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FULL LENGTH ARTICLE

NOTCH3 is expressed in human apical papilla and in subpopulations of stem cells isolated from the tissue

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Abstract NOTCH plays a role in regulating stem cell function and fate decision. It is involved in tooth development and injury repair. Information regarding NOTCH expression in human dental root apical papilla (AP) and its residing stem cells (SCAP) is limited. Here we investigated the expression of NOTCH3, its ligand JAG1, and mesenchymal stem cell markers CD146 and STRO-1 in the AP or in the primary cultures of SCAP isolated from AP. Our *in situ* immunostaining showed that in the AP NOTCH3 and CD146 were co-expressed and associated with blood vessels having NOTCH3 located more peripherally. In cultured SCAP, NOTCH3 and JAG1 were co-expressed. Flow cytometry analysis showed that 7%, 16% and 98% of the isolated SCAP were positive for NOTCH3, STRO-1 and CD146, respectively with a rare 1.5% subpopulation of SCAP co-expressing all three markers. The expression level of NOTCH3 reduced when SCAP underwent osteogenic differentiation. Our findings are the first step towards defining the regulatory role of NOTCH3 in SCAP fate decision.

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Introduction

Since the discovery of stem cells from apical papilla (SCAP), new clinical treatment concepts have emerged. A clinical regenerative protocol has been proposed based on some research studies and many clinical case reports.[1–5] The theory behind this protocol is that SCAP in the apical papilla may be induced to regenerate damaged or lost pulp tissue in the canal space.[6–8] This possibility is further supported by the capacity of SCAP to regenerate pulp-dentin-like tissues *in vivo* in animal models.[6,9] However, despite such clinical endeavors to practice regenerative treatments, the understanding of the biology of SCAP is still limited.

NOTCH signaling pathway plays a key role in the development and morphogenesis of different organs and tissues in various species. It promotes or suppresses cell proliferation, initiates or inhibits cell differentiation and determines the fate of different types of stem cells.[10] There are four NOTCH receptors (NOTCH1–4), which are activated by direct contact with their membrane-bound ligands (JAGGED (JAG) 1, JAG2, Delta-like (DLL) 1, DLL3 and DLL4) on neighboring cells. Upon activation of NOTCH receptors, enzymatic activities are triggered resulting in the cleavage of NOTCH intracellular domain (NICD), which is then translocated into the nucleus to activate the transcription of target genes, such as HEY1 and HEY2.[10,11] NOTCH signaling pathway is also believed to be involved and play an important role in the process of tooth development and pulp regeneration and repair after injury.[12–14] NOTCH3 and its ligand JAG1 are upregulated during tooth development in the vicinity of blood vessels and in the subodontoblast layer, but not in odontoblasts. Similar expression pattern was found in pulp tissue undergoing repair and regeneration after pulp exposure and capping.[12,15,16] Interestingly, NOTCH3 is expressed in the cervical loop (stem cell niche) of continuously erupting teeth (mouse incisors and vole molars), suggesting its possible role in maintaining the undifferentiated state of cells within that niche.[13]

Little is known regarding the expression of NOTCH3 and its ligand JAG1 in apical papilla (AP) and SCAP. The aim of this study was to investigate whether and where NOTCH3 is expressed in AP and its expression along with JAG1 in cultured SCAP, as well as its co-expression with mesenchymal stem cell markers CD146 and STRO-1.

Material and methods

Sample collection

This study was approved by the Human Research Ethics Committee of United Arab Emirates University (#11/10) and Boston University Medical Institutional Review Board (#H-28882). Freshly extracted, intact human teeth were obtained from 10 to 24 years old consented healthy patients ($n = 5$). The teeth were caries-free and had incompletely formed root apices. The root apical papillae were microdissected from extracted teeth and SCAP were isolated and cultured as described below based on a previous report.[6]

Immunohistochemical staining of apical papillae

Apical papillae were obtained as mentioned above and processed for cryosectioning; 8 μm-thick sections were fixed with cold acetone at −20 °C for 15 min, washed in PBS, treated with 1.5% hydrogen peroxide for 30 min and blocked with 2.5% normal horse serum (Vectastain Elite ABC kit; Vector laboratories) for 1 h. Tissue sections were then incubated with mouse monoclonal anti-NOTCH3 antibodies (dilution 1:100, Abcam, USA) for 1 h at room temperature, followed by washing and incubation with biotinylated anti-mouse immunoglobulin G (secondary antibody) for another 1 h. After washing, avidin-peroxidase complex was added and incubated for 30 min, followed by washing and the addition of peroxidase substrate solution for 5 min. Sections were counterstained with hematoxylin solution (Sigma, USA). Negative control slides were prepared in parallel without adding the primary antibody.

For immunofluorescence staining, frozen sections of AP were fixed with cold acetone at −20 °C for 15 min, washed and blocked for 1 h. Sections were then incubated with primary antibodies: NOTCH3 (dilution 1:100, Abcam, USA) and CD146 (dilution 1:50, Invitrogen, USA) for 1 h at room temperature, followed by washing and incubation with the appropriate fluorophore-conjugated secondary antibodies for another 1 h.

Isolation and culture of SCAP

The papillae were minced and digested in a physiological solution containing 3 mg/mL collagenase type I (GIBCO/Invitrogen) and 4 mg/mL dispase (GIBCO/Invitrogen) for 45 min at 37 °C. Isolated cells were then plated in culture dishes containing alpha-modification of Eagle's medium (GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (GIBCO/Invitrogen), 2 mM L-glutamine (GIBCO/Invitrogen), 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO/Invitrogen) and incubated in a humidified incubator (Thermo Scientific) at 37 °C in 5% CO_2. Once the cells reached ~80% confluence, they were trypsinized and passaged.

Immunocytochemical and immunofluorescence staining of SCAP

For immunocytochemistry, isolated SCAP of passage 2 were seeded on sterile coverslips. At 80% confluence, cells were fixed using 4% paraformaldehyde (PFA) for 30 min, washed with phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton-X100 (Sigma, USA). To inhibit endogenous peroxidase activity, cells on coverslips were incubated in 1% hydrogen peroxide in PBS for 35 min. Nonspecific binding was blocked by incubating cells in 1% bovine serum albumin (BSA) containing 0.5% Tween-20 in PBS for 45 min. Cells were then incubated with goat polyclonal anti-NOTCH3 antibody (dilution 1:25, clone M-20, Santa Cruz Biotechnology Inc., USA) overnight at 4 °C. Cells on coverslips were washed with PBS and then incubated with biotinylated donkey-anti-goat immunoglobulin G (dilution 1:500, Jackson ImmunoResearch Laboratories Inc., USA) for 1 h and then were incubated in extravidin/peroxidase conjugate (dilution 1:1000, Sigma, USA) for 1 h. The antigen–antibody binding sites were