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Characterization of Apical Papilla and its Residing Stem Cells from Human Immature Permanent Teeth –A Pilot Study

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Abstract

Mesenchymal stem cells (MSCs) have been isolated from the pulp tissue of permanent teeth (dental pulp stem cells or DPSCs) and deciduous teeth (stem cells from human exfoliated deciduous teeth or SHED). We recently discovered another type of MSCs in the apical papilla of human immature permanent teeth termed stem cells from apical papilla (SCAP). Here we further characterized the apical papilla tissue and stem cell properties of SCAP using histological, immunohistochemical and immunocytofluorescent analyses. We found that apical papilla is distinctive to pulp in terms of containing less cellular and vascular components than those in pulp. Cells in apical papilla proliferated 2- to 3-fold greater than those in pulp in organ cultures. Both SCAP and DPSCs were as potent in osteo/dentinogenic differentiation as MSCs from bone marrows while weaker in adipogenic potential. The immunophenotype of SCAP is similar to that of DPSCs on the osteo/dentinogenic and growth factor receptor gene profiles. Double staining experiments showed that STRO-1 co-expressed with dentinogenic markers such as bone sialophosphoprotein (BSP), osteocalcin (OCN) and growth factors FGFR1 and TGF β RI in cultured SCAP. Additionally, SCAP express a wide variety of neurogenic markers such as nestin and neurofilament M upon stimulation with a neurogenic medium. We conclude that SCAP are similar to DPSCs but a distinct source of potent dental stem/progenitor cells. Their implications in root development and apexogenesis are discussed.

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Keywords

Apical papilla; immature teeth; MSCs; SCAP; DPSCs; PDLSCs; BMMSCs; apexogenesis; immunohistochemistry; immunocytofluorescence

Introduction

Previously, we isolated and characterized human dental pulp stem cells (DPSCs) from the dental pulp of permanent teeth and stem cells from the pulp of human exfoliated deciduous teeth (SHED) (1,2). DPSCs are capable of differentiating into odontoblast-like cells and produce ectopic dentin in the subcutaneous space of immunocompromised mice. SHED appear to be capable of forming odontoblast- and osteoblast-like cells and generating dentin and bone respectively when transplanted into immunocompromised mice. Very recently, we discovered another type of human MSCs from apical papilla (SCAP) which appear to be a different population of stem cells from DPSCs (3). We utilized SCAP to engineer bio-roots using minipigs as an animal model and demonstrated that these cells are a promising cell source to regenerate bio-roots for future clinical applications (3).

Little, if any, information on human apical papilla is available in the literature. In developing teeth, root formation starts as the epithelial cells from the cervical loop proliferate apically and influence the differentiation of odontoblasts from undifferentiated mesenchymal cells and cementoblasts from follicle mesenchyme (4). This apically extending two-layered epithelial wall (merging of the inner and outer enamel epithelium) forms Hertwig's epithelial root sheath (HERS) which is responsible for determining the shape of the root(s) and forms cementum through epithelial-mesenchymal transition (EMT) (5). It has been known that dental papilla contributes to tooth formation and eventually converts to pulp tissue (4,6,7). As the root continues to develop after the bell stage, the location of the dental papilla becomes apical to the pulp tissue. The histological features at the junction of the pulp and apical papilla have not been well described. We report herein that apical papilla appears to be histologically distinct from pulp and contains unique potent MSCs.

Materials and methods

Sample collection

Normal human impacted third molars (n= 10) with immature roots were collected from healthy patients (6 donors aged 16–24 yrs) in the Dental Clinics at the University of Southern California and the University of Maryland. Bone marrow was obtained from the long bone of a healthy patient (aged ~20) undergoing orthopaedic surgery at Walter Reed Army Medical Center. Sample collection conformed to approved protocols by the respective Medical Institutional Review Boards and National Institute of Health. The informed consent of all human subjects who participated in the experimental investigation reported or described in this manuscript was obtained after the nature of the procedure and possible discomforts and risks had been fully explained. Tooth or bone samples were stored in medium and transported to labs for sample processing.

Cell culture

Root apical papilla was gently separated from the surface of the root, minced and digested in a solution of 3 mg/ml collagenase type I (Worthington Biochemicals Corp., Freehold, NJ) and 4 mg/ml dispase (Roche Diagnostic/Boehringer Mannheim Corp., Indianapolis, IN) for 30 minutes at 37°C. Single cell suspensions of SCAP were obtained by passing through a 70 µm strainer (Falcon, BD Labware, Franklin Lakes, NJ), seeded at 1×10^4 into 10-cm culture dishes

(Costar, Cambridge, MA), and cultured with α -Modification of Eagle's Medium (GIBCO/Invitrogen, Carlsbad, CA) supplemented with 15–20% fetal bovine serum (FBS) (Equitech-Bio Inc., Kerrville, TX), 100 mM L-ascorbic acid 2-phosphate (WAKO, Tokyo, Japan), 2 mM L-glutamine (Biosource/Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO₂. To assess colony-forming efficiency, day-10 cultures were fixed with 4% formalin, and then stained with 0.1% toluidine blue. Aggregates of ≥ 50 cells were scored as colonies. DPSCs and bone marrow mesenchymal stem cells (BMMSCs) were isolated and cultured as previously described (1,8,9). DPSCs were grown in the same medium for SCAP and BMMSCs were cultured in expansion medium containing Dulbecco's Modified Eagle's Medium (DMEM), 100 units/ml penicillin, 100 μ g/ml streptomycin, 25 ng/ml Fungizone, and 10% preselected FBS. Cells were subcultured at 1:3 when they reached ~85% confluence. All primary cells used in this study were at 1–3 passages. For each experiment, same passage of cells were used.

