# Involvement of Notch Signalling Pathway in Senescence of Human Dental Pulp Cells

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**Objective:** *To evaluate whether the Notch signalling pathway is involved in the senescence of human dental pulp cells.*
**Methods:** *Human dental pulp cells were isolated and cultured. The Notch signalling pathway was blocked by adding DAPT (N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester, γ-secretase inhibitor, 5 μmol/L) into the culture medium. Cell proliferation was evaluated by MTT assay. Cell senescence was evaluated through expression of senescence-associated β-galactosidase (SA-β-Gal) using β-galactosidase staining.*
**Results:** *The proliferation of the human dental pulp cells decreased dramatically after treatment with DAPT, compared with the control. SA-β-Gal positive staining cells were seen in the DAPT group, but not in the control group.*
**Conclusion:** *The Notch signalling pathway is involved in the senescence of human dental pulp cells.*

**Key words:** *dental pulp cells, senescence, Notch signalling pathway*

Age-related changes in human dental pulp tissue include: decrease in cell components, increase in fibre components, change towards lipid or voids in pulp cells or fibroblasts[1], decrease in cell growth rate, and decrease in activity of alkaline phosphatase[2] and osteocalcium[3]. Due to these changes, functions of pulp cells weaken, and the cell itself becomes senescent. Cell senescence is defined as when normal somatic cells enter a state of irreversibly arrested growth and altered function[4]. There are several salient features of senescent cells: (i) arrested growth with G1 DNA content before S phase; (ii) selected cell-specific changes, such as cell volume becomes larger than normal; (iii) increased lysosome activity with expression of senescence-associated ß-galactosidase (SA-ß-gal)[5]; and (iv) an association in cultured cells with augmented p53 activity. Abrogation of p53 activity can delay *in vitro* senescence[6].

Various signalling pathways could be involved in cell senescence. The Notch signalling pathway, as recent studies have shown, is essential in human muscle cell regulation. Carey et al[7] reported that lower expression of Notch1, Jagged1 and Delta-like1 were evident in muscle biopsies from older men (60 to 75 years old) compared with those from younger men (18 to 25 years old). Conboy et al[8] demonstrated that inhibition of Notch can impair regeneration of young muscle, whereas forced activation of Notch can restore the regenerative potential of old muscles. Conboy et al also suggested that Notch signalling might be a key determinant in muscle regenerative potential that declines with age. Furthermore, the Notch signalling pathway may contribute to the cell senescence mechanism[8].

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The Notch signalling pathway plays an essential role in proliferation, differentiation and apoptosis of cells[9]. The Notch receptor can be activated through direct interaction with membrane-bound ligands, which are expressed by adjacent cells. Upon receptor-ligand binding, the intracellular domain of Notch (NICD) is proteolytically cleaved by γ-secretase, and translocates to the nucleus, where it interacts with transcription factors such as CBF1, Su(H) and the Lag1 (CSL) family. A CSL-NICD complex modulates the transcription of different targets, and is frequently linked to the expression of the E(spl)/HER/HES (Enhancer of split, Hairy/Enhancer of split–related, Hairy and Enhancer of split) family of transcriptional regulators[9]. DAPT (N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester) is a γ-secretase inhibitor. Previous studies demonstrated that it could efficiently block the presenilin/γ-secretase complex[10] and, as a consequence, prevent activation of the Notch response[11].

More recent studies have shown that Notch2 is also expressed during dentin repair processes in odontoblasts and sub-odontoblastic cells. Notch signalling is activated in response to injury and associated with the differentiation of pulp cells into perivascular cells and odontoblasts[12-14]. In addition, Notch signalling can inhibit odontoblastic differentiation of dental pulp stem cells[15]. However, whether the Notch signalling pathway is involved in senescence of dental pulp cells remains unknown. Therefore, the purpose of the present study was to evaluate the effect of the Notch signalling pathway on senescence of human dental pulp cells.

