J Periodont Res 2009; 44: 289–296

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01091.x

Putative signaling action of amelogenin utilizes the Wnt/β-catenin pathway

M. Matsuzawa[1,2], T.-J. Sheu[1], Y.-J. Lee[3], M. Chen[1], T.-F. Li[1], C. T. Huang[4], J. D. Holz[1], J. E. Puzas[1]

[1]Department of Orthopaedics, Center for Musculoskeletal Research, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA, [2]Department of Craniofacial Growth and Development Dentistry, Division of Pediatric Dentistry, Kanagawa Dental College, Kanagawa, Japan, [3]Department of Biomedical Imaging and Radiological Sciences, National Yang-Ming University, Taipei, Taiwan and [4]Department of Dentistry, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA

Matsuzawa M, Sheu T-J, Lee Y-J, Chen M, Li T-F, Huang CT, Holz JD, Puzas JE. Putative signaling action of amelogenin utilizes the Wnt/β-catenin pathway. J Periodont Res 2009; 44: 289–296. © 2009 John Wiley & Sons A/S

Background and Objective: While it has long been known that amelogenin is essential for the proper development of enamel, its role has generally been seen as structural in nature. However, our new data implicate this protein in the regulation of cell signaling pathways in periodontal ligament cells and osteoblasts. In this article we report the successful purification of a recombinant mouse amelogenin protein and demonstrate that it has signaling activity in isolated mouse calvarial cells and human periodontal ligament cells.

Material and Methods: To determine the regulatory function of canonical Wnt signaling by amelogenin, we used TOPGAL transgenic mice. These mice express a β-galactosidase transgene under the control of a LEF/TCF and β-catenin-inducible promoter. To investigate in greater detail the molecular mechanisms involved in the β-catenin signaling pathway, isolated osteoblasts and periodontal ligament cells were exposed to full-length recombinant mouse amelogenin and were evaluated for phenotypic changes and β-catenin signaling using a TOPFLASH construct and the *LacZ* reporter gene.

Results: In these *in vitro* models, we showed that amelogenin can activate β-catenin signaling.

Conclusion: Using the TOPGAL transgenic mouse we showed that amelogenin expression *in vivo* is localized mainly around the root, the periodontal ligament and the alveolar bone.

J. Edward Puzas, PhD, Department of Orthopaedics, Box 665, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642, USA
Tel: +1 585 275 3664
Fax: +1 585 756 4727
e-mail: edward_puzas@urmc.rocheter.edu

Key words: amelogenin; bone formation; osteoblast; β-catenin; periodontal ligament; cell signaling

Accepted for publication January 30, 2008

Although the oral sciences have made substantial advances in understanding the biology and materials required for the repair or replacement of damaged teeth, attempts to regenerate the tissue and structure of mature dentition have not been successful (1–3). This is a result, in part, of incomplete knowledge of the developmental patterns and regulatory factors involved in the genesis of a tooth. In this report we provide evidence of a new regulatory factor that may play an important role in the maturation of ameloblasts, periodontal ligament cells and bone cells. We have found that amelogenin may not only be important as a structural protein for matrix deposition but may also contribute differentiating signals to cells in the immediate environment of a developing tooth.

Amelogenin is a protein that is known to be critical to the structural formation and mineralization of enamel, the hardest substance in the human body (4,5). Amelogenin isoforms comprise 90% of the mineralizable matrix that covers the crown of the tooth. As these proteins are cleaved and degraded, mineral deposition in the form of crystals occurs in a well-ordered pattern (6). Thus, it has been assumed that the major function of the amelogenins is as a structural support for the initiation of enamel mineralization (7). During the course of its formation, enamel undergoes changes from an organic, protein-rich substance, of which amelogenin comprises over 85%, to a nearly completely mineralized architecture of hydroxyapatite. Ameloblasts differentiate from the oral ectoderm and deposit a proteinaceous extracellular matrix, as well as calcium and phosphate ions, at the

dentin–enamel junction (8). Crystallization within the deposited matrix follows. Finally, hydroxyapatite crystals form organized structures of bundles that comprise the ultrastructure of enamel rods and inter-rods, and once these processes are complete, the protein portion of the matrix is degraded, leaving mineralized enamel (9).

