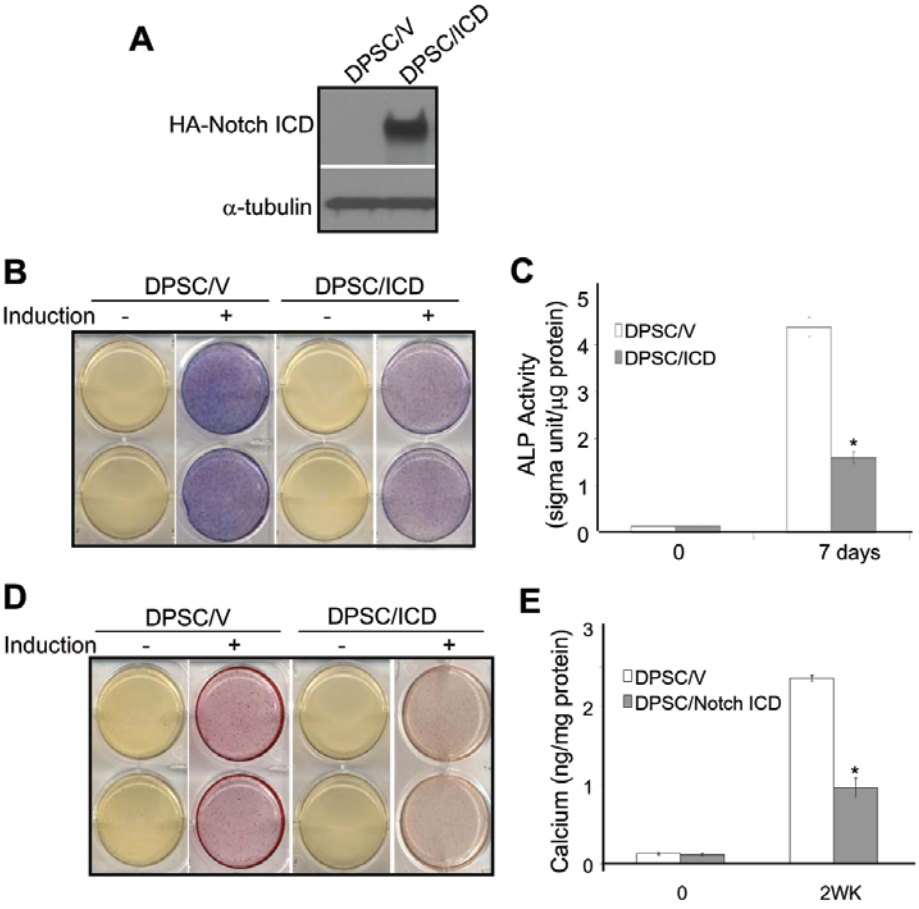

Figure 4. Notch-ICD inhibits DPSC differentiation. **(A)** DPSCs were stably infected with retroviruses expressing HA-Notch-ICD or control vector. **(B)** Both DPSC/V and DPSC/ICD cells were induced to differentiate for 7 days. ALP activity was stained with 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue BB. **(C)** ALP activity assay was determined with an ALP assay kit. **(D)** Both DPSC/V and DPSC/Jag cells were induced to differentiate for 2 wks and stained with 2% Alizarin red. **(E)** Cells were de-stained, and calcium concentration was determined by absorbance measurement at 562 nm in a multiplate reader. We performed Student's *t* test to determine statistical significance. *p < 0.01.

collagen, OCN, and ALP. In contrast to these studies and to our work reported here, Nobta *et al.* (2005) demonstrated that Notch-1, Delta1, and Jagged-1 were expressed in maturing osteoblast cells during bone regeneration. The forced expression of Jagged-1 or Delta1 enhanced osteoblast differentiation induced by BMPs. This contradictory result may be due to cell type and the stimuli used for inducing cell differentiation.

Currently, we do not know how Notch signaling inhibits the odontoblastic differentiation of DPSCs. It is known that the transcription factor Runx2 plays a critical role in odontoblastic differentiation and tooth development (Gaikwad *et al.*, 2001). Recently, it has been reported that Runx2 transcriptionally induced the expression of dentin sialophosphoprotein (DSPP), which encodes both DSP and dentin phosphoprotein (DPP) (Chen *et al.*, 2005). Since Notch signaling inhibited DSPP expression in DPSCs according to our results, it raises a possibility that Notch signaling may inhibit Runx2 transcriptional activities. Studies have demonstrated that Runx2 interacted with the Notch target gene Hes1 and the transcriptional co-repressor Groucho (McLarren *et al.*, 2001). Therefore, Notch signaling may induce Hes1 to inhibit Runx2-dependent gene transcription in DPSCs. In future studies, it will be interesting to examine whether Notch signaling and Runx2 cross-talk in DPSCs during the induction of odontoblastic differentiation.

ACKNOWLEDGMENTS

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