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Augmentation of enamel mineralization by APin

조선대학교 대학원

치의공학과

박 종 태

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*APin*에 의한 법랑질 석회화 증진

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ABSTRACT

Augmentation of enamel mineralization by APin

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APin was previously suggested to have a functional role in the mineralization and maturation of enamel, which is mediated by MMP-20 and tuftelin expression. In the present study, the biological function of APin was examined during ameloblast differentiation and enamel mineralization *in vitro*.

An APin recombinant protein was produced and stable APin transgenic cell lines were also established using ameloblast-lineage cells (ALCs). The cells were cultured for 2 weeks with or without the APin recombinant protein in a differentiation and mineralization medium. The alkaline phosphatase (ALP) activity was measured and the level of mineralization was determined by staining the

calcified nodules with Alizarin red S stain. A high-density protein microarray was also performed to identify the other proteins interacting with APin. A subset of genes that reacted strongly with APin was selected. The expression of these genes was evaluated by RT-PCR after the inactivation or overexpression of APin.

Mineralization was augmented by the ALCs treated with the APin recombinant protein and the sense APin overexpressing cells. The ALP activity was also increased markedly in the sense APin overexpressing cells and the ALCs treated with APin recombinant protein. The inactivation of APin in the ALCs down-regulated the expression of BMP receptor 1B (BMPR-1B) and the EF hand calcium binding protein 2 (CBP2), whereas its expression was up-regulated markedly when APin was overexpressed. However, APin did not affect the mineralization of osteoblasts and periodontal ligament cells (PDL cells).

These results provide deeper insights into the process of ameloblast maturation and in enamel mineralization. It also suggested that APin augmented enamel mineralization.

I. INTRODUCTION

Tooth development occurs through a series of complex steps that are controlled by an epithelium-mesenchymal interaction between the dental epithelium and ectomesenchyme (1). The production of the enamel matrix by ameloblasts is essential for the formation of enamel. However, little is known about the differentiation, maturation and mineralization of ameloblasts during amelogenesis.

Dey *et al.* reported OD314 (APin), which was not expressed in calvarial osteoblasts and dental papilla cells, is expressed in odontoblasts and pulpal cells (2). The *APin* cDNA transcript (FLJ20513) was originally cloned from the human KATO III cell line. It has also been identified in calcifying epithelial odontogenic tumor (CEOT)-associated amyloids and designated the "APin" protein (3). APin was also reported to be a gastric cancer-specific gene based on the serial analysis of the gene expression (SAGE) data (4). In addition, *EO-009*, a rat homologue of the human APin protein, was identified from the secretome profile of rat enamel organ cells based on the signal trap methodology (5). Recently, it was reported that APin was primarily involved in the maturation of the enamel for the proper mineralization of enamel during tooth development and APin was mediated by the expression of matrix metalloproteinase-20 (MMP-20) and tuftelin (6). It was shown that the organic matrix is removed and a high degree of mineralization proceeds during the maturation stage (7). However, the process of enamel mineralization and the function of MMP-20 and tuftelin are not completely understood.

Originally, proteins cooperate with each other throughout the interaction. The protein microarray has only recently been applied to the diagnosis of disease, the discovery of biomarkers, expression/functional studies of proteins, examinations of the interactions of proteins and the development of miraculous medicine in the field

of medical science, pharmacology, biotechnology and *etc.* (8, 9). The protein microarray using a protein chip might be a core technique for various protein studies including interactions between APin and other protein.

In this study, it was aimed to investigate the APin expression and uncover the biological function of APin during ameloblast differentiation and mineralization *in vitro*.

In the initial step of examining the role of APin in enamel mineralization, the APin recombinant protein was produced and stable APin transgenic cell lines were also established using ameloblast-lineage cells (ALCs) (10). The cells were cultured for 2 weeks with or without the APin recombinant protein in the differentiation and mineralization medium. Alizarin red S staining was performed to evaluate the level of mineralization and the Alkaline Phosphatase (ALP) activity was also measured.

The next step of identifying the gene that interacts with APin high density protein microarray was carried out. Of the proteins identified, BMPR-IB (11-14) and EF hand calcium binding protein 2 (CBP2) (15-19), which showed a high affinity for the APin protein, were evaluated their expression after inactivation and overexpression of the APin protein.

Ⅱ. *MATERIALS AND METHODS*

All the experimental procedures were reviewed and approved by the Animal and Human Subjects Research Committee at Chosun University (Gwang-Ju, Korea).

A. Tissue preparation and fluorescence microscopy

Embryonic (E18, E20) and postnatal (PN4, PN7, PN12, P17, PN22, PN26) rat tissues were fixed in 4% paraformaldehyde, decalcified in a 10% EDTA (ethylenediaminetetra acetic acid, pH 7.4) solution at 4°C for 4 weeks, embedded in paraffin, and cut into identical mesio-distal sections. The tissue sections were blocked with 1% BSA in PBS for 30 min, incubated for 1 h with the anti-APin (1:150) antibody and then incubated for 1 h with a fluorescent-labeled secondary antibody (Vector Lab, Burlingame, CA). After rinsing, the cells were examined by fluorescence microscopy (Olympus, Tokyo, Japan).

B. Construction of APin-expressing and APin siRNA-expressing plasmid and transfection

The immortalized ALCs were kindly provided by Dr. T. Sugiyama (Akita University School of Medicine, Akita, Japan) and cultured as previously described (10). The construct of pCMV APin and U6-APin siRNA was constructed and transfected in the ALCs, as previously described (6). The experiments were performed three times in duplicate. Forty-eight hours after transfection, the level of the mRNAs for *APin, CBP2, BMPR-IB* and *ALP* expression were assessed by RT-PCR using the primers and PCR conditions described below. The primer sequences used were: *APin: 5'*-cca gca ggc tag tcc tat gtc cta tgt gg/*5'*-cgc gtc

gac atg aga tca gtg, *CBP2: 5′*-act ttg tgg agc aca tgg gag act/*5′*-tgt tgg tgc ctt ctt cat ccg aga, *ALP: 5′*-ctc tcc gag atg gtg gag gt/*5′*-gtc ttc tcc acc gtg ggt ct, *BMPR-IB: 5′*-ggg ttg gtg tca ctg gta gga tta/*5′*-acc tga cag atc aga tgc ttc ctc and *GAPDH: 5′*-acc aca gtc cat gcc atc ac/*5′*-tcc acc acc ctg ttg ctg t (forward/reverse). The following PCR conditions were used: predenaturation at 94°C for 5 min, followed by 30 cycles each of denaturation at 94°C for 45 s, primer annealing at 58~64°C for 45 s, and product extension at 72°C for 45 s, with a final extension at 72°C for 7 min. The PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. The relative amount of each mRNA was determined at the 50% level of the PCR product and normalized using the relative amount of GAPDH mRNA.

C. Stable APin transgenic cell lines

The cells were transiently transfected with 2 μg of the pCMV driven APin-expressing plasmid and co-transfected with 2 μg of the U6-APin siRNA-expressing plasmid and pPUR (BD Biosciences Clontech, Palo Alto, CA) in each. After the transfection, the cells were grown for 2 days without selection. Selection was carried out using 200 μg/㎖ geneticin (Invitrogen, Grand Island, NY) in MEM containing 5% FBS and 1% penicillin/streptomycin for 2 weeks. The colonies were isolated using a colony separator and expanded first in a 24-well (1 ㎖) then in a 6-well (3 ㎖) and finally in 60 ㎜ (5 ㎖) dishes. Western blotting was used to assess the presence of the transfected gene in the selected clones.