In the absence of amelogenin its fundamental importance to tooth development becomes apparent (10). In the pernicious disease amelogenesis imperfecta, a mutated allele renders amelogenin nonfunctional and results in disorganized hypomineralized enamel as well as in malformed teeth (11). Analogously, in amelogenin knockout mice, similar results are observed as well as lower migration and proliferation rates of cementoblast/periodontal ligament cells, leading to increases in cementum and root dentin defects around the molars (12,13). While these structural aberrations are probably caused, at least in part, by a deficiency of scaffolding function, there is an increasing body of evidence suggesting that amelogenin also has a signaling function (14–16).

In 1993, Wang found that amelogenin implantation in muscle resulted in ectopic bone formation. He concluded that mesenchymal cells were induced to differentiate into osteocytes by amelogenin (17). Evidence that amelogenin was functioning in a signaling role was suggested by the characterization of a plasma membrane-binding protein that some investigators feel could be a cell-surface receptor. Two of the smaller splice products of amelogenin (M73 and M59) have been shown to bind with saturation kinetics to a 95 kDa transmembrane protein identified as lysosomal membrane protein-1. It has not yet been directly shown what downstream signals are mediated by lysosomal membrane protein-1 under amelogenin activation; however, this report supports a more expanded role of amelogenins as regulatory proteins (18). Perhaps the most compelling evidence that amelogenin may act as a regulatory protein is that it was found to alter the expression of a number of phenotypically relevant genes (19,20). Type X collagen and Sox9 were upregulated with a subsequent increase in mineralization and differentiation in pre-odontoblasts. In periodontal ligament cells, type I collagen, bone morphogenetic protein and osteocalcin were all modulated by amelogenin.

Our findings demonstrate that a full-length recombinant amelogenin protein may stimulate cells in the immediate environment of a tooth (i.e. osteoblasts and periodontal ligament cells) to induce a more differentiated phenotype. Moreover, because amelogenin appears to colocalize with β-catenin, it may accomplish this function by influencing the Wnt/β-catenin signaling pathway.

The Wnt signaling pathway functions in many tissues of the body. In the canonical pathway, Wnt causes the stabilization of β-catenin and prevents its degradation through a ubiquitin-mediated proteosomal pathway. This occurs when the activated Wnt receptor (frizzled) catalyzes the phosphorylation of disheveled, which, in turn, leads to inhibition of the action of GSK3β. Inhibition of the GSK3β kinase prevent phosphorylation of axin and β-catenin. As a result, β-catenin translocates to the nucleus where it binds TCF/LEF proteins and elicits transcription of its target genes (21). In our study we also used a β-catenin reporter mouse (TOPGAL) to show the spatial distribution of Wnt signaling in postnatal tissue.

Material and methods

Gateway cloning expression vector

The recombinant mouse amelogenin (rM179) expression vector (pET11a) was a gift from Dr J. P. Simmer, University of Michigan (Ann Arbor, MI, USA) (22). The construct was used as a template for the Gateway cloning system (Invitrogen, Carlsbad, CA, USA). The polymerase chain reaction (PCR) was performed using a high-fidelity Platinum *Pfx* polymerase (Invitrogen). PCR products were purified using a gel extraction kit (Qiagen Inc., Valencia, CA, USA), cloned into pENTR/SD/D-TOPO vector (Invitrogen) and verified by sequencing using M13 primers. One miniprep of each cloned gene was chosen as the Entry Clone. LR clonase (Invitrogen) reactions to transfer DNA fragments from entry clones to a destination vector (pDEST17) (Invitrogen) were carried out according to the manufacturer's instructions (Invitrogen). The product of recombination reactions (LR reactions) was used to transform competent *Escherichia coli* DH5α using heat shock. Miniprep DNAs of the resulting expression clones were prepared from one colony from each reaction.