In situ cell proliferation analysis

The root tips from extracted teeth containing pulp and apical papilla were cultured in a regular BMMSC culture medium and labeled with bromodeoxyuridine (BrdU) using a Zymed BrdU staining kit (Zymed/Invitrogen, Carlsbad, CA). The concentration of BrdU used was the same as for labeling cultured cells described previously (3,10). After 3.5 and 18 hrs of BrdU incorporation, the tissues were fixed, demineralized and embedded. Samples were then sectioned and deparaffinized for immunohistochemical staining of BrdU positive cells using anti-BrdU antibodies. Positively stained cells were counted in randomly selected areas under a light microscope.

Differentiation stimulation

Conditions for the induction of calcium accumulation were as reported previously (1,11). Osteo/dentinogenic medium contains the above basic elements plus 10 nM dexamethasone, 10 mM β -glycerophosphate, 50 μ g/ml ascorbate phosphate and 10 nM 1, 25 dihydroxyvitamin D3. Calcium accumulation was detected by 2% Alizarin Red S (pH 4.2) staining. The induction of adipogenesis was as previously reported (2,3,12). Adipogenic medium contains basic elements plus 1 μ M dexamethasone, 1 μ g/ml insulin and 0.5 mM IBMX (3-isobutyl-1-methylxanthine). Oil droplets accumulated within cells after adipogenic differentiation were stained by oil red O reagent. For neurogenic induction, cells were cultured in the presence of B27 supplement, bFGF (40 ng/ml) and EGF (20 ng/ml) (2).

Antibodies

Mouse antibodies included anti-DSP (LF-21) (Dr. Larry Fisher, NIDCR/NIH); anti-STRO-1 (Dr. Stan Gronthos, Institute of Medical and Veterinary Science, Australia), nestin, neurofilament M (NFM), neuronal nuclei (NeuN), and 2', 3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) from Chemicon; anti- β III-tubulin from Promega and monoclonal anti-CD146/MUC18 (melanoma-associated glycoprotein) from Chemicon. Rabbit antibodies included anti-basic FGF (bFGF), TGF β R1, TGF β R2, NSE (neural-specific enolase), Flt-1 (VEGF receptor 1), Flg (FGFR1) and FGFR3 from Santa Cruz Biotechnology, Santa Cruz, CA; bone sialoprotein (BSP, LF-120), alkaline phosphatase (ALP, LF-47), osteocalcin (OCN, LF-32), anti-alkaline phosphatase (ALP) (LF-47), and matrix extracellular phosphoglycoprotein (MEPE) (LF-155) from Dr. L. Fisher; and anti-endostatin, glutamic acid decarboxylase (GAD) from Chemicon. Rabbit and murine isotype-matched negative control antibodies were obtained from Caltag Laboratories (Caltag/Invitrogen).

Immunohistochemistry and immunocytofluorescence

Deparaffinized sections were immersed in 3% H₂O₂/methanol for 15 minutes to quench the endogenous peroxidase activity, and incubated with primary antibodies (1:200 to 1:500 dilutions). Isotype-matched control antibodies were used under the same conditions for 1 hour. For enzymatic immunohistochemical staining, Zymed SuperPicTure polymer detection kit (Zymed/Invitrogen) was used according to the manufacturer's protocol. Subsequently, sections were counterstained with hematoxylin. For immunocytofluorescent analysis, cells were subcultured into eight-chamber slides (2 × 10⁴ cells per well) (Nunc). The cells were fixed in 4% formaldehyde for 15 min and then blocked and incubated with primary antibodies (1:200–1:500 dilutions) for 1 h, respectively. The samples were subsequently incubated with goat secondary antibodies of either IgG-rhodamine red or IgG-Cy2 (Jackson ImmunoResearch) for 45 min followed by counterstaining with DAPI (Chemicon) according to the manufacturer's instruction.

Results

Histological characteristics of apical papilla

A smooth-surfaced soft tissue was noted at the apex of extracted immature teeth and with a pair of pliers, this tissue (apical papilla) was easily detached from the apex exposing pulp tissue in the canal space. As shown in Fig. 1A, C, the apical papilla appears to be readily separated next to the pulp. Histological views of the longitudinal sections revealed that there is a cell rich zone between pulp and apical papilla (Fig. 1B). To distinguish the cell rich zone in mature pulp, here we term it "apical cell rich zone". Apical papilla appears to contain less blood vessels and cellular components than do the dental pulp and the apical cell rich zone.