D. Purification of APin recombinant protein

The coding region of *APin* was amplified by PCR using the primers, *5′*-caggctgctagcatgtcctatgtggttcc-*3′* and *5′*-gtaaactgcagcttatggttctcttaggctatc-*3′*, and

cloned into pRSET A (Invitrogen) *NheI* and *PstI* sites. The new plasmid was named pRSETa-APin. The E. colistrain, BL21 (DE3)pLysS, that was transformed with pRSETa-APin was cultured in LB medium containing ampicillin (50 ㎍/㎖) and chloramphenicol (12.5 ㎍/㎖) at 37°C. When E. coli grew to OD_{600} between 0.6~0.8, isopropyl-β-D-thiogalactopyranoside (IPTG, Elpis-Biotech., Inc., Taejeon, South Korea) was added to a final concentration of 1 mM and incubation was continued for 3 hours. Cell lysis was performed by sonicating the cells in a phosphate lysis buffer with the lysozyme, followed by purification on Ni-NTA agarose (1 ㎖/ 4 ㎖ of lysate, Qiagen, Inc., Hilden, Germany). Elution was carried out using high concentration of imidazole (250 mM) after first washing with a low concentration of imidazole (50 mM). The composition of all the buffers used was in accordance with the QIAexpressionist manual (Qiagen) with the addition of protease inhibitors (PMSF, Roche Applied Science, Mannheim, Germany) and β-mercaptoethanol (5 mM, Fluka and Riedel, Buchs, Switzerland) in the native buffers. The separated fractions were analyzed by SDS-PAGE and Western blotting. Electrophoresis gels were stained in coomasie blue and the membranes with the blotted proteins were analyzed with the anti-His Tag monoclonal antibody (EMD Biosciences, Inc., CA). The protein concentration was determined using the Bradford method (Bio-Rad Laboratories, CA).

E. Alizarin red S staining and ALP activity

The confluent ALCs were cultured for 2 weeks in a DMEM-based medium containing 3 ㎍/㎖ APin the recombinant protein. The stable APin transgenic cell lines were also cultured for 2 weeks using 400 ㎍/㎖ geneticin (Invitrogen) in MEM supplemented with the same components (differentiation and mineralization medium). For the mineralization assay, the calcified nodules of the ALCs and stable

APin transgenic cell lines were stained with an Alizarin red S solution. After the culture, the dishes were washed three times with phosphate-buffered saline (PBS, pH 7.4) and fixed by adding a 70% ethanol solution for 20 min. The cells were washed three times with distilled water and stained with a 1% Alizarin red S (Sigma-Aldrich, St. Louis, Mo) solution in 0.1% NH_4OH at pH 4.2~4.4. The Alizarin red associated with the cells was dissolved in 0.1 ml of cetylpyridinium chloride for 10 min, which resulted in the release of calcium-bound Alizarin red into solution (20). After the solution was collected, the absorbance (OD_{570}) was read on a spectraMAX microplate reader (Molecular Devices Corporation, Sunnyvale, CA). For the detection of the ALP activity, ALCs and the stable APin transgenic cell colonies were fixed in a 60% acetone/40% citrate working solution, and then stained with a Fast Blue RR Salt capsule and naphthol AS-MX Phosphate Alkaline Solution (Sigma). The ALP activity in the lysates was assayed by incubation for 30 min at 37°C in a buffer (pH 10) containing 0.1 M 2-amino-2-methyl-1-propanol and 2 mM $MgCl_2$ using p-nitrophenylphosphate (Wako, Osaka, Japan) as a substrate (21). The expression of *APin* and *ALP* mRNA was assessed by RT-PCR using the same primer and PCR conditions as described above. Alizarin red S staining and the ALP activity was also evaluated in other cells, such as MDPC-23 cells (Dr. Hank, Michigan University, Michigan) for odontoblast, NIH3T3 cells for fibroblasts and MG-63 cell (ATCC, Rockville, MD) for osteoblasts, PDL cells were isolated and cultured as described Cho *et al.* (22). These cells were also cultured in DMEM with or without the APin recombinant protein.

F. Identification of the protein interactions

The protein interactions were identified using a Protoarray™ human protein microarray PPI complete kit v3.0 (Invitrogen, Carlsbad, CA). Twenty milliliters of a

fresh blocking buffer was placed in a Nalgene tray and Protoarray™ protein microarray was then added to the tray. The tray was incubated with gentle agitation at 4°C for 1 hour. One hundred and twenty microliters of the diluted DNA (50 ㎍ /㎖) was placed on the top of the slide and covered with Hybrislip™. The slide was inserted into a 50 ml conical tube with the array facing up. The slides were incubated at 4-6°C for 90 min (no shaking). After washing, 30 ml of a Streptavidin conjugated Alexa Fluor® 647 antibody solution (See Reagent/ Stock preparation) was added immediately and incubated at 4-6°C for 30 min (no shaking). After washing, the slides were allowed to dry in a dark place with the lid of the slide box open. The slides should dry in less than 1 hr. Once the slides were completely dry (no translucent areas), they were scanned with GenePix 4000B (Molecular Devices Corporation, Sunnyvale, CA) at 635 nm with a PMT gain of 600, a laser power of 100% and a focus point of 10 ㎛. This is done to keep signals within the linear range. The image data was analyzed using GenePix Pro 6.0 (Molecular Devices) and Prospector 4.0 (Invitrogen). The data was analyzed using the appropriate analysis algorithm.

G. Fluorescence microscopy and Western blotting

The pCMV driven APin-expressing plasmid was transfected into C2C12 cells with the Lipofectamine Plus (Invitrogen) as described above, in six-well culture plates for Western blots or Lab-Tek chambered coverglasses (Nunc, Rochester, NY) for fluorescence microscopy. Cells were treated with 0.1% DMSO as vehicle alone or 10 ㎍/㎖ brefeldin A (Sigma).

For fluorescence microscopy, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with Triton X-100 in PBS, and quenched with NH_4Cl (50 mM) in PBS. Cells were incubated for 1 h with the anti-APin antibody,

washed with PBS, and then incubated for 1 h with a fluorescent-labeled secondary antibody (Vector). The cells were counterstained with PI (propidium iodide) to visualize the nucleus. After rinsing, the cells were examined with fluorescence microscope (Olympus).

Western blotting analysis was performed to analyze APin expression. The proteins were extracted from the cell lysates after the cells had been lysed in a NP-40 lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM EDTA, pH 7.4, 10 ㎍/㎖ leupeptin, and aprotinin). The conditioned media were also collected and centrifuged at 14,000 rpm for 5 min at 4°C to remove dead cell debris. The supernatant were precipitated for 1 h on ice with 10% trichloroacetic acid. The precipitated proteins were lysed with lysis buffer as described above. Samples were loaded on a denaturing 10-20% Tris-HCl polyacrylamide gel and transferred to nitrocellulose membranes. APin was detected using the anti-rabbit APin antibody (1:1500), goat anti-rabbit-IgG (1:10,000), and ECL detection system (Amersham Pharmacia Biotech., Piscataway, NJ).

