Histological study and localization of stem/ progenitor cells in human dental follicle

(ヒト歯嚢の組織学的研究および幹細胞分布について)

日本大学大学院松戸歯学研究科歯学専攻 [1]

日本大学松戸歯学部 顎顔面外科学講座 [2]

日本大学松戸歯学部　病理学講座 [3]

日本大学松戸歯学部　口腔科学研究所 [4]

加藤　有悟 [1]

末光　正昌 [3, 4]

髙橋　康輔 [2, 4]

(指導 ： 近藤　壽郎　教授)

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Yugo Kato,[1] Masaaki Suemitsu,[3, 4] and Kosuke Takahashi[2, 4]

[1] Nihon University Graduate School of Dentistry at Matsudo, Maxillofacial Surgery, Matsudo, Chiba 271-8587, Japan

Departments of [2]Maxillofacial Surgery and [3]Pathology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

[4] Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Correspondence to: Yugo Kato

E-mail: yugokatodent@gmail.com

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Abstract

A human dental follicle (hDF) is a connective tissue that comprises ectomesenchymal cells, which originate from the neural crest, and contains stem cells and/or progenitor cells of the periodontium. hDF have the capacity to commit to differentiation into multiple cell lineages, such as osteoblastic, adipogenic, and neurogenic. The aim of this study was to examine the histological findings and localize stem/progenitor cells in hDF. Our findings revealed that the hDF structure comprised 3 layers, including the outer, intervening, and inner layers. The outer layer seen to comprise fibrous connective tissue that contained fibroblasts and some peripheral nerves. The inner layer of the intervening layer comprised dense fibrous connective tissue with many fibroblast - like cells. The intervening layer contained eosin-stained collagen fiber. The collagen fibers were arranged in the interior but were irregular in the outer layer.
The intervening layer comprised slightly dense fibrous connective tissue, which contained The hDF samples were stained for the suggested markers for mesenchymal stem cells (i.e., CD90, CD106, and CD271). The cells positive for CD90 and CD106 were most frequent in the inner layer of the crown side, whereas those that were positive for CD271 were frequent in the outer layer of crown side. The hDF samples were stained for the suggested markers for pluripotent stem cells (i.e., CD15, STRO-1, and Notch-1). The cells positive for these markers were most frequent in the outer layer of root side. In conclusion, hDF comprised a 3-layer structure of outer, middle, and inner layers. Several cells that expressed stem cell markers were found in the outer layer of the hDF.

Introduction

The human dental follicle (hDF) is an ectomesenchymal tissue that surrounds the developing tooth germ and has an embryonic neural crest origin (1). It contains resident stem/ progenitor cells, such as osteoblast/ cementoblast precursor cells, which are commonly

referred to as dental follicle stem cells (DFSCs) (1-3). We have previously reported that human dental follicle cells (hDFCs) had the capacity to differentiate to osteoblastic (1), adipogenic (2), and neurogenic lineages (4) and that hDFCs had gene expression profiles during osteogenic differentiation (5). hDFCs can be easily harvested from patients who undergo extraction of an impacted 3rd molar tooth or supernumerary tooth and are usually discarded after extraction. Therefore, hDFCs could be a source for regenerative medicine and can be collected without additional invasive methods. The use of hDFCs for regenerative medicine has great potential.

Recently, numerous studies have published the potential of stem/ progenitor cells for multilineage differentiation from dental follicles in vitro (1-5). However, only few studies have histologically examined and analyzed the localization of stem/ progenitor cells in hDF. To establish suitable means of identifying progenitor populations in hDF, the histological characteristics and the localization of stem/ progenitor cells have to be investigated. Cell surface markers have been described for the identification of the potential for multilineage differentiation of stem/ progenitor cells isolated from embryonic and adult tissues from multiple species (6-11). The selective use of the combination of markers CD90, CD106, CD271 has been shown to enable isolation of the most potent and genetically stable mesenchymal stem cells (MSCs) (6,7). Notch-1, STRO-1 and CD15 have been reported as the stem cell markers that modulate stem/ progenitor cell differentiation (8-11).

The aim of this study was to isolate potential stem cells from hDF by performing histological study and localization of stem/ progenitor cells by immunocytochemistry and immunohistochemistry of hDF.