Organ culture studies on cell proliferation in apical papilla

We asked whether cells in apical papilla proliferate at a different rate compared to cells in pulp when the extracted teeth are placed in cultures. BrdU was used to label the cells in the tissues in cultures. The results shown in Fig. 2 demonstrate that apical papilla contain 2- to 3-fold more BrdU positive cells than do pulp tissue in the same tooth 3.5 h or 18 h after the BrdU incorporation. Statistical analysis was examined by one-way factorial ANOVA followed by Fisher's protected least significant difference (PLSD) post-hoc test. The data suggest that once the tissues are placed in cultures, cells in apical papilla start to enter the growth cycle more than those in pulp.

In situ immunohistochemical analysis of osteo/dentinogenic markers

We next examined the in situ expression of osteo/dentinogenic markers in apical papilla. DSP, ALP, BSP, and OCN stained positively only in odontoblasts lined against the newly formed dentin (Fig. 3), whereas none of these markers were detected in the apical papilla.

Osteo/dentinogenic and adipogenic potentials

To compare osteo/dentinogenic and adipogenic potencies of SCAP and DPSCs to the most studied mesenchymal stem cells BMMSCs, cells were exposed to respective differentiation media. The results in Fig. 4 are representative data from several independent experiments of consistent results demonstrating that the accumulation of calcium in cultures of SCAP and DPSCs was comparable to that of BMMSCs (Fig. 4B, F, J, Alizarin red stain), whereas the adipogenic potential of SCAP and DPSCs was much weaker than BMMSCs. In Fig 4K, L, oil droplets accumulated in BMMSCs are clearly demonstrated by the oil red O stain and the positively stained adipocytes constituted up to 30–40% of cells in BMMSC cultures. In contrast, only a few cells in SCAP cultures showed the red stain (Fig. 4C, D, arrows) or very faint stain in DPSC cultures (Fig. 4G, H).

Immunophenotype of SCAP in cultures

To further characterize gene expression profiles of SCAP, we selected a list of genes that are important for defining MSCs. Semi-quantitative analysis was performed after the immunocytofluorescent staining and the gene expression profiles of SCAP and DPSCs were compared as shown in Fig. 5A. SCAP and DPSCs express similar osteo/dentinogenic markers and growth factor receptors, however, SCAP express less amount of DSP, MEPE, TGF β RII, FGFR3, Flt-1, Flag, and MUC18. Double staining experiments revealed that STRO-1 co-expressed with osteo/dentinogenic markers such as BSP, OCN, MEPE and growth factors FGFR1 and TGF β R1 (Fig. 5B). Approximately 20–30% of cells in cultures were positive for STRO-1 and ~70–80% positive for other markers. Additionally, we stimulated the SCAP with the neurogenic medium for 4 weeks followed by detection of neurogenic marker expression. The immunofluorescence images in Fig. 6 demonstrate the expression of β III tubulin, GAD, NeuN, nestin, GFAP, neurofilament M, NSE and CNPase.

Discussion

Dental papilla has been considered to be the source of odontoblasts during tooth development. As the differentiated odontoblasts lay down the primary dentin, the dental papilla becomes encased within the dentin structure and evolves into pulp tissue. The apical end of the dental papilla, however, has not been discussed much in the literature. It is generally believed that the formation of root dentin is the result of signaling from HERS to the adjacent undifferentiated mesenchymal cells which then turn into odontoblasts that are responsible for the root dentin formation. The anatomical location of these undifferentiated mesenchymal cells has not been clearly elucidated. They may be residing either in pulp or apical papilla. Dental pulp also harbors undifferentiated mesenchymal cells that are known to be capable of differentiating into new odontoblasts to replace the lost original odontoblasts. To make a clear distinction of the odontoblasts from a different source, we herein term the odontoblasts derived from dental papilla during the developing stage the “primary odontoblasts” that make primary and secondary dentin, as opposed to the “replacement odontoblasts” derived from pulp to replace primary odontoblasts and make tertiary dentin or more specifically, the reparative dentin. Some authors described the replacement odontoblasts as odontoblast-like cells (13–16).

Earlier and recent evidence has shown that replacement odontoblasts derive from underlying mesenchymal cells are located in cell rich zone and cell proper particularly in the perivascular region (17–19). As mentioned above, isolated DPSCs from pulp have shown the capacity to differentiate into odontoblast-like cells and produce ectopic dentin in immunocompromised mice (1). By detecting the expression of STRO-1, one of the markers that defines MSCs, DPSCs were found and considered to reside in the perivascular and perineurosheath regions (20). This finding corresponds to that concerning the source of replacement odontoblasts, which is from the perivascular regions where stem/progenitor cells reside. Currently, however, it is unknown where the DPSCs in the pulp come from.