Ⅲ. *RESULTS*

A. Expression of APin protein during tooth development

The expression pattern of APin was observed during various developmental stages of the maxillary second molar in rats. In the developing rat tooth, APin protein expression was increased gradually according to ameloblast maturation (Fig. 1A-I). APin protein expression was not observed during the cap (Fig. 1A), bell (Fig. 1B) and advanced bell stages (Fig. 1C). At the crown stage (Fig. 1D, E), weak expression of the APin protein was detected in the secretory ameloblasts, and which was located in the cervical region. At the root formation stage (Fig. 1F, G), second molar began to form a root but did not erupt yet. The APin protein was expressed not only in the supranuclear region but also in the ruffle-end of the ruffle-ended ameloblasts. Although APin was expressed mainly in the ameloblasts, it was also expressed at a low level in the odontoblasts (Fig. 1G). During tooth eruption (Fig. 1H), the APin protein was strongly expressed in the post-maturation stage ameloblasts, and its expression was maintained in the junctional epithelium during the protective stage of ameloblasts (Fig. 1I).

B. Mineralization of the ALCs observed by Alizarin red S staining

In the normal ALCs culture, the calcified nodule appeared at day 7, and gradually increased up to day 14 (Fig. 2A, E). Nodule formation of the ALCs was enhanced by the APin recombinant protein (Fig. 2B). In the sense APin overexpressing cells, the nodule tined red began to be observed at day 4, which showed a tendency to increase in number as differentiation progressed (Fig. 2C). Regarding the antisense

APin overexpressing cells, they decreased themselves in the Alizarin red S staining (Fig. 2D, E). After 14 days, the increase in the mineralization of ALCs treated with the APin recombinant protein and sense APin overexpressing cells was 2.4 times higher than that of the normal ALCs (Fig. 2E). Expression of the APin protein was confirmed by Western blotting using the anti-His Tag monoclonal antibody (Fig. 2F).

C. ALP activity of the ALCs observed by ALP staining

The ALCs showed stronger staining for ALP activity when cultured in the APin recombinant protein compared with those cultured in the normal medium (Fig. 3A, C). Staining was also stronger in the sense APin overexpressing cells cultured in the differentiation and mineralization medium (Fig. 3B), whereas it was weaker in the antisense APin overexpressing cell lines (Fig. 3D). The ALP activity in the normal ALCs showed a slight increase at day 4. The ALCs treated with the APin recombinant protein and sense APin overexpressing cells markedly augmented the ALP activity, whereas the antisense APin overexpressing cell lines showed lower ALP activity (Fig. 3E). The effect of APin overexpression and inactivation on the expression of ALP mRNAs *in vitro* was examined by RT-PCR. APin expression itself increased and decreased significantly after its overexpression and inactivation, respectively (Fig. 3F). The inactivation of APin in the ALCs down-regulated the expression of ALP, whereas its expression was markedly up-regulated, as an effect of APin overexpression (Fig. 3F).

D. Mineralization of the other oral tissue cells

Calcified nodule formation of the MDPC-23 cells was enhanced by the APin recombinant protein, which was visualized by Alizarin red S staining (Fig. 4A, B).

The nodule tined with red the MDPC-23 cells treated with APin recombinant protein began to be observed at day 4, which showed a tendency to slightly increase in number as differentiation progressed (Fig. 4B). However, the PDL cell (Fig. 4C, D), MG-63 (Fig. 4E, F) and NIH3T3 (Fig. 4G, H) treated with the recombinant protein differentiated without a distinct change against the normal group.

E. Identification of protein interactions

A schematic diagram of the Protoarray^{TM} protein microarray PPI kits for the epitope-tagged proteins (Fig. 5A). After blocking the ProtoArray^{TM} protein microarray NC, the probe with the protein was incubated with the microarray. After washing, the slides were incubated with the Anti-His-Alexa Fluor 647 Antibody. The slides were washed and dried. The array was scanned to obtain an array image and analyze the results. The results obtained after probing the ProtoArray^{TM}human protein microarray NC v1.0 with 50 ㎍/㎖ of the His-tagged APin probe (Fig. 5B). In the protein microarray, seventy-four proteins were identified using mass spectrometric methods and database searches (Z-Score > 3). Several high-level expressed proteins were related to the functional characteristics, such as mineralization, RNA binding, tumor genesis and *etc*. (Table 1). Fig. 5C shows a high magnification of the green boxed area shown in Fig. 5B. The yellow box indicates the binding to the cognate protein (BMPR-1B). Fig. 5D shows a high magnification of the yellow boxed area shown in Fig. 5B. The yellow box indicates the binding to the cognate protein (CBP2). The effect of APin overexpression and inactivation on the expression of the mRNA of BMPR-1B and CBP2 *in vitro* was examined by RT-PCR. APin expression itself was increased and decreased significantly after its overexpression and inactivation, respectively (Fig. 5E). The

inactivation of APin in the ALCs resulted in the down-regulation of the expression of BMPR-1B and CBP2, whereas the expression was markedly up-regulated as an effect of APin overexpression (Fig. 5E).

F. Subcellular localization of APin protein and their secretion

Immunofluorescence was performed after transfection with the APin-expressing plasmid into C2C12 cells. APin protein was clearly localized to crescent-like structures primarily in the cytoplasm (Fig. 6A). The relative transfection efficiency was approximated visually and was confirmed by a second investigator. APin was barely expressed in normal C2C12 cells. A conservative estimate showed that at least 60% of C2C12 cells were APin-positive when transfected with the APin-expressing plasmid.

Western blotting was performed to semi-quantify the secretion of APin in culture media. The protein detected by Western blotting in the media migrated near their predicted molecular weight, around 30 kDa. When brefeldin, an inhibitor of protein secretion, was added, the APin protein was not detected in the conditioned media of transfected cells (Fig. 6B), whereas APin showed a strong signal, around 30.6 kDa, in the total cell lysate. There were two signals of APin protein, around 30.6 and 30 kDa, in the cell lysate when brefeldin was added (Fig. 6B).

Ⅳ. *DISCUSSION*

Recently, there has been considerable interest in cell-specific genes in an attempt to understand the various cell differentiation mechanism and to separate the related factors (23). However, little is known about the specific genes for ameloblast, nor is there any information on the molecular biological mechanisms of ameloblast differentiation and enamel mineralization. As a possible mechanism for ameloblast differentiation and enamel mineralization, it was previously suggested that APin plays a functional role in the mineralization and maturation of enamel (6).

This study aimed to investigate the biological function of APin during ameloblast differentiation and mineralization *in vitro*.

In this study, fluorescence microscopy was used to confirm the APin protein distribution within the surrounding organization of the developing second molar of rats. APin was expressed more strongly in ameloblasts than in odontoblasts and it was also strongly expressed in maturation ameloblasts. During the enamel maturation stage, the organic matrix is removed from the enamel, and mineralization continues up to a composition of 95% mineral and only 5% organic matrix and water (7). It was assumed that enamel maturation stage is associated with enamel mineralization. Therefore, the specific APin expression in the maturation stage ameloblast suggests that APin plays an important role in enamel mineralization.

