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Crosstalk between miR-34a and Notch Signaling Promotes Differentiation in Apical Papilla Stem Cells (SCAPs)

ABSTRACT

Stem cells from the apical papilla (SCAPs) are important for the formation and regeneration of root dentin. Here, we examined the expression of Notch signaling components in SCAPs and investigated crosstalk between microRNA miR-34a and Notch signaling during cell differentiation. We found that human SCAPs express NOTCH2, NOTCH3, JAG2, DLL3, and HES1, and we tested the relationship between Notch signaling and both cell differentiation and miR-34a expression. NOTCH activation in SCAPs inhibited cell differentiation and up-regulated the expression of miR-34a, whereas miR-34a inhibited Notch signaling in SCAPs by directly targeting the 3′UTR of *NOTCH2* and *HES1* mRNA and suppressing the expression of NOTCH2, N2ICD, and HES1. DSPP, RUNX2, OSX, and OCN expression was consequently up-regulated. Thus, Notch signaling in human SCAPs plays a vital role in maintenance of these cells. miR-34a interacts with Notch signaling and promotes both odontogenic and osteogenic differentiation of SCAPs.

KEY WORDS: microRNA, stem cells from apical papilla, tooth development, epigenetic, cell fate determination, tooth regeneration.

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INTRODUCTION

Stem cells from the apical papilla (SCAPs) are mesenchymal stem/progenitor cells residing in the root apex of immature permanent teeth. These postnatal stem cells contribute to the formation of developing radicular pulp as well as the odontoblasts that are responsible for root dentinogenesis. They play a vital role in pulp healing and regeneration. SCAPs can also be used in tissue engineering, an example of which is stimulation of SCAPs to establish root formation in immature teeth (Banchs and Trope, 2004; Chueh and Huang, 2006). Dentin-pulp-like tissues in the empty root canal space and bioengineered roots that can support a porcelain crown have been generated by utilizing SCAPs recombined with biological scaffolds *in vivo* (Sonoyama *et al*., 2006; Huang *et al*., 2009).

The Notch signaling pathway is evolutionarily conserved and controls fate decisions through cell-cell interactions during development and post-natal life. Notch receptors and their ligands Delta/Jagged are single-pass transmembrane proteins expressed on neighboring cells. Notch signaling is activated by ligand-receptor binding, which induces proteolytic cleavage of the intracellular domain of the Notch receptor (NICD). There are 2 possible proteolytic cleavage events: the first is catalyzed by ADAM-family metalloproteases, whereas the second is mediated by γ-secretase. DAPT is one of the major inhibitors of γ-secretase and thus has been widely used as an inhibitor of the Notch signaling pathway. The NICD translocates into the nucleus and binds to members of the CSL (CBF-1, Suppressor of Hairless, Lag-1) transcription factor family. Following NICD binding, CBF-1/RBP-JK becomes a transcriptional activator and induces downstream targets, such as hairy/enhancer of split (*HES*) genes, a family of basic helix-loop-helix (bHLH) transcription factors.

Evidence has been accumulating that Notch signaling plays a critical role in tooth repair and regeneration (Mitsiadis *et al*., 1995). During tooth development, complementary expression of the Delta/Jagged ligands and Notch receptors in adjacent epithelial and mesenchymal cells correlates with ameloblast and odontoblast differentiation. Moreover, NOTCH1 expression is found in the stem cell compartment in continuously growing rodent incisors, whereas it is mainly absent in mouse molars (Harada *et al*., 1999; Thesleff and Mikkola, 2002). NOTCH2 is expressed in odontoblasts and pre-odontoblasts during dentin repair (Mitsiadis *et al*., 2003). Hes1, the downstream effector of

Notch signaling, has been found in stellate reticulum cells and outer dental mesenchyme (Mustonen *et al*., 2002) and is considered important in the maintenance of stemness and cell fate determination in tooth development. Activation of Notch1/Jagged/Hes1 signaling increases proliferation of stratum intermedium cells and contributes to enamel formation (Harada *et al*., 2006). Inhibition of Hes1 results in decreased cell proliferation as well as massive apoptosis in the epithelial stem cell niche (Felszeghy *et al*., 2010). Because the Notch signaling pathway functions in a context-dependent manner, the mechanism of Notch signaling in tooth development and repair/regeneration remains incompletely understood.

