대한구강해부학회지 제 37권 제1호
Korean J Oral Anatomy Vol. 37, no. 1 (2016) pp.9~16

A comparison of odontogenic gene expression in crown and root odontoblasts

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접수: 2016년 11월 22일/ 수정접수: 2016년 12월 20일/ 게재 승인: 2016년 12월 26일/ 출간: 2016년 12월 31일

With the new functional discovery of stem cells from apical papilla (SCAP), there has been identified difference between SCAP and dental pulp stem cells (DPSCs) in gene expression. SCAP and DPSC, which are both human mesenchymal stem cells extracted from two histologically similar but distinct tissues, have different potential regeneration abilities. For deeper understanding of distinct regeneration ability, the difference among SCAP, crown pulp cells, and root pulp cells must be studied. The purpose of this paper is to identify the main differences of gene expression among SCAP, crown pulp, and root pulp and to discover the possibility of origin difference of crown dentin and root dentin. In the Real time PCR gene expression chart, crown pulp showed higher expression level of Runx2 than that of SCAP and root pulp. In the western blot assay, both expression levels c-IAP and CDK2 showed similar pattern. The gene expression levels were higher in SCAP and root pulp cells than in crown pulp cells. In addition, c-IAP and CDK2 expression levels in SCAP and root pulp cells were similar. The results suggested that these comparison data show that the origin of the crown pulp could be different from the origin of the root pulp. Thus, there is a possibility that the crown dentin and root dentin have different origin.

Keywords: Odontoblast, Crown, Root, Gene expression, Comparison

INTRODUCTION

Dentin takes up a large portion of the tooth and dental pulp stem cells (DPSCs) have been used to regenerate dentin[1]. The regeneration ability of stem cells has encouraged researchers to discover the mechanism of pluripotent cell differentiation and application for treatment purposes[2]. There are a number of studies related to stem cells from apical papilla (SCAP) and its potential application to regenerative biotechnologies. With the new functional discovery of SCAP, there has been identified difference

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between SCAP and DPSC in gene expression[1]. Previous reports showed that SCAP and DPSC, which are both human mesenchymal stem cells extracted from two histologically similar but distinct tissues, have different potential regeneration abilities[3]. For deeper understanding of distinct regeneration ability, the difference among SCAP, crown pulp cells, and root pulp cells must be studied. There have been cases where an immature incisor with its pulp exposed formed the tip of the root after the root canal treatment[4,5]. However, scientific reasoning behind this phenomenon should be further discussed.

The root tip that continued to form after pulp extirpation suggests the possibility of different origin between the crown dentin and the root dentin. In addition, gene expression difference among SCAP, crown pulp cells, and root pulp cells supports the possibility of different origin between crown and root dentin. The purpose of this paper is to identify the main differences of gene expression among SCAP, crown pulp, and root pulp and to discover the possibility of origin difference of crown dentin and root dentin.

MATERIALS AND METHODS

Primary Cell Culture

We collected human impacted third molars at Seoul National University Dental Hospital (Seoul, Korea). The experimental protocol was approved by the Institutional Review Board. Informed consent was obtained from the patients. To isolate SCAP and dental pulp cells, we separated the periapical SCAP from the surface of the tooth root and the dental pulp tissue from the pulp chamber and the root canal by cutting around the cemento-enamel junction and separated the root and crown pulp cells respectively with sterilized dental fissure burs. Pulp tissues were then minced to explants and placed in 60-mm culture dishes (Nunc, Rochester, NY). Explants were weighed down with a sterile cover glass and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and an antibiotic-antimycotic agent (Invitrogen, Carlsbad, CA) in a 5% CO_2 atmosphere at 37°C.

Cell proliferation assay

Cells at passage 2 were seeded onto 6-well plates (1000 cells per well) and counted using a Cell-Counting Kit-8 (WST-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions and at the indicated days, as previously described[6]. Each test was conducted three times and results are presented as mean ± standard deviation.