This study examined whether or not APin is indeed related to tooth mineralization *in vitro*. In an initial step for identifying the role of APin in enamel mineralization, Alizarin red S staining was performed and the ALP activity was examined. It was found that ALCs treated with the APin recombinant protein and the sense APin overexpressing cells markedly augmented the calcified nodule

formation and increased the ALP activity, whereas there was less calcified nodule formation in the antisense APin overexpressing cell lines. The overexpression of APin in the ALCs up-regulated the expression of ALP mRNA, whereas their expression was markedly down-regulated by its inactivation. ALP transports phosphate or nutrients in the stratum intermedium, which is characterized by the high activity of the enzyme, ALP (24, 25). The ALCs were prepared from an organ culture of tooth germs including the stratum intermedium (10). Although APin is believed to regulate the expression of ALP involved in enamel mineralization, its molecular mechanism to ALP requires further investigation.

Alizarin red S staining and ALP activity were proceeded in order to determine the role of APin in the mineralization of other oral cells, *e.g.* MDPC-23, MG-63, NIH3T3 and PDL cells. The odontoblast-like cells (MDPC-23) treated with the APin recombinant protein showed slightly enhanced formation of calcified nodules visualized by Alizarin red S staining against normal MDPC-23 cells, whereas the osteoblast-like cells (MG-63) and PDL cells treated with the recombinant protein differentiated without any distinct change compared with the normal group. Interestingly, among the oral tissues, the enamel and dentin showed augmented mineralization by APin. This unique pattern in the ALCs and odontoblast cells supports the hypothesis that dentin is adjacent to the enamel and that the differentiation and mineralization of ameloblasts and odontoblasts are regulated by the reciprocal epithelial-mesenchymal interaction (26, 27). It would be interesting to determine if APin has any other unique function(s) in augmenting the dentin mineralization.

The protein chip is an automatic machine system that fixes thousands of proteins to a small chip. The protein chip system also analyzes the binding of proteins, and consists of a sensor chip and an analyzing system for protein

binding. In this study, NC slide chip system was used, which was made from nitrocellulose coated on glass. All the proteins on the chip, the gateway system, made a full-length clone and the proteins were expressed at the insect cell. Because all the produced proteins had a GST tag, the proteins were extracted by a GST column and were spotted using a pin spot method. In the protein microarray of APin, seventy-four proteins with a Z-score over 3 were identified using mass spectrometric methods and database searches. Several strongly expressed proteins were related to the functional characteristics, such as mineralization, RNA binding, tumor genesis and *etc.*. Two proteins (BMPR-IB and CBP2), which have high affinity and noticeable roles in mineralization, were found among several binding proteins.

Bone morphogenetic proteins (BMPs), which are the member of the transforming growth factor β (TGF β) superfamily, may control cell differentiation and proliferation in the development stage, such as in tooth and/or hair follicle development (11). In the development stage, BMP can induce apoptosis, proliferation and differentiation, which are controlled by two types of BMP receptors. The type Ⅱ receptors, which are active in serine/threonine kinases, transfer the characteristics of the Gly-Ser segment to the type Ⅰ receptor through transphosphorylation. After the activated type Ⅰ receptor recruits, they phosphorylate the downstream signaling molecules of the Smad family (12). For optimal binding and signal transduction, BMPR-1B is required the dimerization by BMP2 (28). Ameloblasts express BMP2 during their terminal differentiation. BMP2 was also expressed by the secretory ameloblasts (13). It was reported that MMP-20, in which is expressed in the secretory stage, is controlled by *APin* (6). BMP2, which is a morphogenetic signal, increases the level of *MMP-20* expression, and the mineralization was reported to be markedly augmented in the

BMP2-treated pellet (29, 30). This suggests that APin regulates the expression of MMP-20 and induces the biomineralization of the enamel through BMP2 and BMPR-1B.

EF-hand calcium binding protein parvalbumin has been studied widely in muscle and neural cells (15). However, recent studies have shown that it plays an important role in the mineralization of the tooth development (15-19). When the Tomes' process is developing, parvalbumin is expressed near the nucleus in differentiating ameloblasts. After the development of the Tomes′ process, parvalbumin is distributed widely in the cells. The expression of parvalbumin varies in the rough-end and smooth-end ameloblasts. In the case of odontoblasts, the level of parvalbumin expression in odontoblast cells is lower than that in ameloblasts. CBP2 was detected using the protein microarray, which have an affinity for the APin protein. The overexpression of APin in the ALCs up-regulated the expression of the CBP2 mRNA, whereas their expression was down-regulated by its inactivation. In a previous report, APin was strongly expressed in the ruffle-ended ameloblast, which is related to the function in calcium transport that is strongly associated with this data (6). It was also reported that APin might control the expression of tuftelin during enamel maturation (6), which is supported by Deutsch *et al.* (16). Deutsch *et al.* (16) proposed that the EF-hand calcium-binding domain is the consensus sequence found in tuftelin. The cDNA sequence has a single N-glycosylation consensus site and several O-glycosylation sites. It also contains 7 phosphorylation sites and is located in the N-terminal region. This fact suggests that tuftelin plays a key role in motivating enamel mineralization.

A previous study examined whether or not APin was secreted (6). APin tended to show division into two molecular weights. One was located in the cytosolic

compartment. Another was secreted and post-translationally modified. In this study, the APin protein was localized in the cytoplasm of the ameloblasts including the supranuclear region and ruffle-end of the ruffle-ended ameloblasts. The experimental data corroborates the finding that APin is secreted efficiently. The main issue raised by this study is whether the signal is transmitted through this signaling pathway after the secreted protein has attached to BMPR-1B or the secreted protein is attached directly to the CBP2 *etc.*.

Further studies will be needed to understand the role of several genes expressed in the protein microarray data.

In Conclusion, these results provide deeper insights into the process of ameloblast maturation and in enamel mineralization. It also suggested that APin augmented enamel mineralization. In addition, this is the first study to show using a protein microarray that the protein interacts with APin.

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Ⅵ. *TABLE*

Table 1. List of high expression proteins

Database ID	Z-Score	Proteins	Function
NM_006775.1	12.3754	KH domain	RNA binding protein
BC016979.1	**8.17742**	**EF-hand calcium binding protein 2**	**Regulate cellular activity and mineralization**
BC054520.1	**7.92155**	**MEF2 (MADS box transcription enhancer factor 2)**	**- Coregulator for myogenic cell proliferation - Tumor proliferation and tumorigenesis.**
NM_003621.1	5.81057	PTPRF interaction protein, binding protein 2	Protein tyrosine phosphatase gene, adhesion, epitheial
BC001280.1	**5.39477**	**Aurora kinase A**	**- Overexpression and amplification of human tumor**
BC033758.1	4.95498	centaurin	Amplification of human cancer, differentiation exchaning factor
BC053557.1	4.21134	zinc finger protein	DNA-binding motif
BC032485.1	4.07541	apoptosis-inducing factor	
NM_003975.1	3.85951	SH2 domain protein	Regulating apoptosis, cell proliferation
NM_001203.1	**3.57965**	**BMPR-IB**	**Organ development**

Ⅶ. *FIGURE LEGENDS*

Fig. 1. Immunofluorescent localization of APin in developing rat teeth The tissue sections were fixed with paraformaldehyde and incubated with the polyclonal anti-APin antibody. Cap stage (A), bell stage (B), advanced bell stage (C), crown stage (D)(E), root formation stage (F)(G) and tooth eruption stage (H)(I). (A)(B)(C) show that the APin proteins are not expressed in the ameloblast. Note the weak APin localization in the cytoplasm (arrow)(D)(E)(F)(G), contrasting with the strong staining in the enamel matrix deposited adjacent to the Tomes' processes (thick arrow). The strong APin staining is evident in the supranuclear region (thick arrow) and ruffle-end (arrow) of the ruffle-ended ameloblasts (G). In addition, APin was also expressed weakly in the odontoblast (arrow head), but the expression was stronger in ameloblasts. APin was also expressed in the junctional epithelium (arrows) of a postnatal 26-day-old rat (H). Tb, tooth bud; Am, ameloblast; Od, odontoblast. Scale bars of A, B, D, F and G indicate 20 ㎛. Scale bars of C, E, H and I are 50 ㎛.