In the present study, we first discovered the loose physical connection between the pulp and the apical papilla. The latter tissue can be easily detached or peeled off from the apex. Under the histological view, we observed a layer of densely populated cell rich zone (apical cell rich zone) between the two tissues. The vascular density in apical papilla appears lower than that of pulp. More accurate measurement using labeled antibodies to stain vascular antigens such as the endothelial marker CD146 and/or CD34 is needed to confirm this observation. When we placed the apical papilla in cultures, cells within the tissue showed more BrdU incorporation than those in the pulp in cultures. Previously we studied the proliferation rates of SCAP and DPSCs and found that the former proliferate faster with greater population doublings than the latter (3), which finding corresponds to that in the present study. One property of adult MSCs is that they normally remain in a non-proliferative, quiescent state in vivo until stimulated by

the signals triggered by tissue damage and remodeling (21). They enter the cell cycle when being isolated and exposed to cultural environments. It appears that similar activities in terms of proliferation are occurring when cells are still residing inside of the tissue which is being placed in cultures.

BMSCs are the gold standard MSCs in terms of multipotentiality. SCAP and DPSCs, on the other hand, appear to have a different profile of multipotency and are more committed to osteo/dentinogenicity (1,3,8). We confirmed the adipogenic aspect of the multipotentiality. Despite SCAP expressing many osteo/dentinogenic markers following *ex vivo* expansion, they express lower levels of DSP, MEPE, TGF β RII, FGFR3, Flt-1, Flg and MUC18 than do DPSCs. The *in situ* immunohistochemical staining of various osteo/dentinogenic markers revealed that only odontoblasts in the pulp express these genes, not in the apical papilla (Fig. 3).

Two criteria are normally used to define stem cells: i) capable of self-renewal and ii) give rise to progenitor cells that eventually differentiate into specialized cells. One approach to test the self-renewal property of stem cells is the population doubling (PD). The PD of SCAP was measured previously to be up to 80 (3), a number that is very high among identified postnatal stem cells. As a newly identified population of postnatal stem cells, SCAP exhibit heterogeneous nature by showing i) co-expression of STRO-1 with a variety of osteo/dentinogenic markers and ii) there are low percentage of STRO-1 positive cells while high percentage of cells positive with osteo/dentinogenic markers in cultures. In addition, SCAP show a positive staining for several neural markers including β III tubulin, GAD, NeuN, nestin, GFAP, neurofilament M, NSE, and CNPase by immunocytochemical staining. It is possible that SCAP are derived from neural crest cells or at least associated with neural crest cells, analogous to dental stem cells such as DPSCs and SHED (2,8).

The capacity of SCAP to differentiate into functional dentinogenic cells has been verified using animal models (3). *Ex vivo* expanded SCAP were transplanted into immunocompromised mice using hydroxyapatite/tricalcium phosphate (HA/TCP) particles as a carrier. A typical dentin structure was regenerated in which a layer of dentin tissue was formed on the surface of the HA/TCP along with connective tissue (3). To verify the functional role of root apical papilla, one approach is to remove this tissue and determine how that would affect the root development. We performed a preliminary study using minipigs as a model in which the apical papilla of one root of a molar was surgically removed at early developing stage and this halted the development of that particular root, despite the pulp tissue being intact. In contrast, other roots of the tooth containing apical papilla showed normal growth and development (data not shown). Based on our current and previous findings, it is tempting to speculate that SCAP, not DPSCs, are a cell source for the primary odontoblasts that produce root dentin as depicted in Fig. 7.

The discovering of stem cells in apical papilla may also explain a clinical phenomenon that was presented in a number of recent clinical case reports showing that apexogenesis can occur in infected immature permanent teeth with periradicular periodontitis or abscess (22–24). It is likely that SCAP residing in the apical papilla survived the infection due to their proximity to the periapical tissues. Therefore, after endodontic disinfection, under the influence of the survived HERS, these cells give rise to primary odontoblasts to complete the root formation.

In view of our present studies along with what we recently reported, we conclude that apical papilla harbors multipotent MSCs that express various MSC markers. They are capable of forming odontoblast-like cells and produce dentin *in vivo* and are likely to be the cell source of primary odontoblasts for the root dentin formation. Further research is needed to elucidate the relationship between cells in apical papilla and those in the apical cell rich zone between pulp and apical papilla, and their potential roles in the odontoblast differentiation.