Materials and Methods

Collection of human dental follicle

The hDF were obtained from individuals aged 15-20 years, after obtaining informed

consent, and bases on a previously reported method (1). Briefly, impacted mandibular 3rd molars were surgically removed and collected from humans. All the extracted tooth roots were immature. Experiments using hDF were performed in accordance with the guidelines established by the Ethics Committee of Nihon University School of Dentistry at Matsudo (Recognition number: EC17-15-040-1).

Specimen preparation and staining

The excised samples were fixed in 10% neutral-buffered formalin solution and embedded in paraffin. Semi-serial 4-μm- thick sections were obtained in a sagittal orientation; stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) alcian blue (AB) ; and observed on light microscopy.

Cell cultures

Impacted teeth were surgically removed and collected. The hDF tissues were digested in a solution of 0.1 U/ mL type 1 collagenase and 1 U/ mL dispase (Roche, Mannheim, Germany) for 1 hour at 37 °C. The attached hDFCs were cultured in 100-mm dishes using mesenchymal stem cell (MSC) growth medium, which comprised an MSC base medium supplemented with fetal bovine serum, penicillin/ streptomycin, and L-glutamine (Lonza, Walkersville, MD, USA) in an incubator (MCO-175M; Sanyo, Osaka, Japan) with 5 % CO_2 in air at 37 °C.

Immunocytochemistry

The hDFCs were seeded at a density of 5×10^4 cells/ well on 2-well chamber slides (Thermo Fisher Scientific, NY, USA) and were cultured in growth medium for 24 hours. The culture medium was then removed and the cells were washed 3 times with phosphate-buffered

saline (PBS). The cells were fixed with 10% formalin neutral buffer solution for 30 minutes at room temperature, in 0.1% Triton X-100 (Thermo Fisher Scientific, NY, USA) and blocked with 10% normal goat serum (Thermo Fisher Scientific, NY, USA) in PBS. Immunostaining of the hDFCs was carried out using the ChemMate ENVISION KIT (Dako Gloustrup, Denmark) and anti-human CD90 monoclonal antibody (Thy-1, 1:500; abcam, Cambridge, UK); mouse CD106 monoclonal antibody (VCAM-1, 1:10; Dako Gloustrup, Cambridge, UK); mouse CD271 monoclonal antibody (LNGFR, 1:50; abcam, Cambridge, UK); mouse CD15 monoclonal antibody (SSEA-1, 1:200; abcam, Cambridge, UK); mouse STRO-1 monoclonal antibody (STRO-1, 1:50; Santa Cruz, California, USA); and rabbit Notch-1 monoclonal antibody (1238Y, 1:100; abcam, Cambridge, UK) as the primary antibody. 3,3'-diaminobenzidine was subsequently applied. Specimens were counterstained with Mayer's Hematoxylin.

Histologic analysis and immunohistochemistry

The hDF with tooth were washed in physiologic saline solution and fixed in 10% neutral-buffered formalin solution for 48 hours; decalcified in 10% ethylenediaminetetraacetic acid (pH 7.0); and embedded in paraffin. Semi-serial 4-μm-thick sections were obtained in the sagittal orientation, stained with H&E, and observed on light microscopy. Immunostaining of the hDFCs was carried out using the ChemMate ENVISION KIT (Dako Gloustrup, Denmark) and anti-human CD90 monoclonal antibody (Thy-1, 1:500; abcam, Cambridge, UK); mouse CD106 monoclonal antibody (VCAM-1, 1:10; Dako Gloustrup, Denmark); mouse CD271 monoclonal antibody (LNGFR, 1:50; abcam, Cambridge, UK); mouse CD15 monoclonal antibody (SSEA-1, 1:200; abcam, Cambridge, UK); mouse STRO-1 monoclonal antibody (STRO-1, 1:50; Santa Cruz, CA, USA); and rabbit Notch-1 monoclonal antibody (1238Y, 1:100; abcam, Cambridge, UK) as the primary antibody.

Measurement of immunohistochemical staining

The staining intensity was measured by an upright microscope (System bio microscope, BX51-P, Olympus, Japan). Cells with cytoplasm or nucleus that stained brown were considered positive. The hDF were divided into 9 regions for the purpose of comparing the distribution attitudes by region.