Emerging evidence suggests that microRNAs (miRNAs) function as key regulators of various biological and pathologic processes (Xie *et al.*, 2011). Recently, it has been reported that transfection of miR-34a into glioma cells led to decreased expression of Notch-1, Notch-2, and CDK6 (Li *et al*., 2009), and that over-expression of miR-200b significantly inhibited Notch-1 expression (Wang *et al*., 2010). Previous studies suggested that miR-34a plays an important role in dentinogenesis by targeting *NOTCH1* and *BMP7* (Wan *et al*., 2012). Furthermore, Notch signaling and miR-34a interact in colon cancer stem cells (CCSCs) during self-renewal. miR-34a dampens Notch signaling and promotes asymmetric division, whereas low miR-34a levels retain plasticity in daughter cells (Bu *et al*., 2013). However, the underlying mechanism of the crosstalk between miR-34a and Notch signaling pathway in SCAPs is not known.

In the present study, we investigated the Notch signaling pathway in SCAPs and elucidated the crosstalk between Notch signaling and miR-34a in cell fate determination.

MATERIALS & METHODS

Ethics Statement

All human tissues were collected from patients (ages 12-20 yr) in the Dental Clinics at West China Hospital of Stomatology, Sichuan University under approved guidelines. The study and the consent procedure were approved by Ethical Committees of West China School of Stomatology, Sichuan University and the State Key Laboratory of Oral Diseases.

Cell Culture and Characterization

SCAPs in primary culture were generated from the root apex of immature teeth (third molars or premolars) as previously described (Appendix Fig. A) (Sonoyama *et al*., 2008). Cell-surface molecule characterization was carried out by immunofluorescence (Appendix Figs. B-F). Multi-lineage differentiation was characterized as described previously (Appendix Figs. G-J) (Sonoyama *et al*., 2008). RNA was purified and lineage-specific gene expression level was tested by real-time reverse-transcription polymerase chain reaction (qRT-PCR) before and after induction (Appendix Fig. K). (See Appendix for details.)

Total RNA of SCAPs was isolated and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA synthesis for messenger RNA was performed with the SuperScriptIII First-Strand Synthesis System (Life Technologies, Foster City, CA, USA). Products were amplified with primers for human Notch signaling pathway components. PCR conditions and primer design are included in the Appendix Table. The PCR products were separated on 1.5% agarose gels and photographed.

SCAPs were plated in 6-well plates containing embedded glass coverslips and grown until 70% confluent. Immunofluorescence was performed as described in the Appendix. Primary antibodies against NOTCH2, NOTCH3, JAG2, HES1 (all 1:100, Abcam, Cambridge, MA, USA), and DLL3 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

Functional Blockade

To test Notch signaling activity, we treated SCAPs for 72 hr with recombinant JAG1 protein at concentrations of 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL or with DAPT at 0.2 μM, 1 μM, 2 μM, 5 μM, and 10 μM. For rescue experiments, cells were treated with JAG1 for 7 days and then treated by DAPT for another 7 days. JAG1 and DAPT were added at 50 ng/mL and 10 μM, respectively. SCAPs cultured in basic cell culture medium were referred to as control. Cells treated by either JAG1 or DAPT alone for 14 days were also examined for interpretation of the rescue results. Meanwhile, we also added JAG1/DAPT for 7 days followed by stopping the treatment for another 7 days. JAG1 and DAPT together for 14 days were alternatively referred to as control of JAG1-14 days and DAPT-14 days. To verify the relationship between Notch signaling and cell differentiation in SCAPs, we cultured cells in mineralization medium for 14 days.

Oligonucleotide Transfection

miR-34a mimic and miR-34a inhibitor (anti-miR-34a, chemically modified antisense oligonucleotides designed to specifically target mature miR-34a) were synthesized by Ribobio, Guangzhou, China. Oligonucleotide transfection was performed as described previously (Wan *et al*., 2012) (see Appendix for details). Cells were harvested 72 hr after oligonucleotide transfection. The mRNA levels of Notch signaling molecules (*NOTCH2, NOTCH3, JAG2, DLL3, HES1*) and differentiation markers *DSPP, ALP, RUNX2, OSX, OCN*, and *OPN* were examined by qRT-PCR. Primers and probe sets included an endogenous *GAPDH* control and were purchased from Applied Biosystems (Foster City, CA, USA). miR-34a reverse transcription was performed with the TaqMan® microRNA Reverse Transcription Kit (Life Technologies) and the miR-34a specific RT primer. The reactions were performed as described previously (Wan *et al*., 2012) (see Appendix for details).

Western Blotting

Total cellular protein was extracted with the Reagent kit (KeyGEN, Nanjing, Jiangsu, China) after the same treatment of real-time qRT-PCR. Nuclear proteins such as HES1, N2ICD (NOTCH2 intercellular domain), and H3.1 were extracted with Nuclear and Cytoplasmic Extraction Reagents (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). Protein concentration was determined with the BCA protein assay

reagent (Beyotime Institute of Biotechnology) (see Appendix for details).