Acknowledgments

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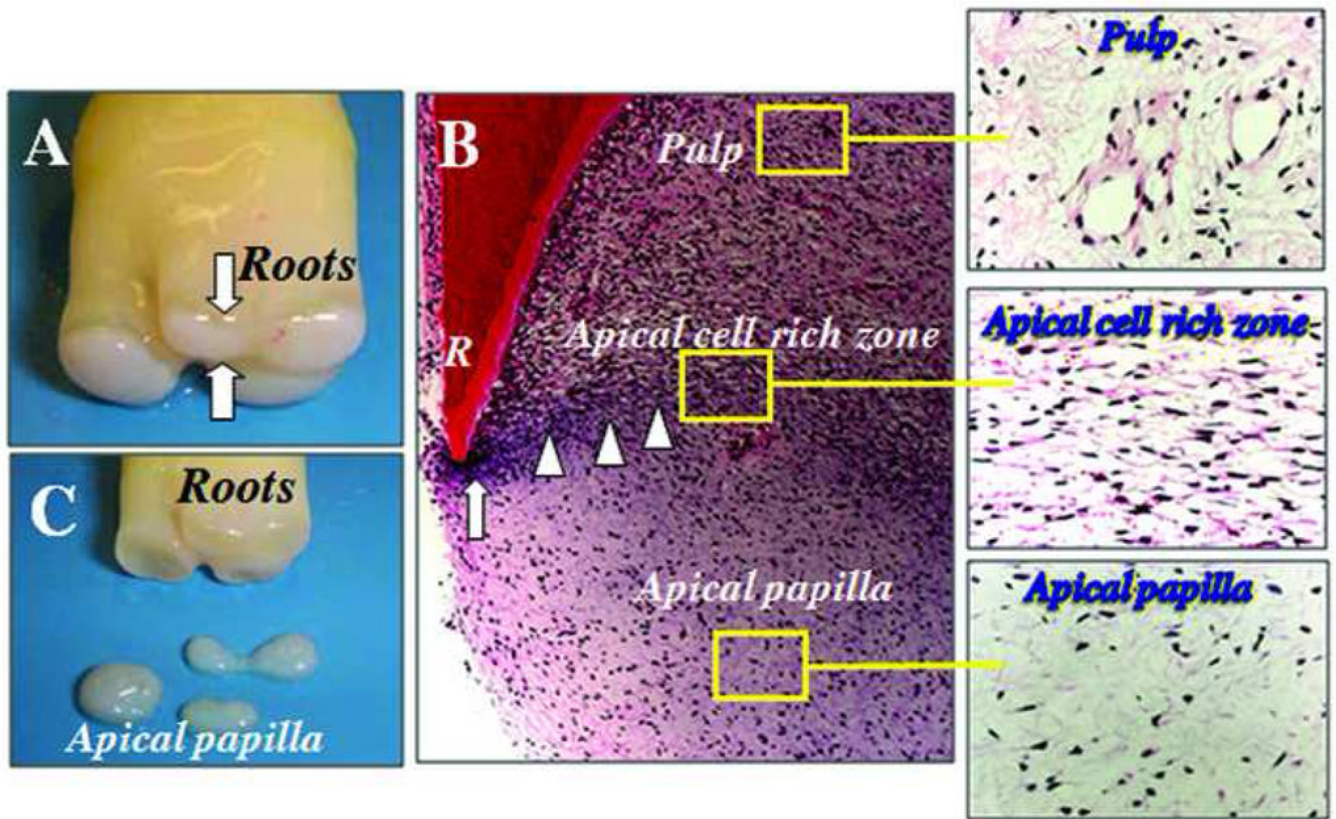


Fig. 1. Anatomy of apical papilla

(A) An extracted human third molar depicting root attached to the root apical papilla (open arrows) at developmental stage. (B) Hematoxylin and eosin (H & E) staining of human developing root (*R*) depicting epithelial diaphragm (open arrows) and apical cell rich zone (open arrowheads). (C) Harvested root apical papilla for stem cell isolation.