As for the positive cells, an area photographed with an objective lens (magnification: 60) was extracted using an image processing software Image J in an area having a certain brightness or less. Then, positive cells per visual field extracted under visual observation were counted. Five fields were photographed from each of the 9 areas, and the average number of positive cells per field was calculated.

Results

Histological findings of the hDF

The outer surface of an hDF had a cap-like shape that was wrapped around the tooth crown; it was thin in the cervical region and thick in the crown region (Fig. 1A). In the sagittal plane section, the hDF had a 3-layer structure, with the outer layer seen to comprise fibrous connective tissue that contained spindle shaped cells and some peripheral nerves (Fig. 1B). Inside of the intervening layer comprised dense fibrous connective tissue with dense fibroblast-like cells. The intervening layer contained eosin-stained collagen fiber. Collagen fiber in outside of the intervening layer were irregular (Fig. 1C). The inner layer was observed to have relatively dense capillaries, odontogenic epithelial islands, and mesenchymal cells with spindle-shaped or elliptical nuclei (Fig. 1D). Single-or double-layered reduced enamel epithelium was observed in the inner layer area adjacent to the tooth crown (Fig. 1E). On PAS and AB staining, the 3-layered hDF was observed to have weakly positive outer layer, positive

intervening layer, and weakly positive inner layer (Fig. 1F).

Immunocytochemical findings of hDF

The immunocytochemical findings for CD90, CD106, CD271, CD15, STRO-1, and Notch-1 are shown in Figure 2. A positive reaction with a dark brown color was demonstrated for STRO-1 in the cell nucleus and for CD15, CD90, CD106, CD271, and Notch-1 on the surface.

Immunohistochemical findings of hDF

The immunohistochemical findings for CD90, CD106, CD271, CD15, STRO-1, and Notch-1 are shown in Figure 3. A positive reaction with a dark brown color was demonstrated for STRO-1 in the cell nucleus and for CD90, CD106, CD271, CD15 and Notch-1 on the cytoplasmic surface.

Number of positive cells in the analytical region of the hDF

Several CD90 - and CD106 - positive cells were found in the superior inner region red area. Several CD271 - positive cells were found in the superior outer region (orange area). Cells that were positive for CD15 and STRO-1 were mostly observed in the middle outer region (blue area). Cells that were positive for Notch-1 were frequently observed in the inferior outer region (green area) (Fig. 4A). CD271 - positive cells were found in the outer crown layer of the area, as shown in orange. CD90 - and CD106 - positive cells were found in the inner crown layer of the area, as shown in red. CD15 - and STRO-1 - positive cells were found in the outer cervical outer layer, as indicated by the blue area, and several Notch-1 - positive cells were found in the outer cervical outer layer of the green area (Fig. 4B).

Discussion

In this study, we observed on the histological findings that hDF was in the form of a cap that was wrapped around the crown. The crown was almost completely formed, but the roots were in the process of being formed, probably because the hDF samples were collected from relatively young individuals aged 15-20 years. Histological examination of the hDF on H&E staining showed that the outer and inner layers, especially the latter, comprised rich collagen fibers. We confirmed that the outer layer comprised sparse fibrous connective tissue among several peripheral nerves as showing in Fig.1B.

Consistent with a previous report (12), the inner layer was observed to have relatively dense capillaries, odontogenic epithelial islands, and mesenchymal cells with spindle-shaped or elliptical nuclei (Fig. 1D)

According to embryological studies, nerve cells are found in the vicinity of the hDF as condensing mesenchyme in the tooth capping stage (13). This suggested that hDF contain nerve cells, even in 15-20-year-old individuals with unmerged teeth. Consistent with the findings of previous reports (14,15), a thin cubic or cylindrical single-or double-layer of reduced enamel epithelium was observed on the surface adjacent to the tooth crown. The reduced epithelium adhered to and covered the enamel just before eruption (16). In terms of embryology, the sheet-like continuity should have been maintained, but in our samples, the continuity was lost in all hDF; this was thought to have been brought about by a weak adhesion between the hDF and the reduced enamel epithelium.

hDF reportedly displayed layer structure(15). In this study, we reviewed again the 3-layer structure of the hDF. Double staining with PAS and AB confirmed the presence of mucus degeneration in the 3-layer structure of the hDF. In particular, PAS staining demonstrated weakly positive inner layer, positive intervening layer, and weakly positive outer layer against a background of weakly positive findings on AB staining. Neutral mucopolysaccharides are stained red with Schiff reagent, and acidic mucopolysaccharides are

stained blue with AB. The positive reaction for PAS staining suggested the presence of neutral mucopolysaccharides in the intervening layer, whereas the weakly positive PAS staining and positive AB staining suggested the presence of acid mucopolysaccharides in the outer and inner layers.