Alkaline Phosphatase and Alizarin Red Staining

To determine whether miR-34a promotes mineralization *in vitro*, we performed alkaline phosphatase (ALP) and alizarin red staining. Briefly, SCAPs were cultured in mineralization induction media plus oligonucleotide transfection. For alkaline phosphatase (ALP) staining, 2 wk 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); 2% Alizarin Red was stained to detect calcification.

Dual Luciferase Assays

Oligonucleotide pairs that contain *NOTCH2* and *HES1* miRNA target seed regions or mismatched sequences were ordered from Takara Biotechnology, Dalian, China (see the Appendix for details). For luciferase activity analysis, the double-stranded miR-34a mimic and mimic negative control were synthesized by Ribobio, Guangzhou, China. The final concentration of miR-34a mimic or mimic negative control and pmirGLO Vector constructs was 50 nM and 100 ng, respectively. For details of the dual luciferase assay, please refer to the Appendix.

Sequence Alignments and microRNA Target Prediction

The potential microRNA target sites within the 3′UTR of *NOTCH2* and *HES1* were identified by the Web-based bioinformatics databases Target Scan (http://www.targetscan.org/vert_42/), microRNA.org (http://www.microrna.org/microrna/home.do), and miRBase (http://microrna.sanger.ac.uk/sequences/). miRNA-mRNA hybridization structures and free energies between microRNA seed sequences and mRNA sequences were determined by RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/).

Statistical Analysis

All experiments were performed independently at least 3 times in triplicate. Numerical data are presented as mean ± SD. The difference between means was analyzed with one-way analysis of variance (ANOVA). Differences were considered significant when $p < .05$. All statistical analyses were done with the software SPSS13.0 (SPSS Inc., Chicago, IL, USA).

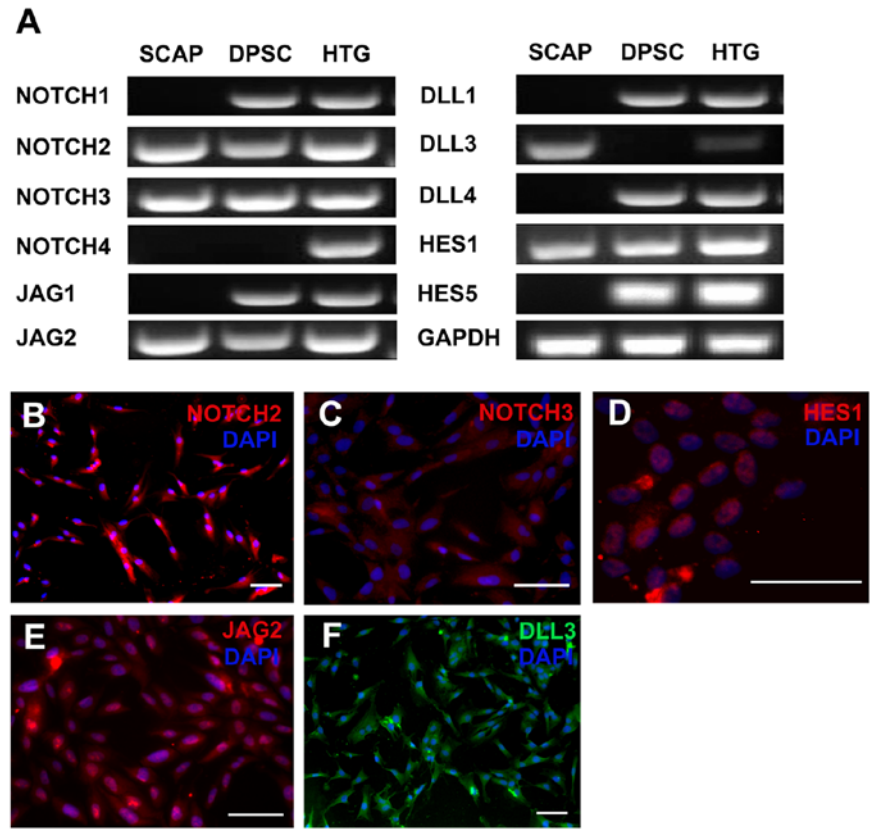

Figure 1. Determination of the Notch signaling components in SCAPs. **(A)** NOTCH2, NOTCH3, DLL3, JAG2, and HES1 were detected in SCAPs, whereas NOTCH1 and JAG1 were detected in DPSCs, and all of these components were detected in human tooth germs (SCAPs, stem cells from apical papilla; DPSCs, dental pulp stem cells; HTG, human tooth germ). B-F: Immunofluorescence staining. **(B)** NOTCH2. **(C)** NOTCH3. **(D)** HES1. **(E)** JAG2. **(F)** DLL3. Scale bar: 20 μm.

