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# Notch1 signalling inhibits apoptosis of human dental follicle stem cells via both the cytoplasmic mitochondrial pathway and nuclear transcription regulation

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ABSTRACT

Dental follicle stem cells (DFSCs) have been considered as promising candidate cells for periodontal tissue regeneration. Understanding the signalling pathways underlying the apoptosis of DFSCs will facilitate its biomedical application. Here we showed that Notch1 signalling could inhibit DFSCs apoptosis because the constitutive overexpression of the intracellular domain of Notch1 (ICN1) promoted proliferation and suppressed apoptosis by inhibiting cytoplasmic mitochondrial membrane depolarization, cytochrome c release and activation of caspase-9 and caspase-3. The survival-promoting effect of Notch1 was also accomplished by up-regulation of the anti-apoptotic proteins Bcl-2 and Mcl-1, down-regulation of the pro-apoptotic proteins Bax and Bad, and blockade of Bax multimerization. Moreover, p-Akt (S473) was significantly increased after ectopic Notch 1 activation. The expression of p53 was also inhibited in Notch1-overexpressing DFSCs, while the ectopic expression of p53 promoted apoptosis even when Notch1 was overexpressed. Meanwhile, all of the opposite phenomena were observed in Notch1 shRNA-silenced DFSCs. Our data strongly suggested that Notch1 signalling inhibited the apoptosis of DFSCs via the cytoplasmic mitochondrial pathway and ICN-Akt signalling pathway, together with nuclear gene expression regulation. These findings would provide molecular cues for the further medical application of DFSCs.



## 1. Introduction

Periodontitis is one of the most common human inflammatory diseases, characterized by progressive destruction of the tooth-supporting tissues and potentially leading to tooth loss. One of the emerging and promising approaches for periodontitis therapy is the delivery of dental follicle stem cells (DFSCs), one type of neural crest-derived stem cells residing in the dental follicle (Angiero et al., 2012; Aonuma et al., 2012; Yao et al., 2008). DFSCs are the precursor cells of periodontal ligament cells, osteoblasts and cementoblasts that form the periodontium and are able to differentiate more efficiently and exhibit higher immunomodulatory effects than other dental stem cells (Yildirim et al., 2016). However, efficient self-renewal and proliferation of stem cell populations after transplantation is influenced by apoptosis. Thus, full elucidation of the signal transduction cascades for apoptosis is required for valid development of the therapeutic potential of DFSCs.

Notch signalling has been shown to play a crucial role in metazoan cell fate determination (Chiba, 2006; Perdigoto and Bardin, 2013). Particularly, its anti-apoptosis activities have been widely observed in multiple cell lines, including immortalized epithelial cells (Nair et al., 2003), T-cell receptor–induced matured cells (Jehn et al., 1999), thymocytes (Deftos et al., 1998) and T cells (Sade et al., 2004). It was reported that the N-terminus of the intracellular domain of Notch (ICN) is predominantly localized in

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the nucleus to regulate the transcription of genes related to anti-apoptosis events (Zeng et al., 2016). Moreover, ICN interacts with p53 to inhibit its phosphorylation and interferes with its DNA binding activity, consequently resulting in the inhibition of p53-dependent transactivation and subsequent apoptosis in human cancer cells (Kim et al., 2007). Also localized in the cytoplasm, ICN can inhibit the conformational changes and multimerization of Bax to block the mitochondrial pathway for apoptosis in HEK cells (Perumalsamy et al., 2010). Other appreciated anti-apoptotic functions of Notch are linked to interactions with the kinase Akt. The ICN-Akt-Mitofusins (Mfn1/2) pathway has been identified to maintain the mitochondrial function, contiguity and prevent consequent nuclear damage in apoptosis (Antico Arciuch et al., 2009; Perumalsamy et al., 2009, 2010).

We have previously shown that Notch1 signalling regulates the proliferation and self-renewal of human dental follicle cells (HDFCs) through the modulation of the G1/S phase transition and through the telomerase activity (Chen et al., 2013). Here, we further revealed the inhibitory roles of Notch1 signalling on the apoptosis of DFSCs, the stem cell subpopulation of HDFCs, via both the cytoplasmic mitochondrial pathway and the nuclear transcription regulation. Our work deepens the understanding of the molecular regulatory mechanisms of DFSCs apoptosis, which would potentially improve future application of DFSCs in periodontal tissue regeneration.