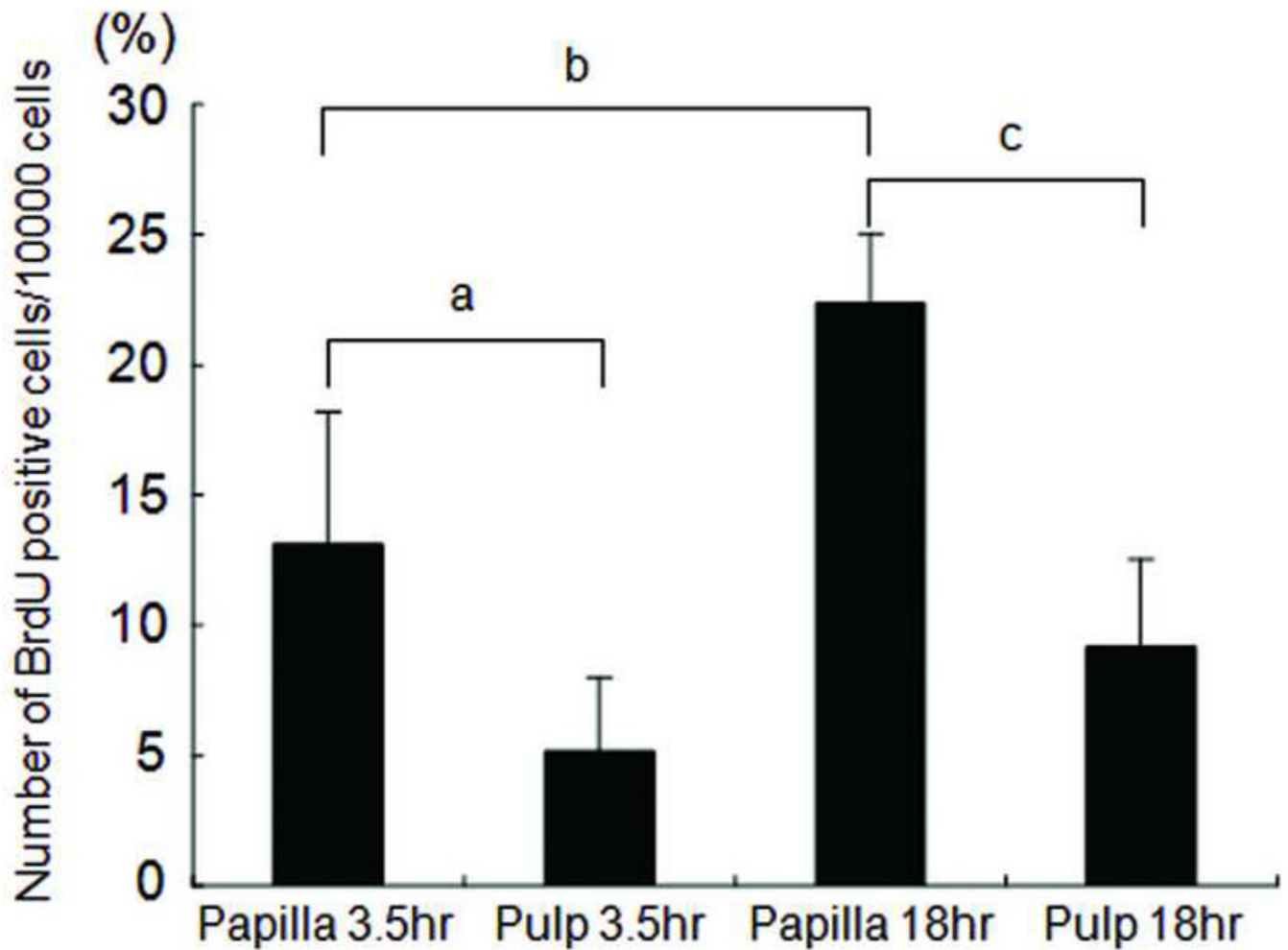


Fig. 2. Cell proliferation in pulp and apical papilla in cultures

In vitro BrdU labeling after 3.5 and 18 hs. Representative data (out of three independence experiments) indicate the numbers of BrdU positive cells in the pulp compared with those in the apical papilla of the same tooth. Four randomly selected fields per sample were used to count the positively stained cells under the microscope. Error bars: SD; a, $p=0.0091$; b, $p<0.0001$; c, $p=0.0002$.

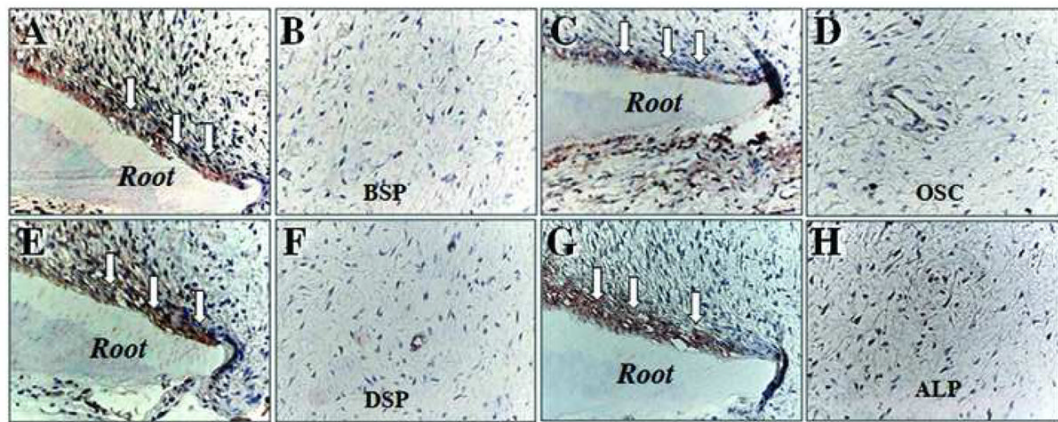


Fig. 3. Immunocytofluorescent staining of osteo/dentinogenic markers in pulp and apical papilla Odontoblasts (open arrows) express osteo/dentinogenic markers including BSP (A), OCN (C), DSP (E), and ALP (G). Apical papilla showed a negative immunohistochemical staining for BSP (B), OSC (D), DSP (F), and ALP (H).