Expression and purification of recombinant protein

For expression in *E. coli*, the expression clone in pDEST17 containing the T7 promoter was transformed into a BL21(DE3)pLysS (Invitrogen) strain. Selected colonies were grown in Luria–Bertani medium at 37°C until the optical density of the culture reached 0.4–0.5 when measured at 600 nm. A final concentration of 0.7 mM isopropyl thio-β-D-galactoside (IPTG) (Sigma, St Louis, MO, USA) was added to induce the expression of rM179. The cells were harvested and resuspended in a bacterial protein extraction reagent (Pierce, Rockford, IL, USA), and the lysed cells were centrifuged for 15 min at 12,000 *g*. The supernatants were applied to a Ni Sepharose 6 Fast Flow column (Amersham Biosciences, Piscataway, NJ, USA) under native conditions. The resin was washed with buffer A [20 mM Tris–HCl, pH 8.0, 400 mM NaCl, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride and 10 mM imidazole] to reduce nonspecific binding, and rM179 was eluted with 50 mM Tris–HCl (pH 8.0) containing 100 mM NaCl, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride and 150 mM imidazole. The eluted protein was further purified to homogeneity by gel filtration chromatography using a Bio-Gel P-60 Gel (Bio-Rad, Hercules, CA, USA) column. The solvent system consisted of 50 mM Tris–HCl (pH 8.0), 100 mM NaCl and 1 mMphenylmethanesulfonyl fluoride. Solvent was run over the column and 1-mL fractions were collected. Purity was confirmed by electrophoresis on 12% sodium dodecyl suphate–polyacrylamide gels.

Cell isolation and culture

Human periodontal ligament cells were isolated from the healthy periodontal ligaments of molar teeth of de-identified individuals undergoing tooth extractions for orthodontic reasons. All the patients gave informed consent before providing the samples. After extraction, the teeth were placed in culture medium [Dulbecco's modified Eagle's medium; Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum, 50 μg/mL of ascorbic acid, 100 units/mL of penicillin and 100 μg/mL of streptomycin (Gibco)]. Healthy periodontal tissue was removed from the center of the root surface using a surgical scalpel. These tissues were cut into small pieces, rinsed with culture medium and placed in 24-well tissue culture plates; and coverslips were then placed over the tissues to prevent floating. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. When confluent, the cells surrounding the explants were passaged to tissue culture dishes by 0.25% trypsin and 0.1% EDTA. In this study, periodontal ligament cells were used between the third and the fifth passages.

ROS17/2.8 cells (a clonal osteoblast-like cell line originating from rat osteosarcoma) were also grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum and antibiotics, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was changed every other day. To study the spontaneous differentiation of ROS17/2.8 cells into osteoblasts, cells were cultured for up to 7 d in Dulbecco's modified Eagle's medium containing rM179.

X-Gal staining and histological analysis

Staining using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for LacZ activity in embryos was performed as reported previously (23). For the embryo whole-mount staining, the pregnant female mice were killed and the embryos were isolated in phosphate-buffered saline and prefixed in 0.2% glutaraldehyde solution containing 2 mM $MgCl_2$, 5 mM EGTA and 0.02% Nonidet P-40 at 4°C for 90 min. The samples were washed three times with 0.02% Nonidet P-40 solution at room temperature for 30 min, transferred to 1 mg/mL of X-Gal solution containing 5 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% Nonidet P-40 and then incubated at 30°C for 3 h. For bone tissue staining, mouse samples were rinsed twice with phosphate-buffered saline, fixed in 0.25% glutaraldehyde for 4 d at 4°C and washed three times with phosphate-buffered saline. The samples were decalcified with buffered EDTA for 3 wk, then embedded and processed to produce frozen sections. X-Gal staining for LacZ activity was carried out at 30°C overnight in 1 mg/mL of X-Gal solution containing 30 mM potassium ferricyanide, 30 mM potassium ferrocyanide and 1 mM $MgCl_2$ (24).