## 2. Materials and methods

### 2.1. Ethics statement

The human dental follicles were separated from impacted third molars extracted for orthodontic reasons from five donors (two 13-year-old boys, one 14-year-old boy and two 13-year-old girls) with written consent signed by the donors and their parents. All five donors had no systemic or oral infections or diseases except presenting with class III malocclusions. The ethical protocol was approved by the local medical ethics committee. All of the experiments in the present study were performed in accordance with the regional and international ethics committee guidelines.

### 2.2. Cell culture

The HDFCs were isolated, cultured and identified as previously described in our lab (Chen et al., 2013). Human dental follicle stem cells (HDFSCs) were cultured based on the method previously described by Honda et al. with some minor modifications (Honda et al., 2011). Briefly, after HDFCs were 80% confluent, cells were suspended at a density of 1 cell per 100 μL and seeded into two 96-well culture plates with the growth culture medium (GCM) consisting of DMEM, 10% FBS and 1% antibiotics (Invitrogen). After 10 days of incubation, 8 colonies (each from a single cell) were obtained and subsequently expanded in GCM. HDFSCs were gradually passaged from 96-well culture plates into 10-cm dishes for subsequent experiments.

Both the human erythroleukaemic K562 and retroviral packaging 293T cell lines were purchased from a cell bank (Chinese Academy of Sciences). The K562 cells were maintained in the RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco). The 293T cells were cultured in DMEM (Hyclone) containing 10% FBS at 37 °C with 5% $CO_2$.

### 2.3. Flow cytometric analysis for HDFSCs phenotype

For phenotypic characterization, HDFSCs were harvested in 0.25% trypsin/EDTA. $10^6$ cells were stained with CD29 (FITC conjugated), CD31 (FITC conjugated), CD34 (PE conjugated), CD44 (FITC conjugated), CD45 (FITC conjugated), CD90 (PE conjugated) in 100 μL of Dulbecco's PBS containing 2.5% BSA and 0.1% sodium azide for 45 min on ice according to the manufacturer's instructions. Isotype controls were run in parallel using the same concentration of each antibody tested. All of the antibodies were purchased from BD Biosciences. Cells were analysed using a FACS Caliber (Becton-Dickinson), and the data were analysed using the CellQuest software (Becton-Dickinson).

### 2.4. Detection of the multipotential differentiation capacity of DFSCs

DFSCs were induced to differentiate into osteogenic and adipogenic lineages. For osteogenic differentiation, the cells were seeded into 6-well plates at a density of $5 \times 10^5$ cells/well and grown in the mineralization-inducing medium containing 100 μM/mL ascorbic acid, 2 mM β-glycerophosphate, and 10 nM dexamethasone for 21 d, then fixed with 4% paraformaldehyde, and stained with 2% Alizarin red (Sigma-Aldrich). For adipogenic differentiation, the cells were seeded into 6-well plates at a density of $2 \times 10^5$ cells/well. At 100% confluence, three cycles of induction/maintenance were used to stimulate optimal adipogenic differentiation according to the manufacturer's instructions (Cambrex). Each cycle consisted of feeding DFSCs with adipogenic induction medium for 3 days followed by 3 days of culture in the adipogenic maintenance medium. The presence of matured adipocytes was assessed by the Oil Red O staining.

### 2.5. Plasmid construction, retrovirus preparation and infection of HDFSCs

The procedures of plasmid construction, retrovirus preparation and infection of HDFSCs were identical to those previously described (Chen et al., 2013). The Geneticin-selected HDFSCs infected with GFP or ICN1 were designated as HDFSC-GFP or HDFSC-ICN, respectively. The uninfected parental HDFSCs were used as negative controls (HDFSC-C).

### 2.6. Notch1 shRNA lentiviral particles transduction

Notch1 shRNA lentiviral particles (sc-36095-V) and control lentiviral particles expressing a scrambled shRNA (sc-108080) were purchased from Santa Cruz Biotechnology. The procedure of Notch1 shRNA lentiviral particles transduction has been described previously (Chen et al., 2013). The puromycin-selected HDFSCs infected with control shRNA lentiviral particles or Notch1 shRNA lentiviral particles were designated as HDFSC-CS or HDFSC-NS, respectively.

### 2.7. Quantitative real-time RT-PCR

The cells in five different HDFSC groups (HDFSC-C, HDFSC-GFP, HDFSC-ICN, HDFSC-CS and HDFSC-NS) were cultured in DMEM containing 10% FBS. At approximately 80% confluence, the cells were starved for an additional 24 h and were subsequently harvested for qPCR (Chen et al., 2013). The primers were listed in Table S1 (in Supporting information).

### 2.8. Western blotting analysis

The expression of target proteins among the five different HDFSC groups was analysed by Western blotting as previously described (Chen et al., 2013). The antibodies used are provided in Table S2 (in Supporting information).