Next, we examined the distribution of stem/progenitor cells in hDF using immunohistochemistry. MSCs or stromal cells are defined as non-hematopoietic, plastic-adherent, self-renewing cells that are capable of in vitro trilineage differentiation into fat, bone, and cartilage. The combination of positive staining for CD90, and CD106, CD271 can be used selectively to isolate the most potent and genetically stable MSCs. We detected and localized the hDFCs that were positive for the markers CD90, CD106, CD271. Although the immunochemical study was performed by single staining, CD90, CD106, and CD271 were expressed in hDFCs. On immunohistochemistry of hDF, the cells positive for CD90 and CD106 were detected on the inner layer of superior region (i.e., crown side of the tooth), and the cells positive for CD271 were detected on outer layer of superior region (Fig. 4). These data revealed a remarkable MSC distribution in the inner and outer layers of the crown side of hDF. A previous study reported that mesenchymal cells in the inner layer were positive for vimentin and α-smooth muscle actin (12). CD15 and STRO-1 have been reported to confirm the promotion of stem cell differentiation and migration (8,9). Notch-1 has been reported to be involved in the cell differentiation of nerves and blood vessels (10,11). In this study, CD15, STRO-1, and Notch -1, which are markers for pluripotent stem cells, were observed in the outer layer of the tooth neck (Fig. 4). At the time of hDF collection for this study, we suggested careful detachment from the surrounding connective tissue, in order to improve the collection of the hDF tissues from the outer layer and obtain a more efficient cell source.

Therefore, We suggested that hDFCs may be one of optimal reserved of regenerative medicine cells that could be collected more efficiently by separating cells from the outer layer

of the hDF tissue. It has been reported that the isolated hDFCs differentiates into various cells by cell culture using bone differentiation medium, fat and nerve differentiation medium. The result of this experiment suggests that when it becomes possible to collect cells, it is suggested that cells for regenerative medicine can be supplied more quickly.

Although the MSCs, which are multipotent and have excellent proliferation ability, were suggested to express all 3 stem cell markers CD90, CD106, and CD271, immunochemical staining was performed by a single staining in this experiment. Further study using double immunostaining is needed for a more detailed examination.