Real-time PCR Analyses

Total RNA was extracted from cells using TRIzol® reagent according to the manufacturer's instructions (Invitrogen). Total RNA (2 μg) was reverse transcribed for 1 h at 50°C with 0.5 mg Oligo dT and 1 μl (50 IU) Superscript III enzyme (Invitrogen) in a 20 μl reaction. One microliter of the RT product was PCR amplified using the primer pairs. For real-time PCR, the specific primers for *SURVIVIN*, *β-CATENIN*, *NFIC*, *RUNX2*, *OSTERIX*, and *COLLAGEN I* were synthesized as listed in Table 1. Real-time PCR was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA)

using SYBR GREEN PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. PCR conditions were 95°C for 1 min, 94°C for 15 s, and 60°C for 34 s for 40 cycles. All reactions were run in triplicate and were normalized to the housekeeping gene GAPDH. Relative differences in PCR results were calculated using the comparative cycle threshold (CT) method.

Table 1. Real-time PCR primer sequences

Gene name		Primer
SURVIVIN	forward	5'-AGG ACC ACC GCA TCT CTA CAT-3'
	reverse	5'-AAG TCT GGC TCG TTC TCA GTG-3'
β-CATENIN	forward	5'-CTG CCA TCT GTG CTC TTC GT-3'
	reverse	5'-TTA TCA GAG GCC AGT GGG AT-3'
NFIC	forward	5'-CGA CTT CCA GGA GAG CTT TG-3'
	reverse	5'-GTT CAG GTC GTA TGC CAG GT-3'
RUNX2	forward	5'-CCA ACC CAC GAA TGC ACT ATC-3'
	reverse	5'-TAG TGA GTG GTG GCG GAC ATA C-3'
OSTERIX	forward	5'-CCC CAC CTC TTG CAA CCA-3'
	reverse	5'-CCT TCT AGC TGC CCA CTA TTT CC-3'
COLLAGEN I	forward	5'-AAC AGC CGC TTC ACC TAC AG-3'
	reverse	5'-GGG AGG TCT TGG TGG TTT TG-3'
GAPDH	forward	5'-CCA TGG AGA AGG CTG GGG-3'
	reverse	5'-CAA AGT TCT CAT GGA TGA CC-3'

Western Blot Analyses

To prepare whole cell extracts, cells were washed three times with PBS, scraped into 1.5 ml tubes, and pelleted by centrifugation at 12,000 rpm for 2 min at 4°C. After removal of the supernatant, pellets were suspended in lysis buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% NP-40, 2 mM EDTA (pH 7.4)] and incubated for 15 min on ice. Cell debris was removed by centrifugation. Proteins (30 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). Membranes were blocked for 1 h with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBS-T), and incubated overnight at 4°C with the primary antibody diluted in PBS-T buffer (1:1000). Rabbit polyclonal anti-NFIC antibody was produced by immunization of rabbit with the synthetic peptides NH_2-RPTRPLQT-VPLWD-COOH (amino acid residues 427~439 of NFIC). C-IAP (sc-12410), CDK2 (sc-163), COL I (sc-59772), N-CADHERIN (sc-7937), p21 (sc-6246), OSTEOCALCIN (sc-30044), and GAPDH (sc-25778) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). β-CATENIN antibody was purchased from Thermo Scientific (Thermo Scientific, Rockford, IL). After washing, membranes were incubated for 1 h with anti-mouse (sc-2031), -rabbit (sc-2004), or -goat (sc-2768) IgG secondary antibodies conjugated to horseradish peroxidase (Santa Cruz). Labeled protein bands were detected using an enhanced chemiluminescence system (Dogen, Cambridge, MA).

Tissue Preparation and Immunohistochemistry

All animal experiments were performed according to the Dental Research Institute guidelines of Seoul National University. Mice teeth were decalcified in 10% EDTA (pH 7.4), embedded in paraffin, and processed for immunohistochemistry. Sections

were incubated overnight at 4°C with primary antibody (dilutions of 1:100–1:200). Secondary anti-rabbit IgG antibody was added to the sections for 30 min at room temperature, followed by reaction with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Signals were converted using a diaminobenzidine kit (Vector Laboratories). Nuclei were stained with hematoxylin[7].