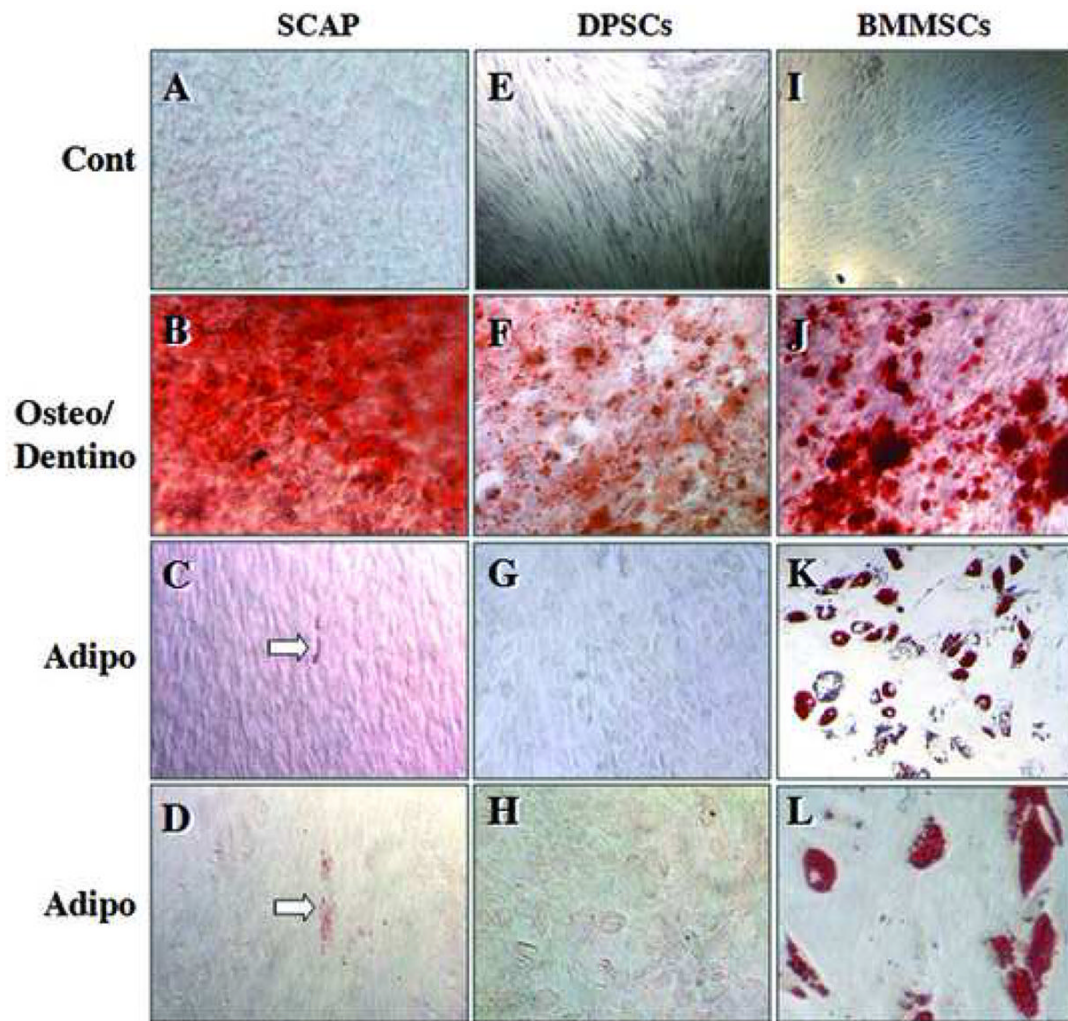


Fig. 4. Osteo-/dentino- and adipo-genesis of stem cells

SCAP (A–D), DPSCs (E–H) and BMMSCs (I–L). Control unstimulated (A, E, I); osteo/dentinogenic stimulation of cells for 8 weeks (B, F, J); adipogenic stimulation for 3 weeks (C–L). Open arrows indicate oil red O stain. Data are representative from several independent experiments [original magnifications, E, I: 50x; B, F, G, J, K: 100x; A, C, D, H, L: 200x]