RESULTS

SCAPs Express *NOTCH2, NOTCH3, JAG2, DLL3*, and *HES1 in vitro*

We performed RT-PCR and found that, in cultured SCAPs, *NOTCH2, NOTCH3, JAG2, DLL3*, and *HES1* were expressed, whereas there was no *NOTCH1, NOTCH4, JAG1, DLL1*, or *DLL4* expression (Fig. 1A). Immunofluorescence confirmed the expression of these Notch signaling components (Figs. 1B-1F).

Notch Signaling in SCAPs Inhibits Cell Differentiation

To determine if the Notch signaling pathway is active in SCAPs, we examined *HES1*, a downstream Notch signaling target and key transcriptional effector of the pathway. The *HES1* mRNA expression level was up-regulated by recombinant JAG1 protein, most dramatically at a concentration of 50 ng/mL (Fig. 2A), and down-regulated by DAPT treatment, most significantly at 10 μM (Fig. 2B). *DSPP, ALP, RUNX2, OSX, OCN*, and *OPN* mRNA expression levels were down-regulated by JAG1 treatment and reversed by DAPT (Fig. 2C). After mineralization induction, mRNA expression levels of *JAG2, DLL3*, and *HES1*

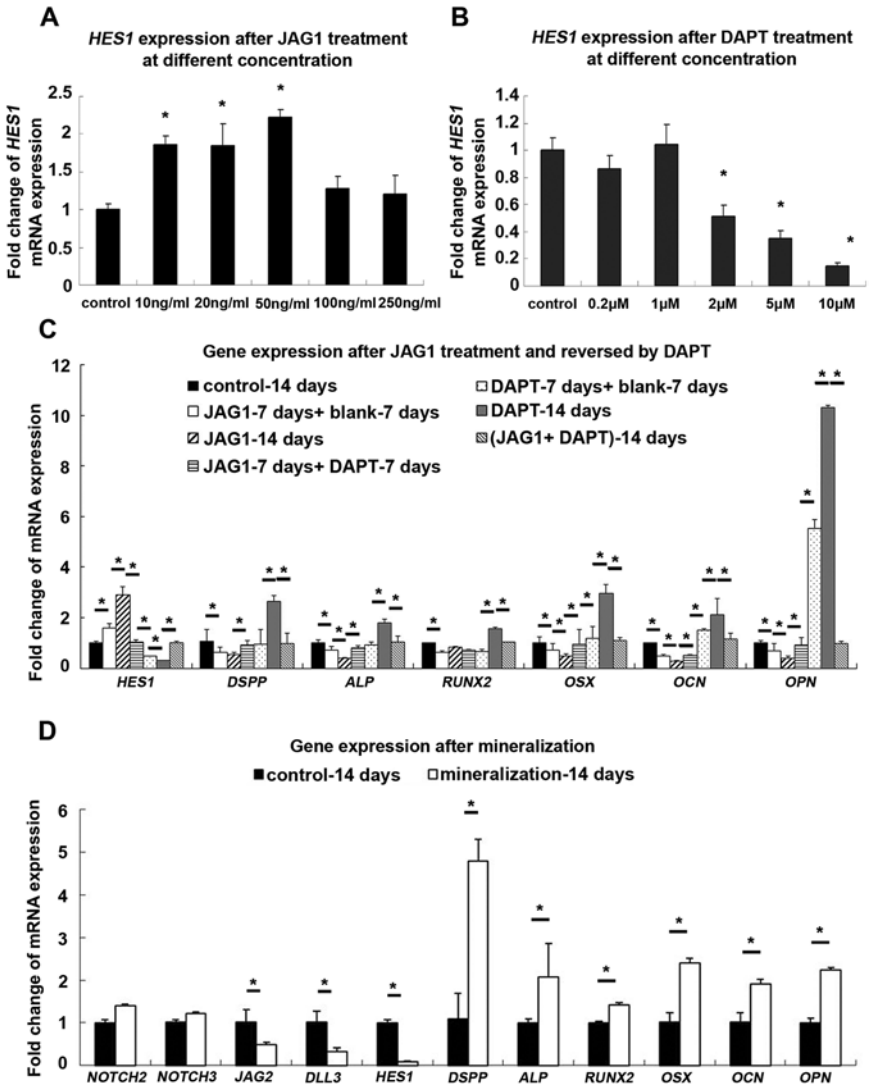

Figure 2. Notch signaling inhibits cell differentiation in SCAPs. (**A**) HES1 mRNA expression was up-regulated after JAG1 treatment for 72 hr (*$p < .05$). (**B**) HES1 mRNA expression was down-regulated after DAPT treatment for 72 hr (*$p < .05$). (**C**) Differentiation marker expression was down-regulated in the group with JAG1 treatment for 14 days, compared with that in the group treated with JAG1 for 7 days followed by DAPT treatment for another 7 days, while the group treated with DAPT for 14 days had the highest expression level (*$p < .05$). (**D**) JAG2, DLL3, and HES1 mRNA expression was down-regulated 14 days after mineralization, while differentiation genes were up-regulated (*$p < .05$).

were significantly down-regulated, and *DSPP, ALP, RUNX2, OSX, OCN*, and *OPN* were up-regulated in the cells (Fig. 2D).

Activation of Notch Signaling Promotes miR-34a Transcription

To study the relationship between miR-34a and Notch signaling, we detected miR-34a expression after treatment. miR-34a was significantly up-regulated after JAG1 treatment and down-regulated by DAPT treatment (Fig. 3A). The miR-34a expression level in SCAPs was decreased after mineralization, most significantly at 2 wks (Fig. 3B).

miR-34a Targets the *NOTCH2* and *HES1* 3′ UTR in SCAPs