RESULSTS

Characterization of SCAP, crown pulp, and root pulp cells

Morphological characteristics of SCAP and DPSC are different. DPSCs were heterogenous, containing cells ranging from narrow spindle-shaped to large polygonal. In contract, SCAP were smaller size and stellate in shape. Crown and root pulp cells showed heterogenous and narrow shape, whereas SCAP showed small stellate shape (Fig.1).

Cell proliferation of SCAP, crown and root pulp cells was investigated *in vitro* using the MTT assay. The proliferation rates of SCAP was a little bit higher than crown pulp and root pulp cells at early differentiation stages from day 1 to day 5. However, there was no significant differences among them (Fig. 2).

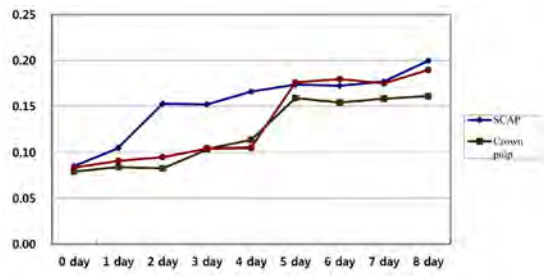

Figure 2. Cell proliferation assay. SCAP, crown pulp, and root pulp cells were seeded on 48-well plates at a density of 5×10^3 cells/well and cultured. Cell proliferation was evaluated by the MTT assay at 1-, 2-, 3-, 4-, 5-, 6-, 7-, and 8-day (Absorbance 540 nm).

Expression of odontoblast differentiation markers in SCAP, crown pulp, and root pulp cells

It is known that the expression level of survivin between SCAP and DPSC is different. Here, we checked the gene expressions of survivin, β-catenin, NFIC, Runx2, OSX, and Col 1 in SCAP, crown pulp, and root pulp by Real time PCR. In addition, we checked the gene expressions of c-IAP, CDK2, Col 1, β-catenin, NFIC, N-cadherin, p21, OC, and GAPDH in SCAP, crown pulp, and root pulp by western blot assay. In the Real time PCR gene expression chart, crown pulp showed higher expression level of Runx2 than that of SCAP and root pulp. Runx2 in SCAP showed similar gene expression level with that in root pulp. For

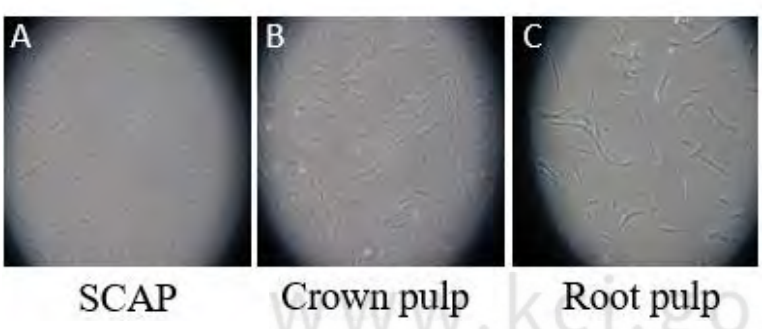

Figure 1. Morphology of cultured SCAP and pulp cells. SCAP (A), crown pulp (B), and root pulp (C) cells were cultured and observed under an optical microscope (X100).

the NFIC gene, SCAP, crown pulp, and root pulp were all different in gene expression level. Among six gene expression charts, survivin, Runx2, and OSX showed clear difference between the crown portion and the root portion and the genes expressed in root pulp were similar to those expressed in SCAP (Fig. 3). In the western blot assay, both expression levels c-IAP and CDK2 showed similar pattern. The gene expression levels were higher in SCAP and root pulp cells than in crown pulp cells. In addition, c-IAP and CDK2 expression levels in SCAP and root pulp cells were similar (Fig. 4). In immunohistochemistry, NFIC protein was expressed both in crown pulp and root pulp odontoblasts. However, NFIC protein expression was hardly detected in CAP. The expression levels of NFIC were higher in root odontoblasts than crown odontoblasts (Fig. 5).