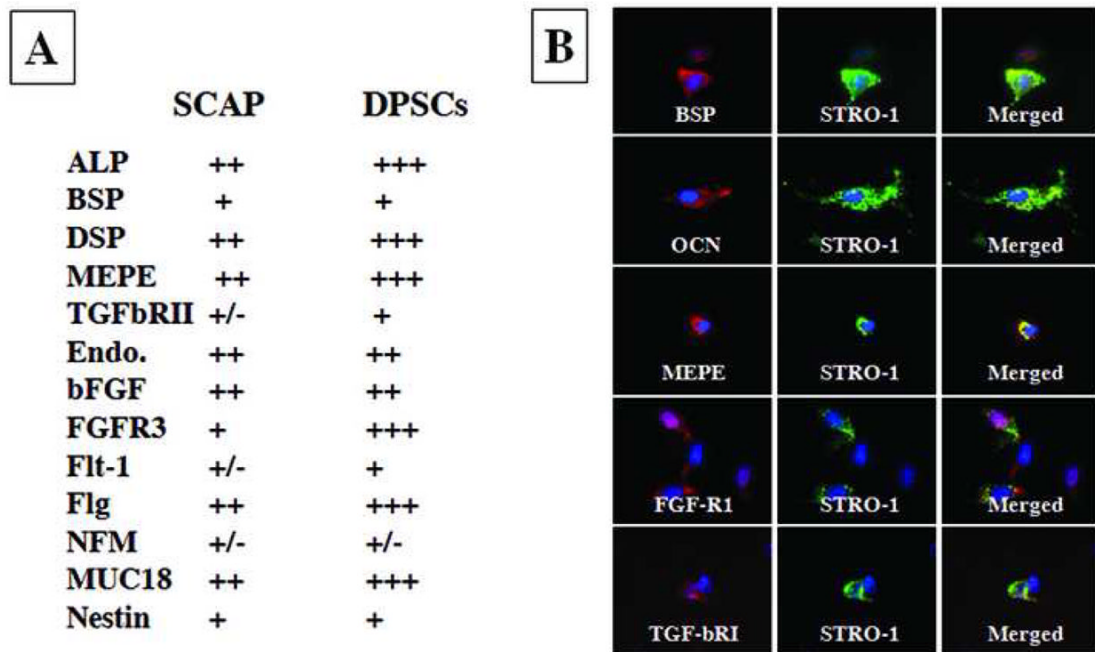


Fig. 5. Immunophenotype of SCAP

(A) SCAP and DPSCs express similar osteo/dentinogenic markers and growth factor receptors. SCAP express less amounts of DSP, MEPE, TGFβRII, FGFR3, Flt-1, Flg, and MUC18. +++, strong; ++, moderate; +, weak; -, negative. (B) Double staining of SCAP showing STRO-1 co-expressed with osteo/dentinogenic markers such as BSP, OCN, MEPE and growth factor receptors FGFR1 and TGFβRI. Cell nuclei exhibit in blue fluorescence by DAPI counter stain. Expression of markers showing either in red of green fluorescence. Images are representative data of independent experiments with consistent results.

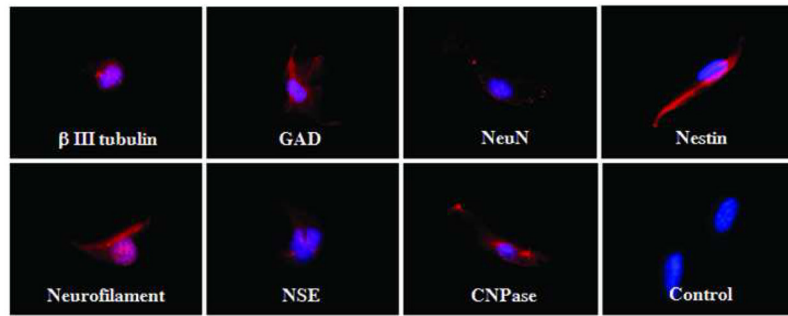


Fig. 6. Expression of neural markers in SCAP

Immunocytofluorescent staining of cultured SCAP expressing a variety of neural markers (red fluorescence) including β III tubulin, GAD, NeuN, nestin, GFAP, neurofilament M, NSE, and CNPase when cultured in neurogenic medium for 4 weeks. Blue fluorescence is the cell nucleus. Images are representative data of independent experiments with consistent results.

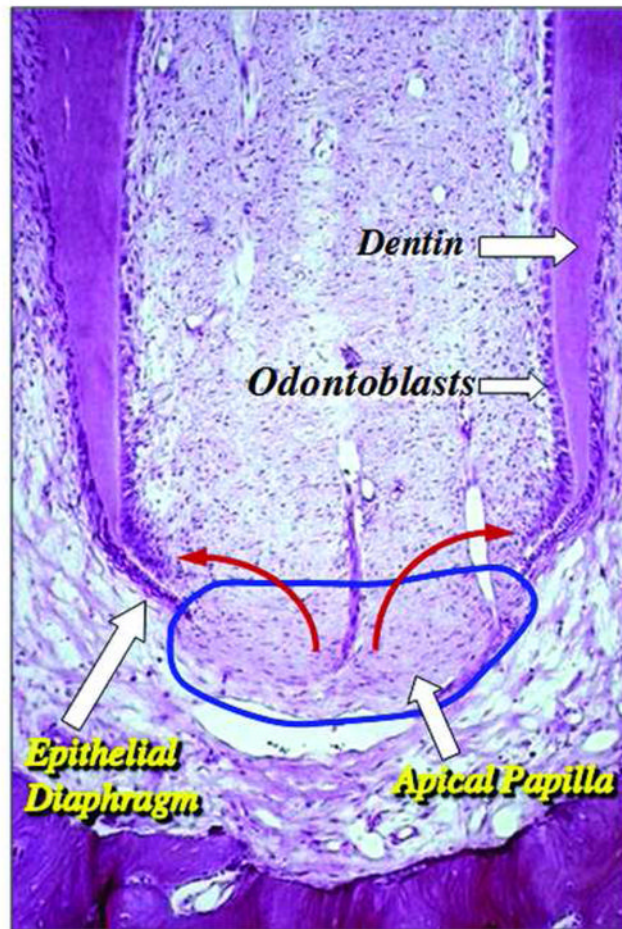


Fig. 7. Diagram of hypothetical cell source for root dentin development

Apical papilla (blue circle) contains stem/progenitor cells which may be the source of the odontoblasts that produce root dentin (red arrows). [Original histological image of immature tooth from *Macaca irus* monkey, courtesy of Dr. C. Torneck; original image published in *Principles and Practice of Endodontics*, 3rd ed., chapter 2, page 6, Fig. 2--2; permission of image reuse obtained from Elsevier Ltd.]