Methods for immunofluorescence histology

Mice were anesthetized and perfusion-fixed with paraformaldehyde–glutaraldehyde in phosphate-buffered saline. The mandibles were dissected and immersed in fixative for 24 h at 4°C. Hard tissues were decalcified at 4°C in a 14% (w/v) sodium–EDTA solution (pH 7.4) for 21 d before being processed for paraffin embedding. Sections (5 μm) were cut and mounted on poly+ glass slides, then deparaffinized, rehydrated and blocked for 1 h in phosphate-buffered saline containing 10% (w/v) normal goat serum. Sequential incubations with the amelogenin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by a secondary goat anti-rabbit Alexa Fluor® 488 immunoglobulin (Molecular Probes, Eugene, OR, USA), were performed with 1:50 dilutions in phosphate-buffered saline at room temperature for 3 and 1 h respectively. After each incubation, the slides were washed three times for 10 min each with phosphate-buffered saline containing 0.05% (v/v) Tween 20, pH 7.4. Nuclei were stained with 10 mg/mL of 4′,6-diamidino-2-phenylindole (Molecular Probes) in phosphate-buffered saline and mounted using ProLong Gold antifade reagent (Molecular Probes). Fluorescence was examined under a Zeiss Axiophot microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) equipped with an Olympus PD70 digital camera (Olympus America Inc., Center Valley, PA, USA).

Protein analysis

Western blotting was carried out on total cell lysates from human periodontal ligament cells. The cultured cells were harvested in mammalian protein extraction reagent (Pierce) using Complete Mini protease inhibitor, and the protein concentrations were measured using a Coomassie Plus Protein Assay kit (Pierce). Fifty micrograms of each extract was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane, the blots were probed with the total anti-β-catenin antibody (Sigma). Horseradish peroxidase-conjugated goat anti-mouse polyclonal antibodies (Bio-Rad) were used as the secondary antibody. The immune complexes were detected using ECL+ Plus with X-OMAT AR film (Kodak, Rochester, NY, USA).

Transient luciferase assays

Human periodontal ligament cells were plated at a density of 3.0×10^4 cells/cm^2 in 12-well plates. Following incubation for 24 h, the medium was changed to Opti-MEM (Gibco) I for 12 h before transfection. Cells were transfected with a pTOPFLASH-Luc reporter construct, βcat (s33y), and with rM179 at a concentration of 0.5 μg/well, using the *Trans*IT-LT1 (Mirus, Madison, WI, USA) transfection reagents according to the manufacturer's instructions. A Renilla luciferase expression plasmid, pRL-SV40, was cotransfected at a concentration of 10 ng/well as an internal control for transfection efficiency. After 48 h, cells were lysed with lysis buffer and assayed for luciferase activity using a dual luciferase assay kit (Promega, Madison, WI, USA). Each experimental condition was measured in triplicate, and the values given represent the mean ± standard deviation from two or three experiments.

Measurement of β-galactosidase activity *in vitro*

For pTOPGAL reporter assays, primary calvaria osteoblasts were isolated from 5-d-old neonatal mice. After 48 h, the cells were lysed (100 μL) using the Cytobuster protein extraction reagent. Twenty-microliter cell lysates were used to measure the β-galactosidase activity with the BD Luminacent β-Gal kit (BD Biosciences Clontech, Palo Alto, CA, USA). The β-galactosidase activity was normalized by reference to the protein content, which was determined using the Bio-Rad protein assay reagents (Bio-Rad).

Real-time PCR assays

To examine gene expression levels, cells were cultured and total RNA was extracted using the RNeasy kit (Qiagen). Single-stranded cDNA was produced using a reverse transcription kit (Invitrogen). PCR was performed on a Rotor-Gene 3000 real-time amplification instrument (Corbett Research, Mortlake, Australia). The primers for type I collagen, alkaline phosphatase, osteocalcin, receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin were from Invitrogen Corp. The PCR reactions contained a final concentration of 1SYBR Green PCR Master Mix (Bio-Rad), 10 μM specific primers and 2.5 ng of cDNA. The relative levels of mRNA of a specific gene were calculated using the standard curve generated from cDNA dilutions. The mean cycle threshold values from quadruplicate measurements were used to calculate the gene expression, with normalization to actin used as an internal control. Calculations of the relative level of gene expression were conducted according to the instructions from the User's Bulletin (P/N4303859) from Applied Biosystems (Foster City, CA, USA).