## Materials and methods

### *Subjects and cell culture*

Normal human third molars from adults (18 to 29 years of age) extracted for orthodontic or impacted reasons, were collected with patients' informed consent. Whole pulp tissue was obtained. The pulp tissue, except the apical 2 mm, was cut into 1 × 1 × 1 $mm^3$ pieces, digested in 3 mg/ml type I collagenase (GIBCO, USA) for 40 to 60 min at 37°C, with constant soft shaking for 5 min intervals, until no visible pieces remained. The digestions were centrifuged at 1000 rpm for 5 min. The cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 15% fetal bovine serum (GIBCO, Australia), 100 units/ml of penicillin, 100 µg/ml of streptomycin and 2 mM L-glutamine. When a confluent monolayer was formed, the cells were harvested with 0.25% trypsin-EDTA (GIBCO, USA), transferred to a 6 mm diameter plastic culture dish with DMEM in 10% fetal bovine serum and marked as Passage 1. The cells were subcultured at a 1:3 split ratio when they reached confluence. The cells used in the present study were from Passage 6 to Passage 10.

### *Blocking Notch signalling pathway*

DAPT (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) to make a 1 mmol/L stock solution. The stock solution was diluted in culture medium to obtain a given concentration of 5 µmol/L. Human dental pulp cells with a total amount of $3 \times 10^5$ cells were seeded into a 6 mm diameter dish. DAPT was added to the culture medium at a final concentration of 5 µmol/L, which has been demonstrated to block the Notch signalling pathway[16]. The control group was treated with DMSO (vehicle) in a similar way[17,18]. The treating process occurred throughout Passage 6 to Passage 10. During the treating process, cells of the DAPT group and control groups were subcultured at the same time at the same ratio (1:3).

### *MTT assay*

The cells of the blocked group and control group were seeded into 96-well plates, respectively, at a density of $3 \times 10^3$ cells/well and were duplicated in 6 wells. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was carried out at day 1, 3, 5, 7, 9, 11, 13, 15 and 17. Optical density (OD) was measured at a wavelength of 570 nm with a reference wavelength of 630 nm using an ELISA plate reader (ELX808, BioTek, Winooski, VT, USA).

### *Senescence-associated ß-galactosidase (SA-ß-gal) staining*

The cells were washed two times in PBS (phosphate buffer solution), stained with a SA-ß-gal staining kit (C0602, Beyotime, China), fixed for 15 min at room temperature in a fixing solution of the SA-ß-gal staining kit, then washed and incubated in fresh senescence-associated ß-gal staining solution overnight at 37°C. One milliliter of staining solution contained: SA-ß-gal staining solution A, 10 µl; SA-ß-gal staining solution B, 10 µl; SA-ß-gal staining solution C, 930 µl; and X-Gal, 50 µl. The cells were viewed under a light microscope at 100 to 200× magnification.