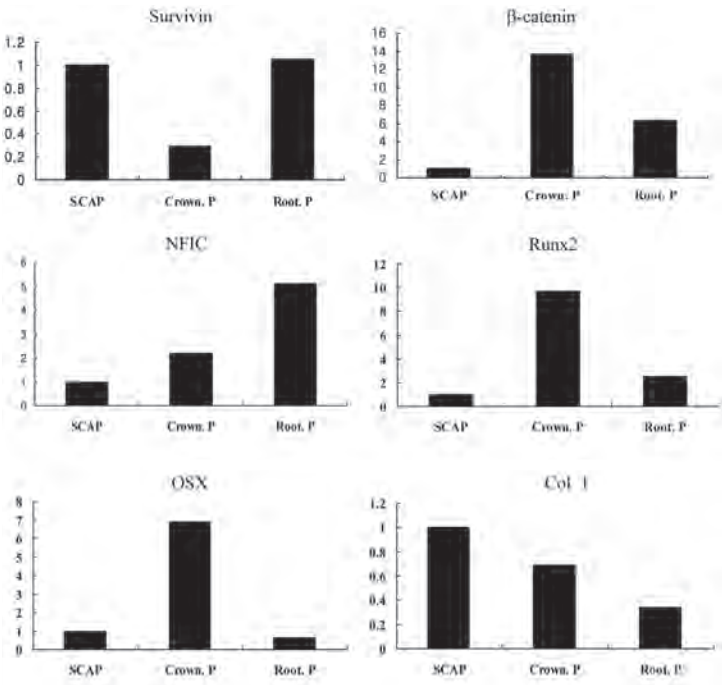

Figure 3. Gene expression of odontoblast differentiation markers in stem cells from apical papillae (SCAP), crown pulp, and root pulp. The expression levels of odontoblast differentiation markers *SURVIVIN*, β-*CATENIN*, *NFIC*, *RUNX2*, *OSTERIX*, and *COLLAGEN I* mRNAs were analyzed by real-time PCR.

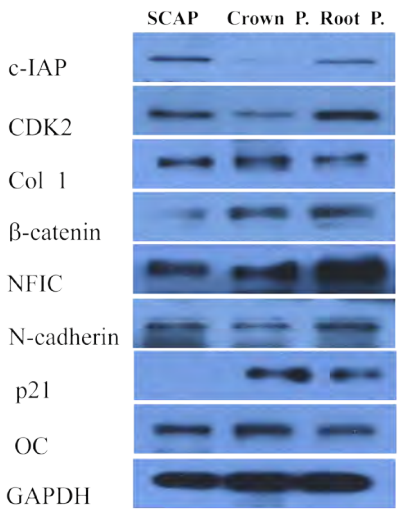

Figure 4. Protein expression of odontoblast differentiation markers in SCAP, crown pulp, and root pulp. The expression levels of odontoblast differentiation markers. C-IAP, CDK2, COL I, β-CATENIN, NFIC, N-CADHERIN, p21, OSTEOCALCIN, and GAPDH proteins were analyzed by western blotting.

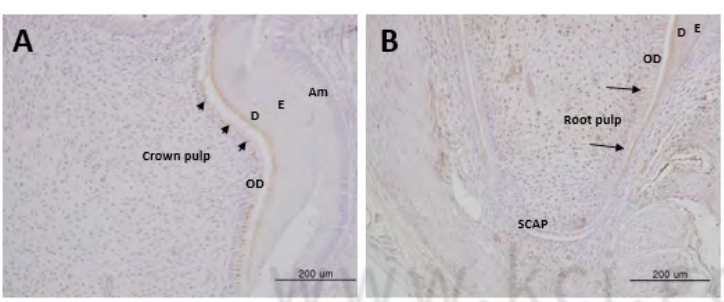

Figure 5. NFIC expression in odontoblasts. NFIC expression was detected by immunohistochemistry during mouse tooth development on postnatal days 10 (P10). NFIC protein was localized in odontoblasts of crown pulp (A, arrowheads) and root pulp (B, arrows). OD: odontoblast, D: dentin, E: enamel, Am: ameloblast, SCAP: stem cells from apical papilla. Scale bars, 200 μm.