Fig. 2. Mineralization of ALCs observed by Alizarin red S staining (A) Alizarin red S staining of the normal ALCs were cultured for 14 days in differentiation and mineralization medium. (B) Alizarin red S staining of ALCs treated with APin recombinant protein. (C) Alizarin red S staining of the sense APin overexpressing cells. (D) Alizarin red S staining of the antisense APin overexpressing cells. ALCs treated with the APin recombinant protein (B) and sense APin overexpressing cells (C) markedly augmented calcified nodule, whereas the antisense APin overexpressing cell lines (D) lowered them. (E) Alizarin red S

dye associated with the cells was dissolved in 10% cetylpyridium chloride, and quantified by measuring the absorbance at 570 nm after 0, 4, 7, 10, 14 days culture. (F) The separated fractions were analyzed using SDS-PAGE (left) and Western Blotting (right). Electrophoresis gels were stained by Coomasie Blue. Lane 1 shows 1 ㎍ of the sample and lane 2 shows 3 ㎍ of the sample. The blotted proteins were analyzed using anti-His Tag monoclonal antibody (lane 3).

Fig. 3. ALP activity of ALCs observed by ALP staining (A) ALP staining of normal ALCs cultured for 14 days in differentiation and mineralization medium. (B) ALP staining of the ALCs treated with APin recombinant protein. (C) ALP staining of the sense APin overexpressing cells. (D) ALP staining of the antisense APin overexpressing cells. (E) ALP activity was measured in the cells cultured for 14 days in differentiation and mineralization medium (□), treated with APin recombinant protein (■), sense APin overexpressing cell (▲), antisense APin overexpressing cell (●). ALCs treated with APin recombinant protein (B)(E) and sense APin overexpressing cells (C)(E) markedly augmented ALP activity, whereas antisense APin overexpressing cell lines (D)(E) lowered them. (a) 0 day, (b) 4 day, (c) 7 day, (d) 10 day, (e) 14 day. (F) RT-PCR analysis of the effect of APin overexpression and inactivation on mRNA expression for ALP.

Fig. 4. Mineralization of the other oral tissue cells (A) Alizarin red S staining of normal MDPC-23 cells cultured for 14 days in differentiation and mineralization medium. (B) Alizarin red S staining of MDPC-23 cells treated with the APin recombinant protein. MDPC-23 cells treated with APin recombinant protein showed the slightly enhanced formation of calcified nodules against normal MDPC-23 cells (A)(B). (C) Alizarin red S staining of normal PDL cells (D) Alizarin red S staining

of the PDL cells treated with APin recombinant protein. (E) Alizarin red S staining of normal MG-63 cells. (F) Alizarin red S staining of MG-63 cells treated with APin recombinant protein. (G) Alizarin red S staining of normal NIH3T3 cells. (H) Alizarin red S staining of NIH3T3 cells treated with the APin recombinant protein. PDL (C)(D), MG-63 (E)(F) and NIH3T3 cells (G)(H) treated with recombinant protein differentiated without a distinct change against the normal group. (a) 0 day, (b) 4 day, (c) 7 day, (d) 10 day, (e) 14 day.

Fig. 5. Identification of the protein interactions (A) A schematic diagram of the antibody cross-reactivity profiling protocol. After probing, an Alexa Fluor® 647 antibody solution was incubated with the microarray. The slides were washed, dried and imaged using an Axon 4000B scanner. (B) Detection of the APin protein using protein microarray. (C) High magnification of the green boxed area in figure B. Binding to the cognate protein (BMPR-1B) is indicated by a yellow box. (D) High magnification of the yellow boxed area in figure B. Binding to the cognate protein (CBP2) is indicated by a yellow box. (E) *In vitro* validation of the targets identified on the protein microarray: RT-PCR analysis of the effect of APin overexpression and inactivation on mRNA expression for BMPR-1B and CBP2. The ALC cells were transiently transfected with the pCMV driven APin-expressing plasmid, U6-APin siRNA-expressing plasmid, and the control vector. Control, normal ALC; Over, APin overexpression; inact, APin inactivation; Mock, ALC expressing empty vector.

Fig. 6. Subcellular localization of APin protein by immunofluorescence (A) and their secretion into culture media (B). The C2C12 cells were transiently transfected with an APin-expressing plasmid. (A) Cells were labeled with an

anti-APin antibody and examined by fluorescence after counterstaining with PI. (B) The cell lysates (lanes 1-2) and conditioned media (lanes 3-4) were analyzed for the presence of APin protein by Western blotting. Expression of APin showed a strong signal in "Over" and two signals in "Over + Brefeldin A"of the cell lysates. Apin protein was detected as a strong signal in "Over" of the conditioned media, while the addition of brefeldin A caused no specific signal in the conditioned media. PI. Propidium iodide; Over, APin overexpression; Over + Brefeldin A, APin overexpression and Brefeldin A treatment.