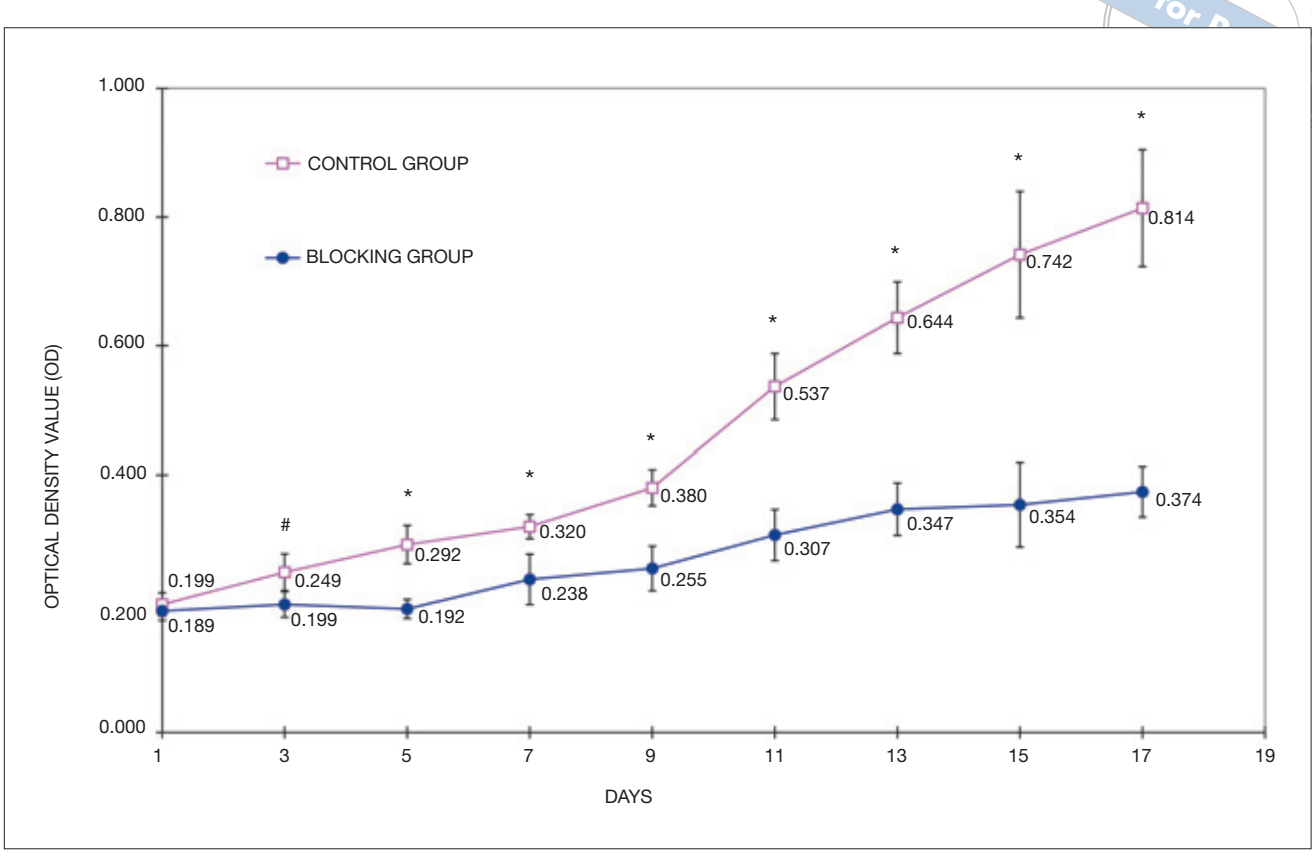


**Fig 1** Cell growth of human dental pulp cells after blocking the Notch signalling pathway by DAPT (5 μmol/L) was significantly decreased from day 3 to day 17, compared to the control cells. # $P < 0.05$, * $P < 0.001$.

*Statistical analysis*

The data was statistically analysed by Student *t* test using SPSS 11.0 for Windows (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered to be significant.

## Results

*State of cultured human dental pulp cells*

Cell clones were formed after 4 days of growth of the first culture and grew outwards. Cells adhered to the culture surface and displayed spindle and polygonal shapes, typically fibroblast-like. The subcultured cells arrayed in order and grew in a swirling pattern.

*Cell proliferation*

The cell growth of the blocking group was significantly decreased from day 3 to day 17 compared with the control group (Fig 1).

*SA-ß-gal expression*

SA-ß-gal positive staining was seen as blue precipitate only in cells of the DAPT group (Fig 2A). The cells of the control group were fibroblast-like, arrayed in order, without blue precipitate (Fig 2B).

## Discussion

Cells, as basic organisational units of living organisms, are also basic units during the process of aging. In the present study, it was hypothesised that the Notch signalling pathway was associated with dental pulp cell proliferation. Notch signalling was blocked with its specific inhibitor, DAPT, in human dental pulp cells. Based on the mechanism of the MTT assay, the OD value indirectly reflected the proliferation condition of cells, such as living cell amount or metabolic activity[19]. The OD value was significantly decreased in the DAPT group compared with the control group from day 3 to 17, suggesting that the cell proliferation of human dental pulp cells was decreased by blocking the Notch signalling path-