Statistical analysis

All data are presented as the mean ± standard deviation. Statistical significance was determined by analysis of variance.

Results

Expression and purification of amelogenin

A recombinant mouse amelogenin (rM179) protein fused to a His-tag was expressed using a pDEST17 expression vector transformed into *E. coli* BL21 (DE3) pLysS. The recombinant protein was expressed after induction with 0.7 mM IPTG. In order to determine whether the fusion protein was soluble, the cell pellet was resuspended with bacterial protein extraction reagent. It was found that more than 50% of rM179 was in a soluble form (data not shown). The lysate supernatant was applied to a Ni-Sepharose column equilibrated for affinity chromatography and rM179 was partially purified in two elution steps. The yield was approximately 2 mg from a 1-L culture of induced cells.

The partially purified protein was run on a sodium dodecyl sulfate–polyacrylamide gel and viewed after staining with Coomassie Brilliant blue (Fig. 1A). The band corresponding to the rM179 protein was identified with an anti-His-tag immunoglobulin (data not shown). The elutant was applied to a size-exclusion chromatography column and the expressed protein was collected. The sodium dodecyl sulfate–polyacrylamide electrophoresis results of this chromatographic step demonstrated the presence of a single band with a purity of greater than 95% (Fig. 1B). We used this protein in subsequent studies.

β-catenin signaling and amelogenin deposition occur at similar tissue sites

During tooth development, Hertwig's epithelial root sheath (25) directs the induction of the dental papilla into odontoblasts. After establishment of root dentin formation, the Hertwig's epithelial root sheath begins to disintegrate and is responsible for the secretion of enamel-related proteins involved in tooth formation. The disintegrating Hertwig's epithelial root sheath tissue remains as Malassez's epithelial rest (26). The complex interplay of cells and factors released in this area contributes to the regulation of enamel formation and alveolar bone remodeling and probably controls ameloblast differentiation. Consistent with this view, a number of reports in the literature, as far back as the 1970s, have described the Hertwig's epithelial root sheath, the periodontal ligament and the epithelial diaphragm to be rich in amelogenin expression (27,28). We also made these observations with antibodies specific to amelogenin (Fig. 2A). Additionally, we found that these regions are sites of active β-catenin signaling. This was determined using the reporter mouse strain, TOPGAL (29). In these animals, β-galactosidase activity is under the

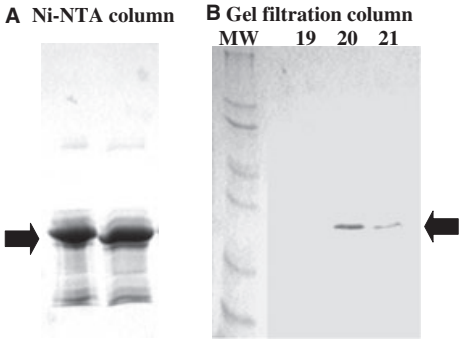

Fig. 1. Coomassie Blue-stained 12% sodium dodecyl sulfate–polyacrylamide gels demonstrating purification of mouse recombinant amelogenin (rM179). Details of the method are described in the *Material and methods* section. (A) The level of purity after elution from a nickel Sepharose (Ni-NTA) column. (B) The level of purity after chromatography on a gel filtration column. MW, molecular weight standards.