After miR-34a mimic transfection, *NOTCH2* mRNA expression was down-regulated, while *HES1* and *DLL3* mRNA expression levels were up-regulated significantly (Fig. 3C). Western blotting showed that NOTCH2, N2ICD, and HES1 were all significantly down-regulated after miR-34a mimic transfection (Fig. 3E). Additionally, we detected up-regulation of both mRNA and protein expression levels of DSPP, RUNX2, OSX, and OCN in SCAPs (Figs. 3C, 3F). After mineralization, SCAPs transfected with miR-34a formed more calcified nodules and showed elevated alkaline phosphatase (ALP) staining compared with control (Figs. 3G, 3H). After miR-34a inhibitor transfection, the mRNA levels of *NOTCH2* and *HES1* were up-regulated (Fig. 3D), and Western blotting showed that NOTCH2, N2ICD, and HES1 protein levels were increased (Fig. 3E). This resulted in down-regulation of both mRNA and protein expression levels of DSPP, RUNX2, OSX, and OCN (Figs. 3D, 3F). The miR-34a expression level during mineralization duration was validated by qPCR and showed consistent effects of transfection through the treatment (Fig. 3I).

To test for a direct interaction between miR-34a and *NOTCH2* and *HES1*, we first located the seed sequences for miR-34a at the 3′UTR of *NOTCH2* and *HES1 mRNA* by TargetScan and miRBase (Figs. 4A, 4B). The oligonucleotide pairs region was synthesized and cloned into the luciferase reporter vector pmirGLO and co-transfected with the miR-34a or a scrambled miRNA negative control in 293T cells. We found that miR-34a effectively suppressed luciferase reporter activity. Furthermore, the mutations significantly impaired the activity of miR-34a, relieving repression of luciferase activity. Analysis of these data suggests that miR-34a directly binds to the 3′UTR of *NOTCH2* and *HES1*, thereby post-transcriptionally regulating mRNA levels (Figs. 4C, 4D).

DISCUSSION

The expression pattern and biological outcome of Notch signaling are context-dependent. JAG1, DLL1, NOTCH1, NOTCH2, and NOTCH3 are all expressed in dental epithelial and mesenchymal cells (Mitsiadis *et al.*, 1997). NOTCH1 has been found

to be expressed in stem/progenitor cells in several cell types in human and mouse teeth. These include human dental follicle cells (Harada *et al*., 1999; Morsczeck *et al*., 2005), human dental pulp stem cells (Ji *et al*., 2008), and stem cells isolated from mouse incisors (Felszeghy *et al*., 2010). In cultured SCAPs, we found that distinct components of the Notch signaling pathway were expressed, such as NOTCH2, NOTCH3, JAG2, and DLL3. In terms of functional effects, previous work has shown that activation of Notch signaling inhibited the odontoblastic differentiation of DPSCs *in vitro* and *in vivo* (Ji *et al*., 2008) and that overexpression of NICD inhibited OCN expression in osteoblastic lineage cells (Nofziger *et al*., 1999). Our results are consistent with these reports, since, in SCAPs, activation of Notch signaling by JAG1 inhibited *DSPP, ALP, RUNX2, OSX, OCN*, and *OPN* expression.