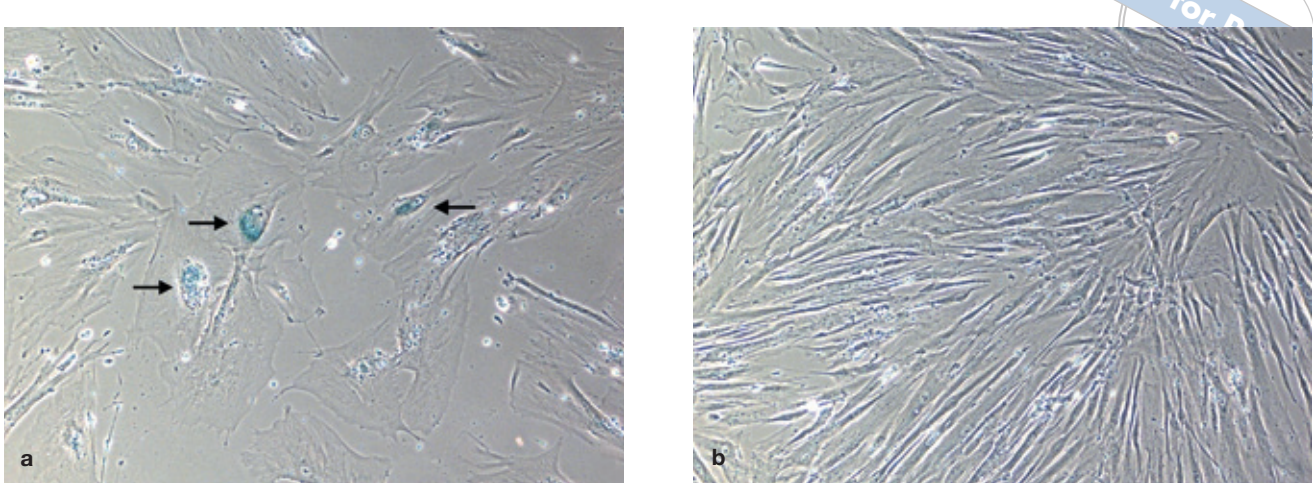


**Fig 2** SA-ß-Gal staining of dental pulp cells. Positively stained cells ([a] DAPT group, Passage 10, 100×) showed blue precipitate (black arrow), larger volume and lower cell number. The control cells ([b] control group, Passage 10, 100×) were fibroblast-like, arrayed in order and without blue precipitate.

way. Similar data was also reported in rat neural stem cells and pancreatic cancer cells[20,21]. Down-regulation of Notch-1 has been shown to inhibit cell growth of pancreatic cancer cells, increase cell population in the G0 to G1 phase and decrease expression of cyclin A, cyclin D1, and cyclin-dependent kinase 2 (CDK2)[21]. Cyclin D1 and CDK2 were found to be involved in the G1/S transition process. It has also been reported that DAPT treatment can regulate CDK5 expression at both transcriptional and protein level[18]. DAPT has also been used to block Notch signalling in rat cortical neurons[18] and mesenchymal stem cells[22].

With regard to the metabolic conditions of cells, SA-β-gal was reported as a biomarker of cell senescence. In skin samples from human donors of different ages, an age-related increase in the frequency and density of SA-β-gal has been found[5]. Additionally, its expression increased with passage in somatic fibroblasts[5]. The present results showed that SA-β-gal positively stained cells were only seen after blocking the Notch signalling pathway with DAPT, but not in the control human dental pulp cells (Passage 10). This suggests that the SA-β-gal positively stained cells were likely more senescent than the cells of the control group. However, activation of the Notch signalling pathway has been shown to inhibit the differentiation of dental pulp cells[15]. Therefore, it was not ruled out that blocking Notch signalling might also increase the differentiation of human dental pulp cells in the present study.

Senescence of dental pulp cells is usually attributed to physiological degeneration, which can affect the ability to conduct dental repair. The present results demonstrate that the Notch signalling pathway is involved in dental pulp cell senescence. Further work needs to be done to understand dental pulp senescence.

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