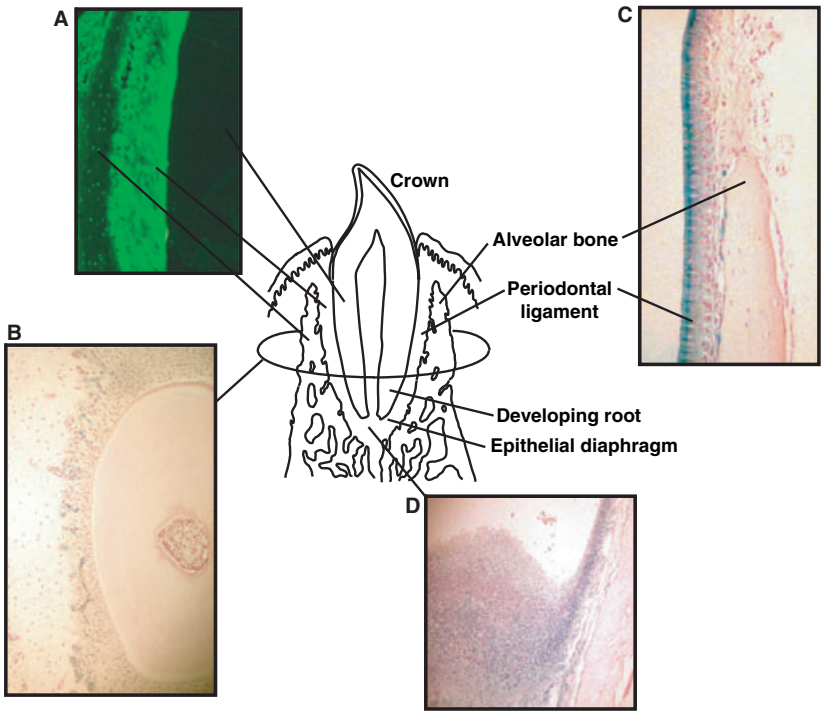

Fig. 2. Amelogenin and β-galactosidase staining in a TOPGAL mouse. (A) Immunofluorescent localization of amelogenin. Strong signals are observed in the periodontal ligament and in the cells within alveolar bone. (B, C and D) The presence of β-galactosidase is shown as a marker of β-catenin signaling in this TOPGAL mouse model. The most intense staining for β-catenin is in the periodontal ligament (B and C) and in the root region. Magnification ×200; nuclear fast red counterstain.

control of a lymphoid-enhancing binding factor, TCF/LEF, and activated β-catenin (30). The transgene is comprised of three consensus TCF/LEF-binding motifs upstream of a minimal c-fos promoter. Immunohistochemical identification of high β-galactosidase activity is representative of β-catenin signaling.

Our results show that β-catenin signaling occurs in a defined region of the tooth root (Fig. 2B–D). The images in these panels are from a vertical and coronal plane through a maxillary molar from a 33-d-old TOPGAL mouse. Intense staining for β-galactosidase activity was observed throughout the periodontal ligament (Fig. 2B,C) and along the surface and base of the root (Fig. 2C). β-Catenin signaling was also noted at the growing apex of the root as well as in the area of Hertwig's epithelial root sheath (Fig. 2D). Although these observations suggest a relationship between amelogenin expression and β-catenin signaling, they do not constitute causal proof of β-catenin signaling by amelogenin. Further *in vitro* results presented below support this contention.

Amelogenin induces β-catenin transcription factor activation

In order to provide a more direct demonstration of the link between amelogenin and β-catenin-mediated signaling, we examined activation of the transcription factor using an antibody that recognizes the activated (i.e. unphosphorylated) form. Figure 3 demonstrates that rM179 (5.0 μg/mL) induces a marked increase in the level of activated β-catenin in periodontal ligament cells. As a positive control, we tested the effect of the paracrine regulatory factor, Wnt3a and found that it also increased the levels of activated β-catenin. Wnt3a plus rM179 induced the activation of β-catenin to a level approximately the same as either of the agents alone. The fold increase for each of these activations was determined by measuring the integrated optical density of the bands, and the results indicated that rM179 is approximately equipotent with Wnt3a in stimulating β-catenin activation. As we used maximal concentrations of Wnt3a and rM179, we did not observe any synergy between the factors. This suggests that their effect may occur through a convergent pathway. Interestingly, neither Wnt3a nor amelogenin changed the total protein levels for β-catenin.