Ⅷ. *FIGURES*

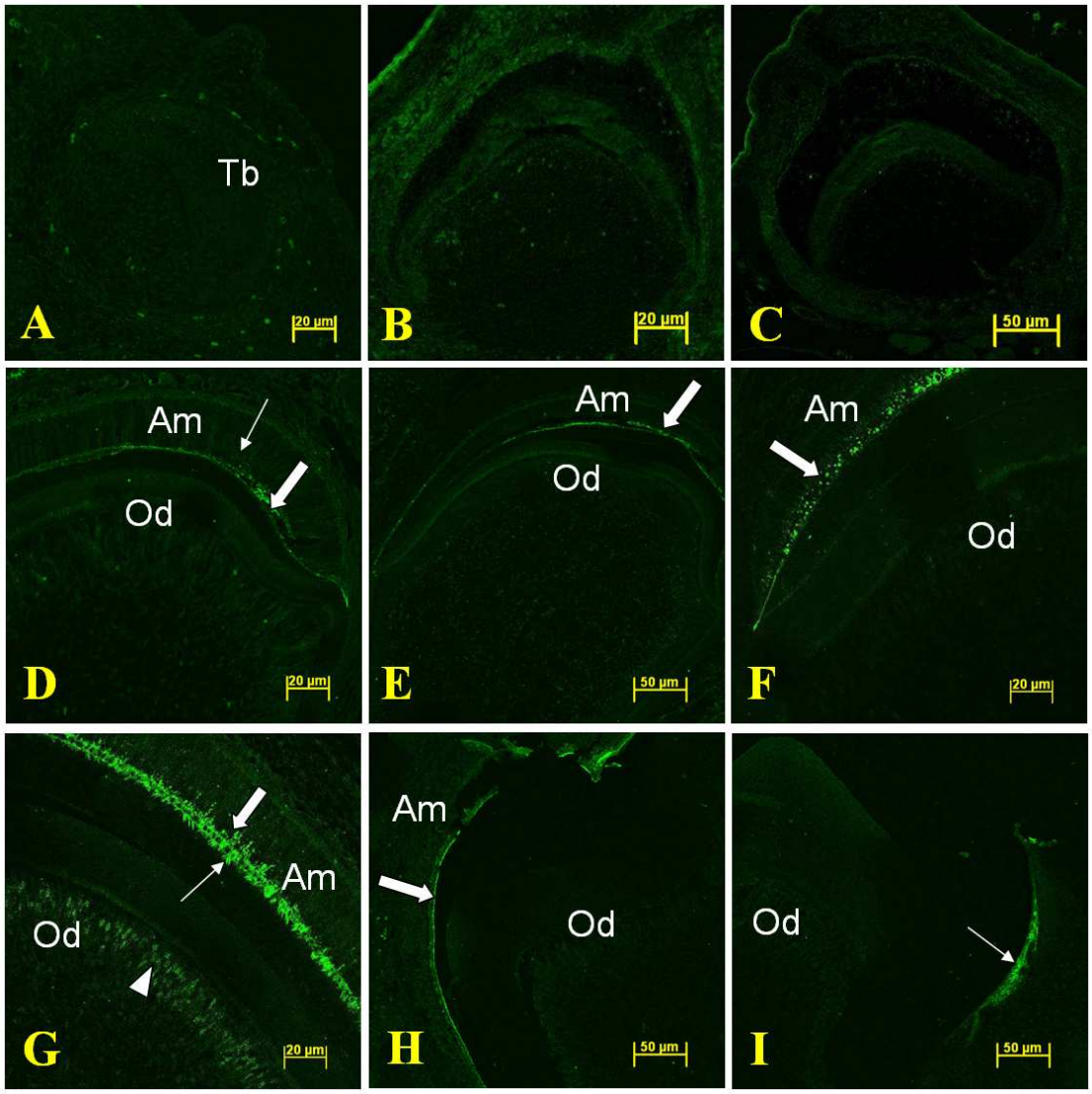

Fig. 1. Immunofluorescent localization of APin in developing rat teeth

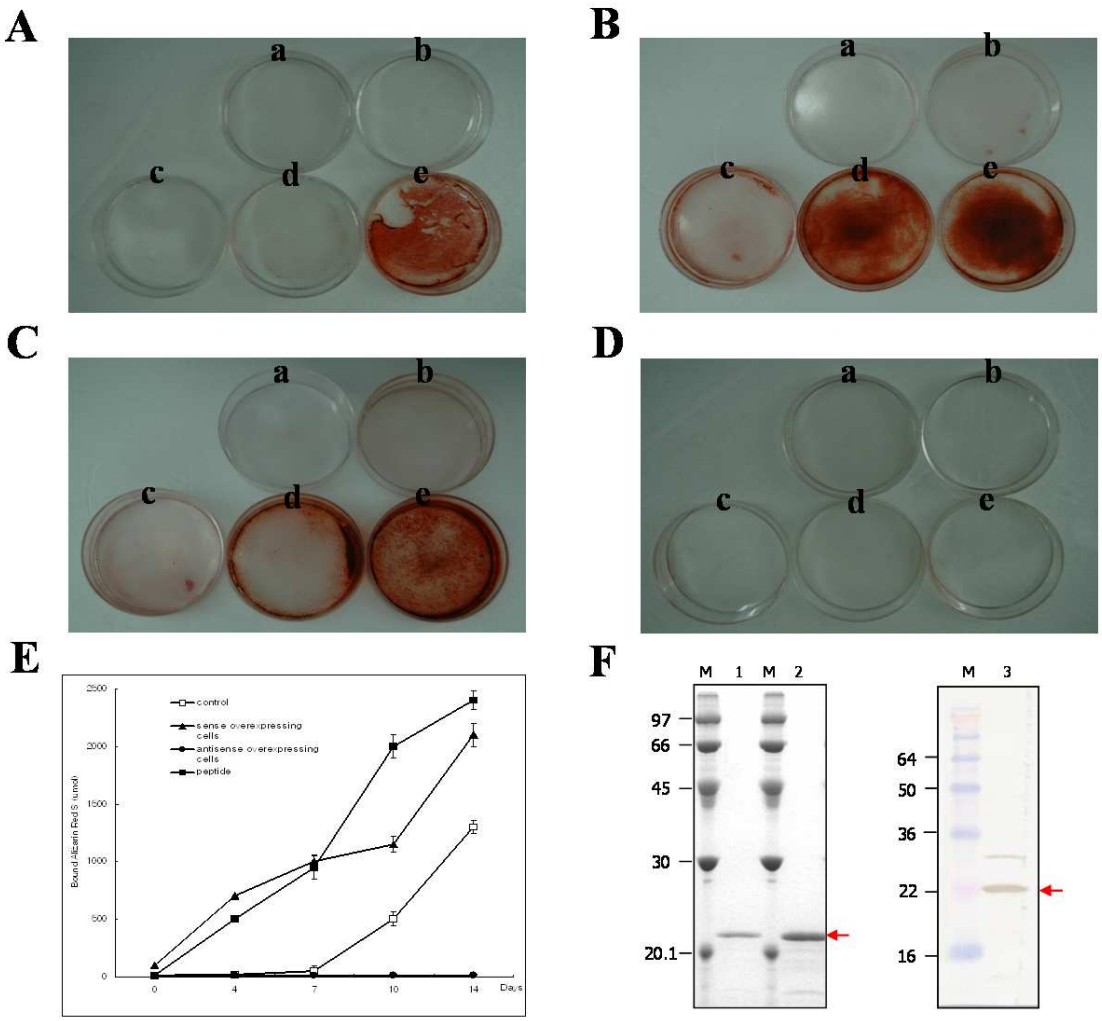

Fig. 2. Mineralization of ALCs observed by Alizarin red S staining

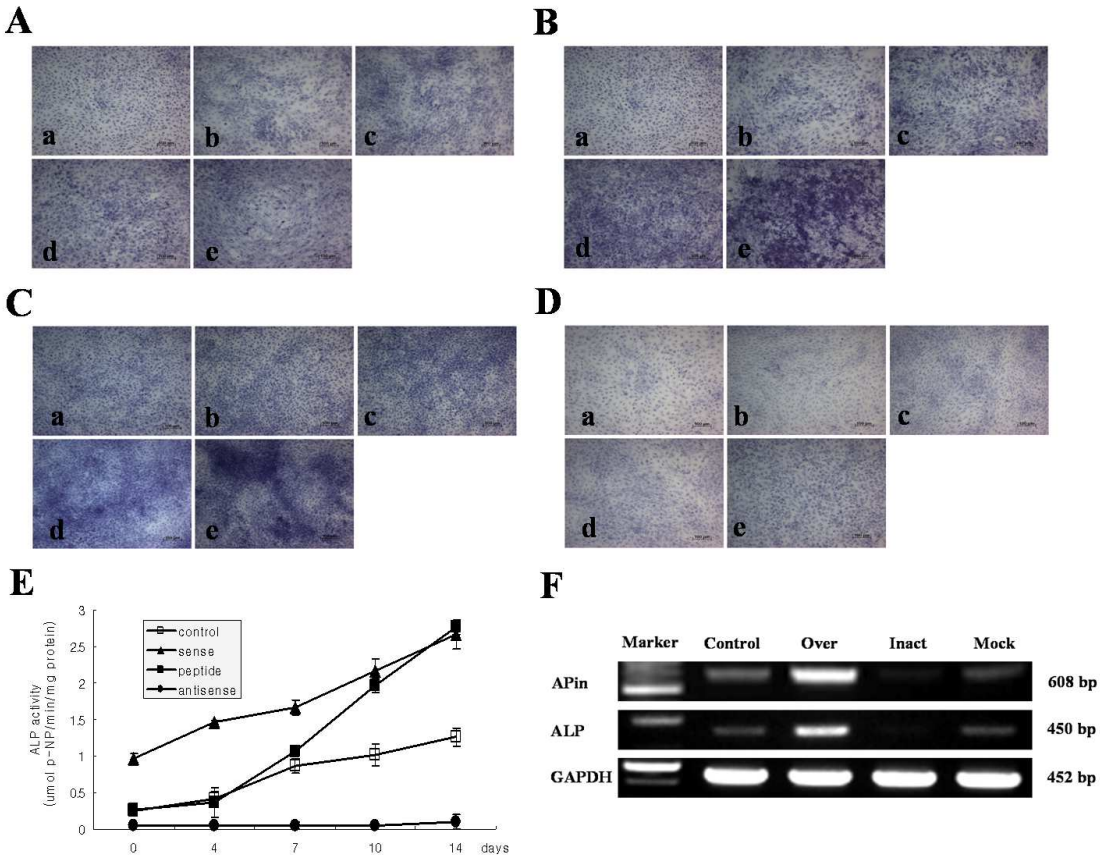

Fig. 3. ALP activity of the ALCs observed by ALP staining

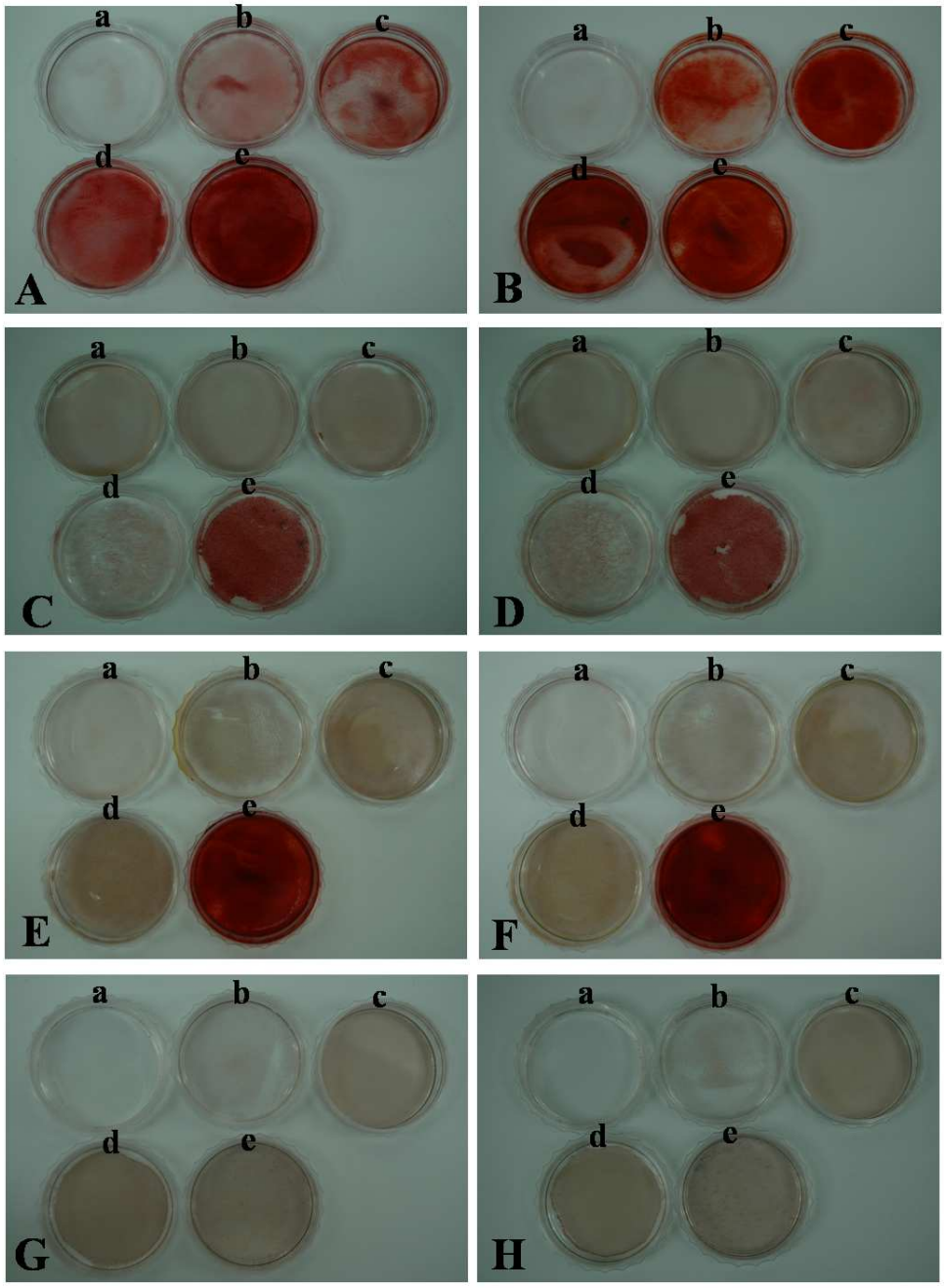

Fig. 4. Mineralization of the other oral tissue cells

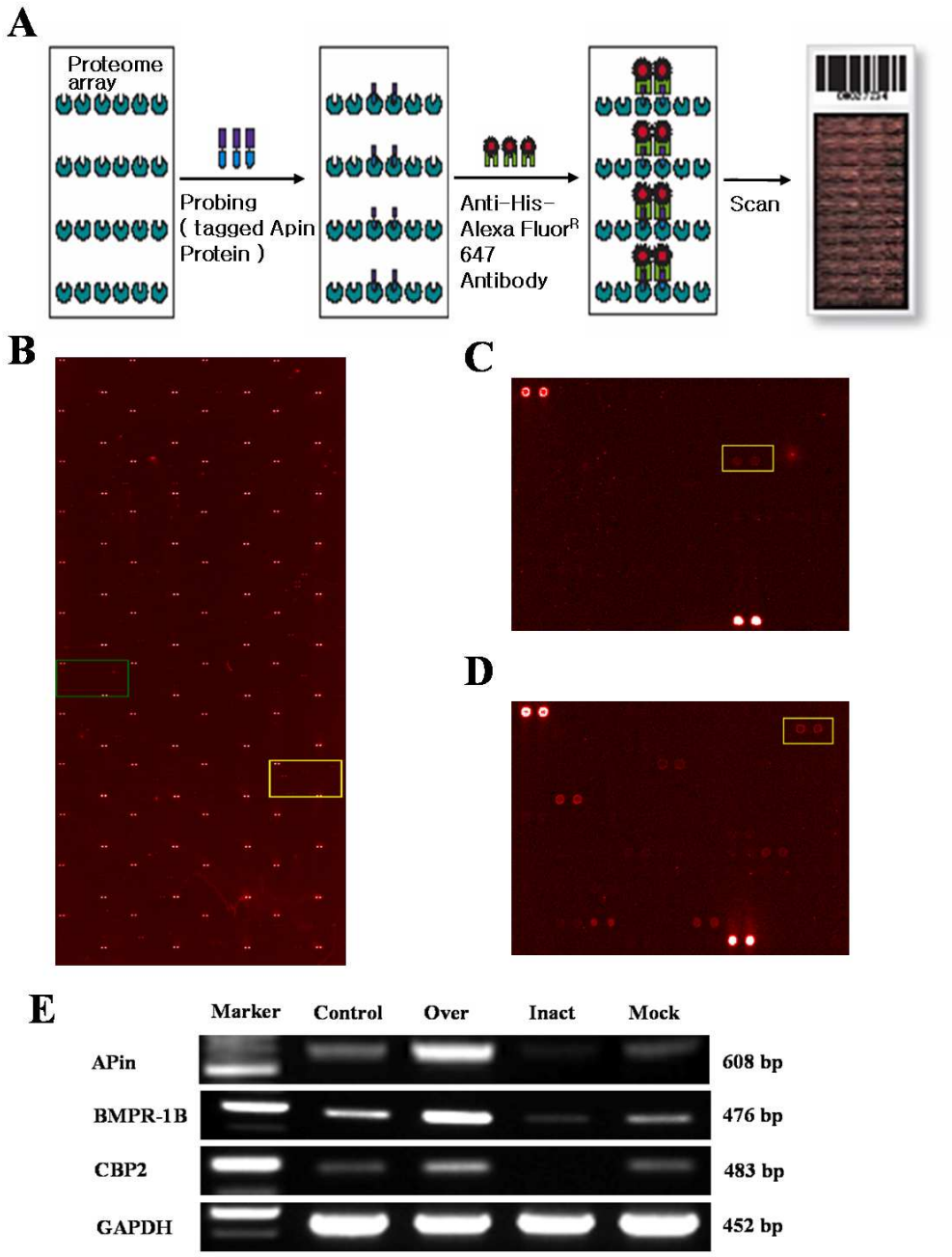

Fig. 5. Identification of protein interactions

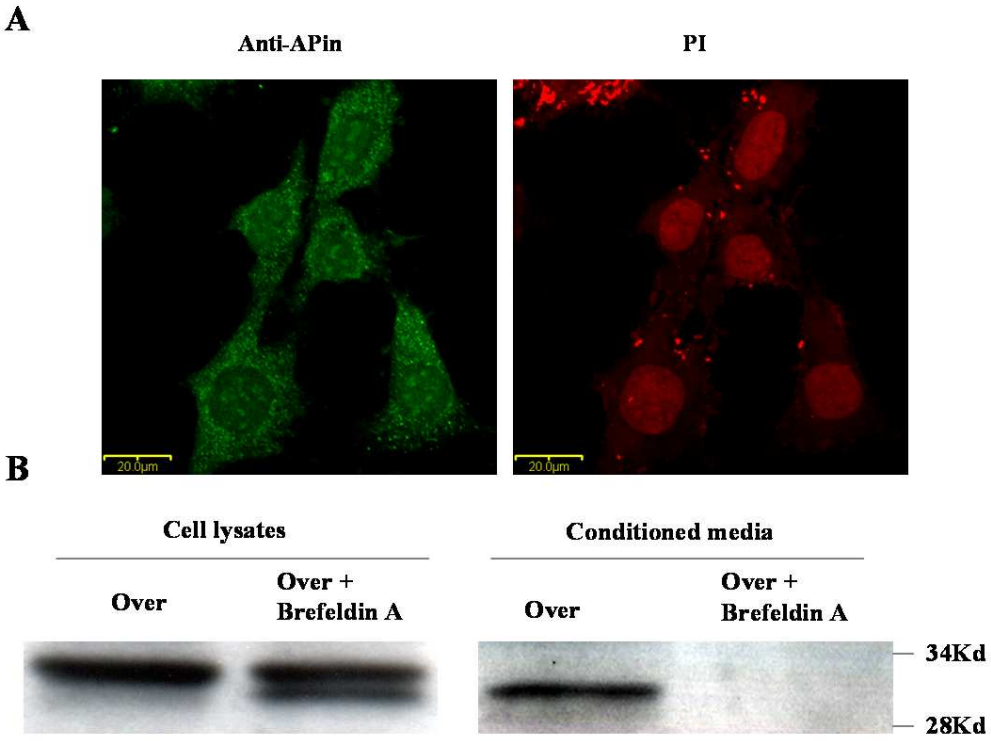

Fig. 6. Subcellular localization of APin protein by immunofluorescence