Recently, it has been reported that miRNAs play critical roles in the regulation of Notch signaling. Several miRNAs have been shown to have crosstalk with Notch signaling (Li *et al*., 2011; Poulton *et al*., 2011). Our previous study (Wan *et al*., 2012) analyzed miRNA expression in the human developing tooth germ (early bell and late bell stages), and we found that miR-34a expression varied significantly during tooth development. A recent study suggested that overexpression of miR-34a inhibited early commitment and late osteoblastic differentiation of hMSCs *in vitro*, whereas inhibition of miR-34a by anti-miR-34a enhanced these processes (Chen *et al*., 2013). It has also been found that 2 members of the miR-34 family—miR-34b and miR-34c—are highly enriched in osteoblasts, and miR-34s have been found to restrict bone formation by targeting a key regulator of osteoblast differentiation, *SATB2* (Ellies and Krumlauf, 2006; Wei *et al*., 2012). These studies indicated a functional role of miR-34a in odontogenesis and osteogenesis, and the function may be tissue- and cell-specific.

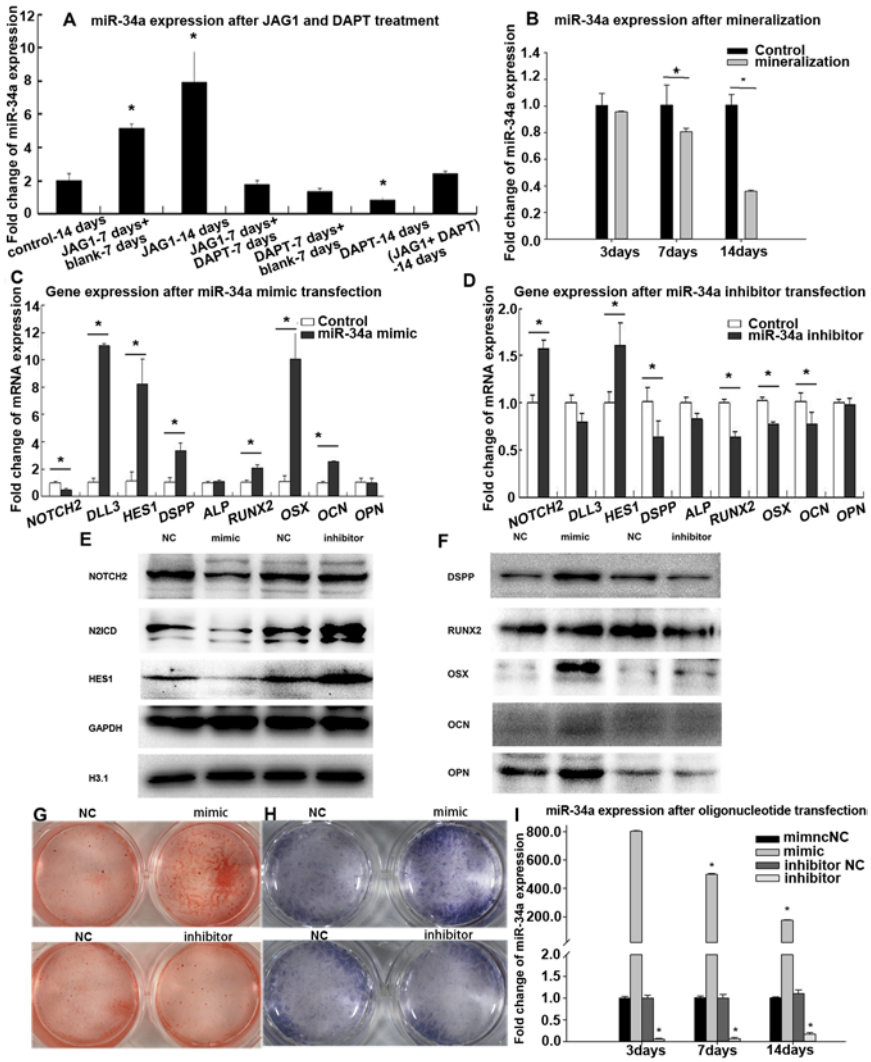

Figure 3. miR-34a crosstalk with Notch signaling pathway, promoting cell differentiation. **(A)** miR-34a transcription was up-regulated after Notch activation by JAG1 treatment, and down-regulated by DAPT treatment. **(B)** After mineralization, miR-34a transcription was down-regulated. **(C)** After miR-34a mimic transfection for 72 hr, the mRNA expression level of NOTCH2 was down-regulated while those of DLL3 and HES1 were up-regulated. DSPP, RUNX2, OSX, and OCN were up-regulated. **(D)** After miR-34a inhibitor transfection for 72 hr, the mRNA expression levels of NOTCH2 and HES1 were up-regulated, while that of DLL3 was down-regulated. DSPP, RUNX2, OSX, and OCN were down-regulated. **(E)** After miR-34a mimic transfection, NOTCH2, N2ICD, and HES1 protein levels were down-regulated, while, after miR-34a inhibitor transfection, NOTCH2, N2ICD, and HES1 protein levels were up-regulated. **(F)** After miR-34a mimic transfection, DSPP, RUNX2, OSX, OCN, and OPN protein levels were up-regulated, while, after miR-34a inhibitor transfection, DSPP, RUNX2, OSX, OCN, and OPN protein levels were up-regulated (NC: microRNA negative control). (G, H) SCAPs transfected with miR-34a mimic formed more calcified nodules **(G)** and showed elevated alkaline phosphatase (ALP) staining signals **(H)** compared with control. **(I)** miR-34a levels were determined in SCAPs transfected with miR-34a mimic negative control, miR-34a mimic, miR-34a inhibitor negative control, and miR-34a inhibitor for 3 days and then mineralization for 7 days and 14 days.

The present study indicated that Notch signaling activation led to up-regulation of miR-34a, whereas mineralization of SCAPs resulted in down-regulation of miR-34a expression (Figs. 3A, 3B). This was further investigated with miR-34a perturbation. These experiments showed that with miR-34a mimic transfection, differentiation markers such as DSPP, RUNX2, OSX, and OCN were significantly up-regulated, and *vice versa* with inhibitor transfection. Thus, miR-34a is a negative regulator of Notch signaling but a positive regulator of mineralization/cell differentiation. This notion was further confirmed by Alizarin red and ALP staining (Figs. 3G,