DISCUSSION

Previous studies have supported that SCAP can be used as unique postnatal stem cells because of its unique tissue regeneration potential. Studies from previous researches have commonly identified higher expression of survivin and uptake rate for BrdU (bromodeoxyuridine) in SCAP compared to DPSC. Additional differences from previous studies were the number of population doublings and STRO-1 positive cells as well as the telomerase activity[8,9,10]. These differences represent SCAP as its unique postnatal stem cell and further gene expression differences among SCAP, crown pulp, and root pulp were studied to reveal the similarity between SCAP and root pulp as well as the disparity between SCAP and crown pulp[11,12]. In the gene expression levels of survivin, Runx2, and OSX, we found that genes were differently expressed in the crown pulp portion and the root pulp portion. In addition, the levels of these genes expressed in the root pulp portion were similar to the gene expression levels in SCAP. The different amount of gene expressed in SCAP and crown pulp suggests that crown pulp could not have been differentiated from SCAP but from another stem cell. According to our survivin gene expression data, the level of suvivin expressed in root pulp was nearly identical to that in SCAP. This result supports that the root pulp could have been differentiated from SCAP. As a result, these comparison data show that the origin of the crown pulp could be different from the origin of the root pulp. Thus, there is a possibility that the crown dentin and root dentin have different origin. However, the gene expression difference could have been caused by the spatial differences between the crown portion and root portion as well as the time difference of when the crown portion and root portion were created. Thus, further studies need to be done to confirm the different origin possibility of the crown dentin and root dentin.

There are a number of studies that revealed the unique regeneration ability of the root portion stem cells. There is a study that used Hertwig's epithelial root sheath to develop root generation[13,15]. There is one study that showed swine root tooth regeneration using SCAP along with periodontal ligament stem cells[14]. In addition, other studies showed that Wnt/β-catenin and insulin-like growth factor promote SCAP differentiation and that basic fibroblast growth factor promotes SCAP proliferation[15,16,17]. Acknowledged as a unique stem cell, SCAP will have an important role in bioroot engineering. In order to develop the potential regeneration ability of SCAP and use it to regenerate a functional tooth, further explanation of the origin and mechanism is necessary. Discovering more differences between the crown portion and root portion will improve ways to use different stem cells accordingly and this will enhance the functional tooth regeneration technology.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Research Foundation of Korea (NRF-2016R1A2B3006584).

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한글초록

치관과 치근 상아질모세포의 치아관련 유전자 발현 비교

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최근에 치근단줄기세포가 새롭게 발견되면서 이 세포의 유전자 발현양상이 치수줄기세포와 다르다는 연구결과가 알려지고 있다. 따라서 치근단줄기세포와 치관의 치수세포 그리고 치근의 줄기세포 사이의 재생능의 차이에 관한 연구가 필요하다. 이 연구에서는 치관 상아질과 치근상아질의 기원의 차이와 이에 따른 특성의 차이를 알아보기 위하여 치근단줄기세포와 치관의 치수세포 그리고 치근의 줄기세포의 유전자 발현 양상을 비교 분석 하였다. 연구결과, real-time PCR 분석에서 치관의 치수세포 가 치근단줄기세포와 치근의 줄기세포 보다 강한 *Runx2* 발현을 나타냈다. Western 분석에서 c-IAP와 CDK 단백질의 발현은 치관의 치수세포에서 보다 치근단줄기세포와 치근의 줄기세포에서 뚜렷하였다. 반면에 치근단줄기세포와 치근의 줄기세포에서 c-IAP와 CDK 단백질의 발현 양상은 유사하였다. 이 연구 결과로 보아 치관의 치수와 치근단의 치수가 서로 다른 특성을 보이는 것은 그들의 발생학적 기원이 다르기 때문임을 시사한다.

주제어: 상아질모세포, 치관, 치근, 유전자, 발현비교