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Figure legends

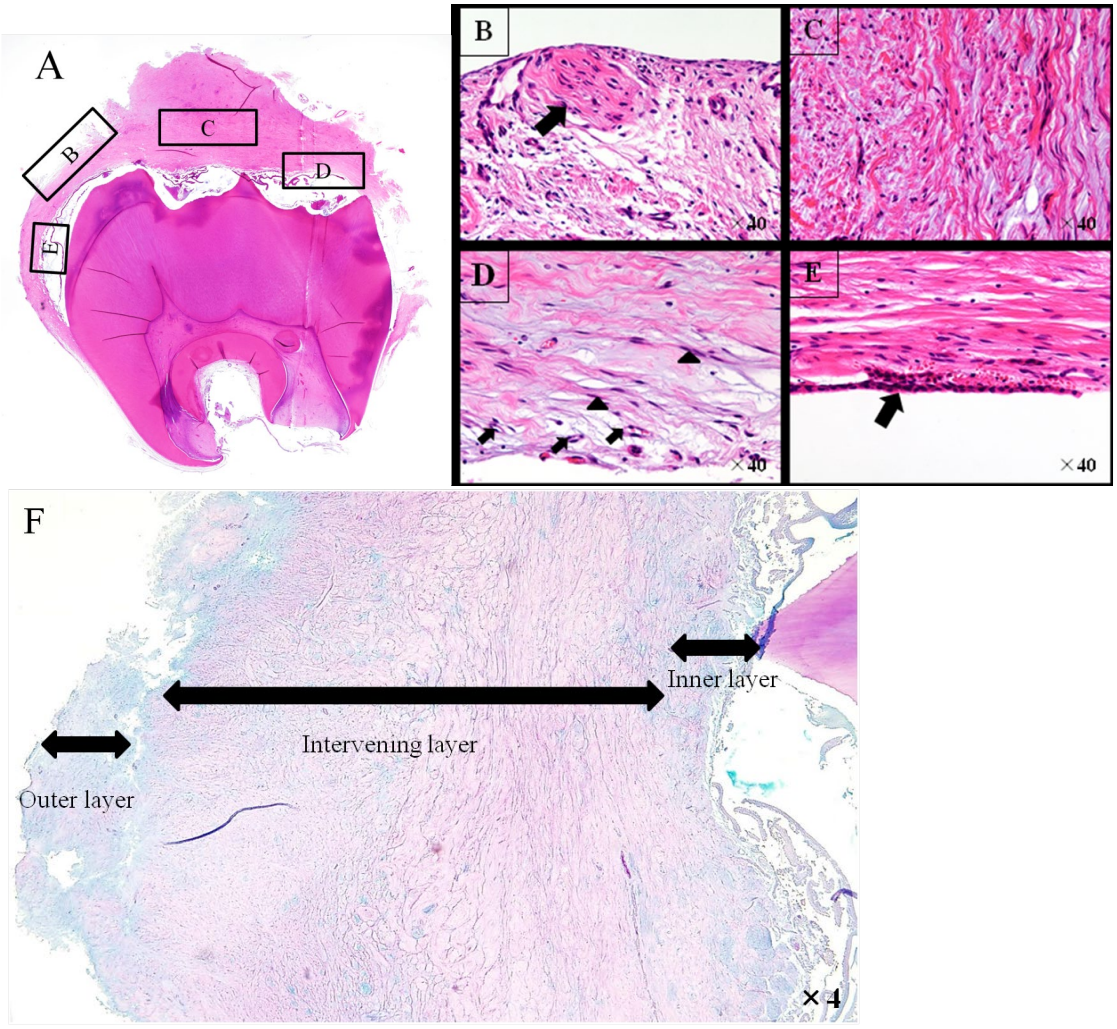


Fig.1 Histological findings of human dental follicle (hDF)

A : Loupe image of H&E-stained hDF and tooth (H&E).

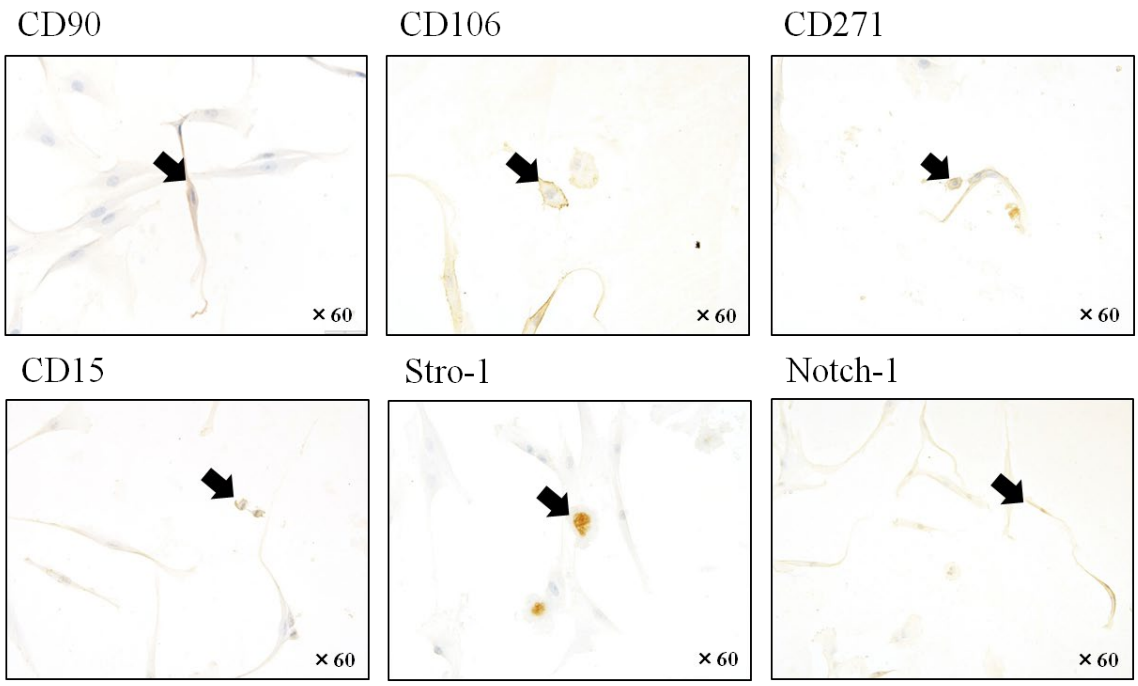
B: Outer layer of the hDF (H&E, ×40).