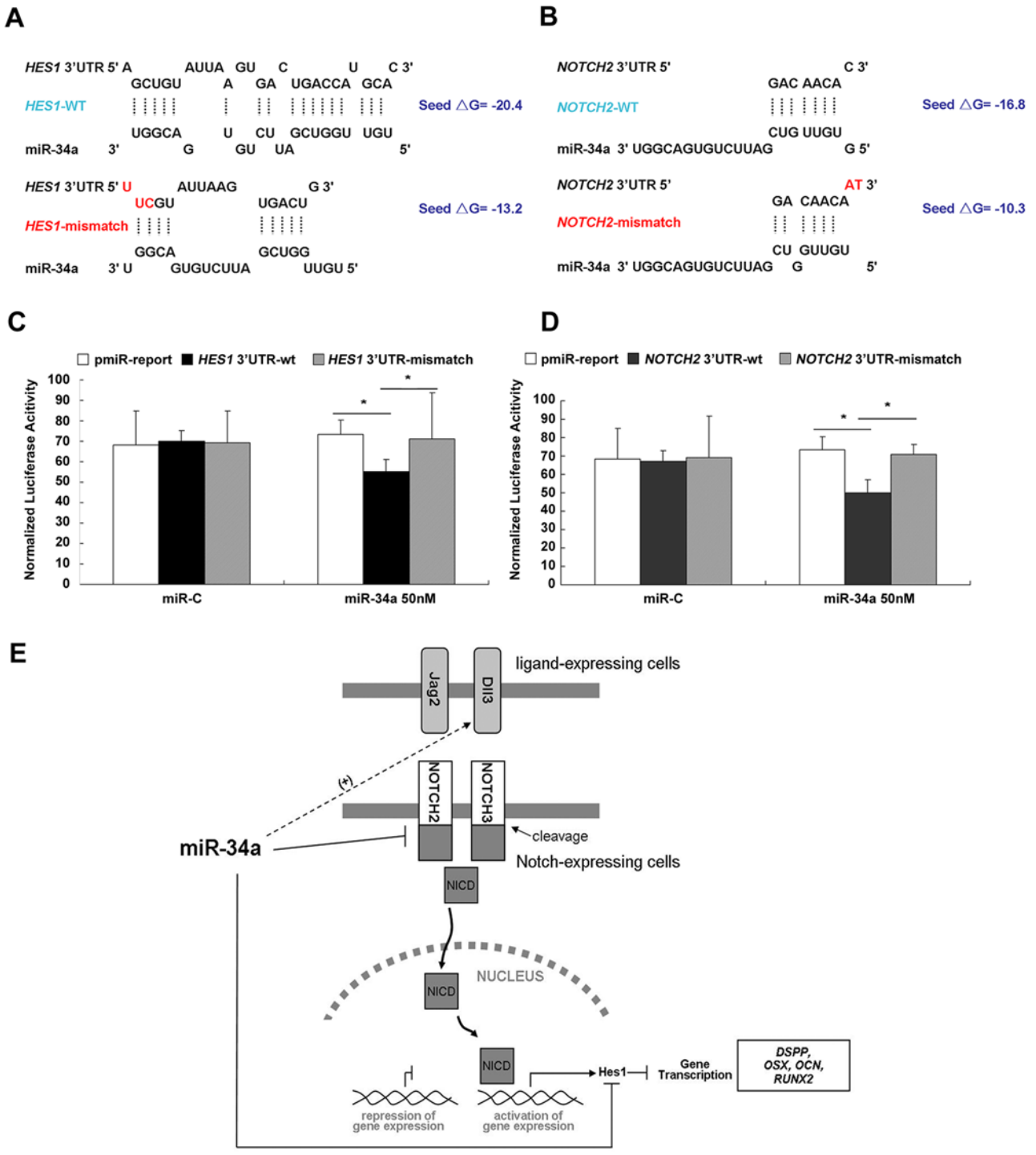

Figure 4. miR-34a suppresses Notch signaling and promotes cell differentiation of SCAPs by targeting NOTCH2 and HES1. (A, B) Schematic representation of luciferase reporter constructs. The predicted structure of each base-paired HES1 **(A)** or NOTCH2 **(B)** 3′UTR/wild-type or mismatched miRNA hybrid is diagrammed, and the calculated free energy (/G) in kilocalories *per* mole of the 5′ seed region of each hybrid is shown on the right. The top strand in each diagram represents 5′ to 3′ target mRNA (HES1 or NOTCH2) 3′ UTR WT and mismatches (nucleotides in color), and the bottom strand represents the miR-34a. **(C)** Control luciferase reporter (pmiR-report), pmiR with wild-type HES1 3′UTR (HES1 3′UTR-wt), or with mismatched HES1 3′UTR (HES1 3′UTR-mismatch) were transfected into 293T cells. 293T cells were co-transfected with either control or miR-34a oligomers. *$p < .05$. **(D)** Control luciferase reporter (pmiR-report), pmiR with wild-type NOTCH2 3′UTR (NOTCH2 3′UTR-wt), or with mismatched NOTCH2 3′UTR (NOTCH2 3′UTR-mismatch) were transfected into 293T cells. 293T cells were co-transfected with either control or miR-34a oligomers. *$p < .05$. **(E)** miR-34a acts directly on and suppresses NOTCH2 and HES1. This results in suppression of Notch signaling and up-regulation of differentiation genes, such as DSPP, RUNX2, OSX, and OCN.