ABSTRACT in KOREAN

*APin*에 의한 법랑질 석회화 증진

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APin은 법랑모세포의 분화와 법랑질의 석회화 과정 및 법랑질 성숙기에서 법랑모세포의 기능에 중요한 역할을 하며, 그 과정들에서 MMP-20과 tuftelin의 발현을 유도하는 것으로 보고되었다.

본 연구에서는 법랑모세포의 분화와 법랑질의 석회화과정에서 APin의 기능분석을 위해 APin 재조합 단백질을 제작하고, 법랑모세포 세포주를 이용하여 APin transgenic 세포주를 확립하였다. 이 세포주를 분화배지에 APin 재조합 단백질을 첨가하여 2주간 배양한 후, Alizarin red S 와 ALP 염색을 하여 ALP 활성화와 석회화 정도를 분석하였다. 또한, 본 연구에서 APin과 다른 단백질의 상호작용을 연구하고자 protein microarry를 시행하였다. APin과 높은 친화성을 보이는 단백질을 선택하여, APin의 과발현과 발현억제를 시킨 후, RT-PCR를 통해 선택된 유전자 발현을 평가하였다.

석회화는 sense APin 과발현 세포와 APin 재조합 단백질을 첨가한 법랑모세포 세포주에서 증진되었다. 또한 ALP 활성화는 sense APin 과발현 세포와 APin 정제된 단백질를 첨가한 법랑모세포 세포주에서 뚜렷하게 증진되었다. 기관발생과 관련이 있는 BMPR-IB와 석회화 과정과 관련된 CBP2는 APin 과발현을 유도한

경우에는 발현이 증가되었으나, APin 발현을 억제시킨 경우에는 발현이 현저히 감소하였다. 그러나 골모세포와 백악모세포의 석회화에는 영향을 주지 않았다.

이상 실험의 결과는 APin이 법랑질 석회화를 증진시킬수 있음을 시사한다. 또한 본 연구는 protein microarray를 이용하여 APin과 상호 작용하는 단백질들을 제시하는 최초의 연구로서, 그 효용가치가 매우 높은 것으로 사료된다.

저작물 이용 허락서

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논문제목	한글 : APin에 의한 법랑질 석회화 증진 영어 : Augmentation of enamel mineralization by APin				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2007 년 6 월 일

저작자: 박 종 태 (서명 또는 인)

조선대학교 총장 귀하