C: Intervening layer of the hDF (H&E, ×40).

D: Inner layer of the hDF. A arrows show capillaries, and arrow heads show mesenchymal cells (H&E, ×40).

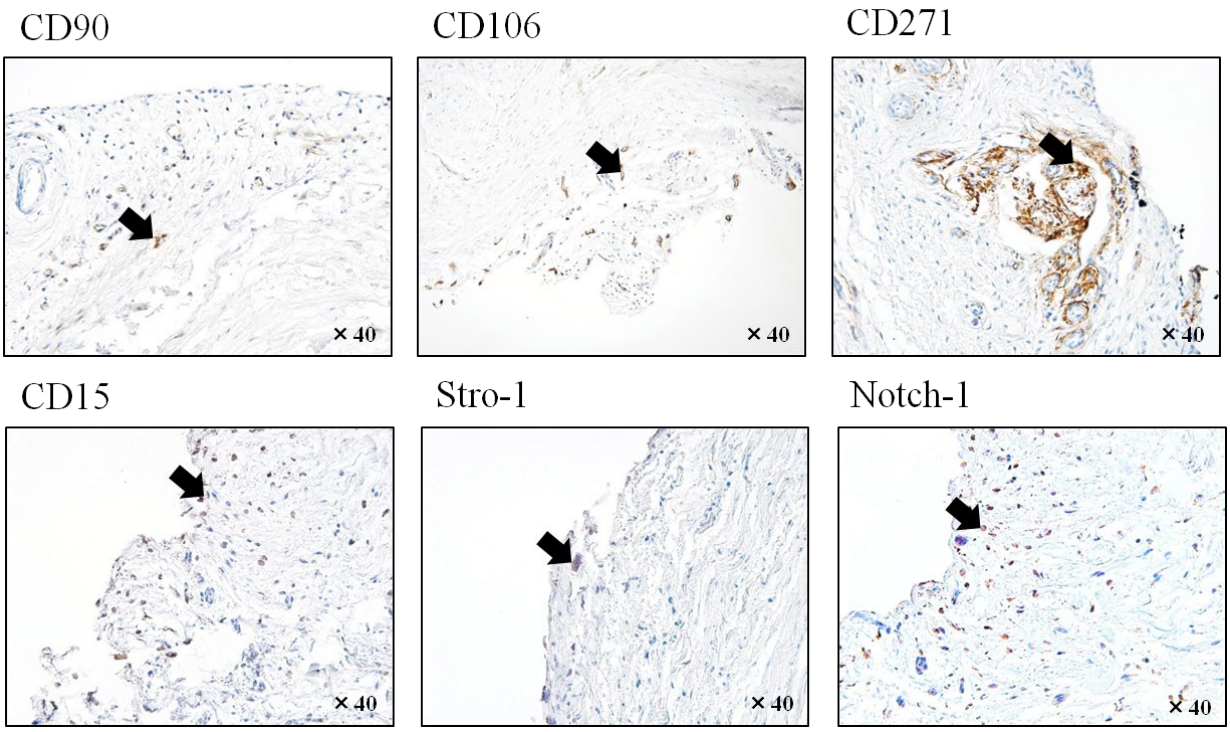
E: Inner layer of the reduced enamel epithelium. A arrow shows reduced enamel epithelium (H&E, ×40).

F: Image of the hDF (PAS and AB, ×4).