3H). By targeting *NOTCH2* mRNA, miR-34a repressed translocation of N2ICD into the nucleus (Figs. 3C, 3E). Although we found up-regulation of *DLL3* in SCAPs after miR-34a mimic transfection, which, in turn, resulted in up-regulation of *HES1* mRNA expression, Western blotting showed that HES1 protein was significantly down-regulated. The inconsistency between the mRNA and protein levels suggested that miR-34a post-transcriptionally regulates HES1 expression by targeting the 3′UTR of *HES1*, thus leading to degradation of *HES1* mRNA, as suggested by dual luciferase assay (Fig. 4C). The overall outcome of miR-34a transfection in SCAPs was inhibition of Notch signaling and induction of cell differentiation (Fig. 4E).

It is known that RUNX2 plays a critical role in odontoblastic differentiation and tooth development (Gaikwad *et al*., 2001), as well as in osteoblastic differentiation (Byers and Garcia, 2004). It has been reported that RUNX2 transcriptionally induced the expression of dentin sialophosphoprotein (DSPP) (Huang *et al*., 2008) and osteogenic differentiation genes (Byers and Garcia, 2004; Zhang *et al*., 2006). The concurrent up-regulation of the lineage-differentiation genes (*DSPP, OSX, OCN*, and *RUNX2*) after miR-34a transfection in the present study was consistent with that in these previous studies. *ALP* and *OPN* expression at the mRNA level was not affected by miR-34a, which may be related to the late-stage expression of these 2 markers or to regulation by other post-transcriptional mechanisms.

Analysis of our data, together, shows that miR-34a-triggered Notch repression results in cell differentiation and that activation of Notch signaling in SCAPs results in elevated miR-34a transcription.

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