Immunocytochemical staining for stem cell marker in dental follicle cell

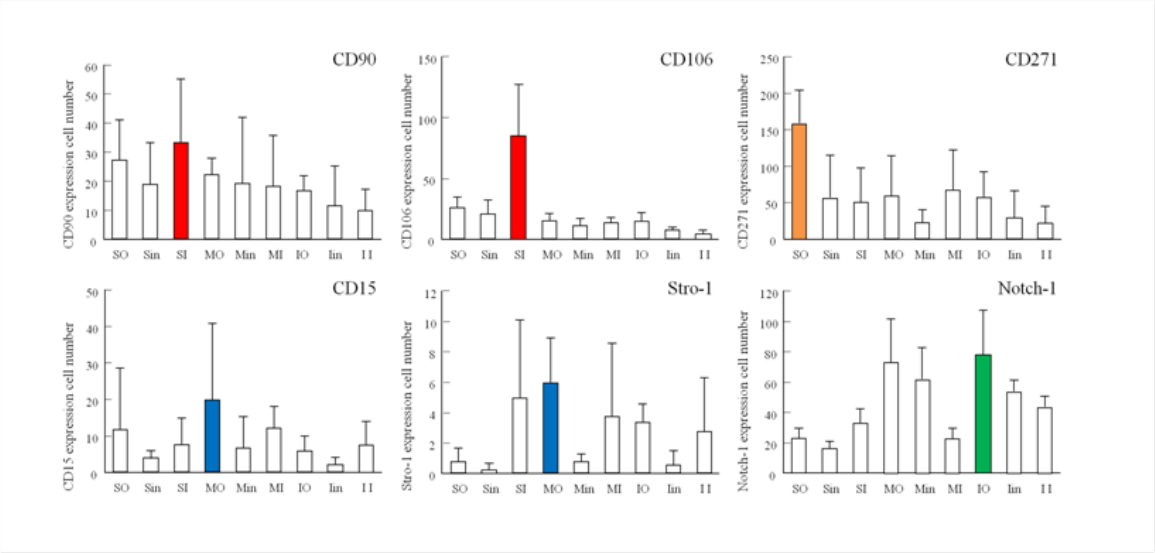
Fig.2 Immunocytochemical staining using anti-CD90, CD106, CD271, CD15, STRO-1 and Notch-1 antibodies (×60).



Immunohistochemical staining for stem cell marker in dental follicle tssiue

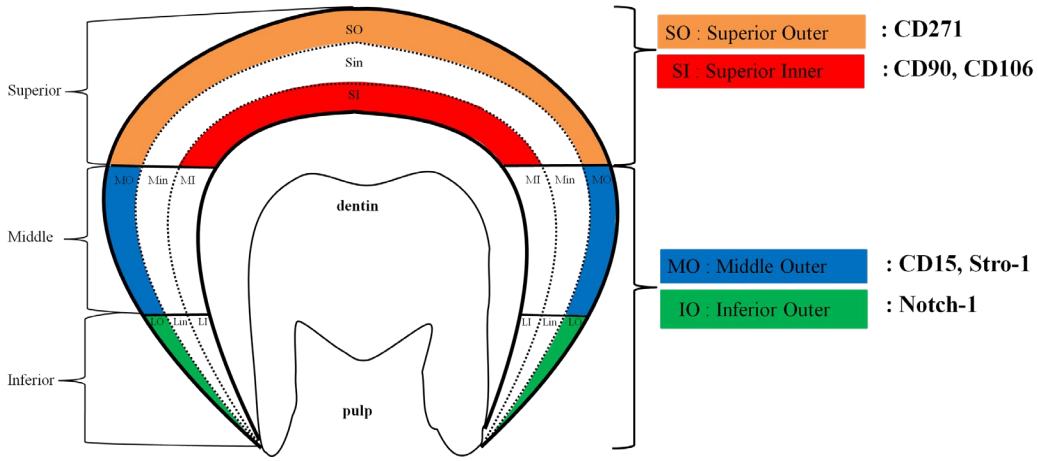
Fig.3 Immunohistochemiscal staining using anti-CD90, CD106, CD271, CD15, STRO-1 and Notch-1 antibodies (×40)

A



SO : Superior Outer
Sin : Superior Intervening
SI : Superior Inner
MO : Middle Outer
Min : Middle Intervening
MI : Middle Inner
IO : Inferior Outer
Iin : Inferior Intervening
II : Inferior Inner

B



Area of mesenchymal and/ or stem cell marker positive cells

Fig.4 Number of CD90, CD106, CD271, CD15, STRO-1 and Notch-1 positive cells in the region of interest (A). Analyzses of hDF and tooth schema, CD90, CD106, CD271, CD15, STRO-1 and Notch-1 (B).