This ability of the Wnt signaling pathway to respond to amelogenin correlates with the histological findings in Fig. 2.

Amelogenin directly activates β-catenin signaling in periodontal ligament cells and osteoblasts

Concomitant with the activation of β-catenin, we also showed that amelogenin can induce transactivating functions of the transcription factor in both periodontal ligament cells and osteoblasts. These results were obtained by measuring the activity of a transfected reporter construct (TOPFLASH) as well as reporter activity from the TOPGAL mice. Figure 4 shows that isolated human periodontal ligament cells, when transfected with the β-catenin TOPFLASH reporter, can be activated in a dose-dependent manner by rM179. A concentration of 5.0 μg/mL of rM179 stimulated the LacZ reporter activity of the TOPFLASH construct by approximately fourfold.

βcat (s33y) is a plasmid containing a constitutively active form of β-catenin. This construct was used as a positive control in the periodontal ligament cells and also demonstrated activation of the TOPFLASH reporter.

Figure 5 shows that rM179 can stimulate β-catenin reporter activity in osteoblasts. Osteoblasts were isolated from TOPGAL mouse calvaria. TOPGAL mice possess a *LacZ* β-catenin reporter gene in all cells. Upon treatment of these isolated cells with rM179 at 3.0 and 5.0 μg/mL, there was a dose-dependent statistically significant increase in reporter activity. Reporter activity was also stimulated with the positive-control agents, Bio and

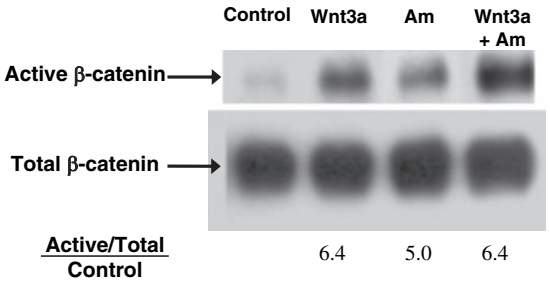

Fig. 3. Human periodontal ligament cells were plated in 60-mm dishes (5.0×10^5 cells/dish) for 16 h. Then, the cells were treated with Wnt3a, mouse recombinant amelogenin (AM) and Wnt3a plus amelogenin (Wnt3a + AM) for another 24 h. The cell lysates were collected for western blot analysis. We used two different antibodies, detecting total β-catenin and active β-catenin, in these studies. The fold increase of the active form of β-catenin was calculated by normalizing densitometric measurements of the spots to total β-catenin and the untreated control. This figure is representative of three separate experiments. Am, mouse recombinant amelogenin.

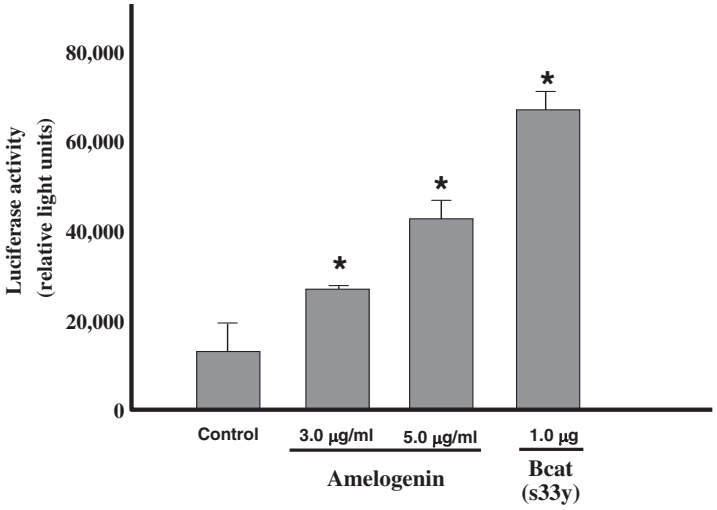

Fig. 4. Human periodontal ligament cells were plated (1.0×10^5 cells/well) for 24 h and then transfected with a TOPFLASH reporter plasmid. Positive control cells were transfected with βcat (s33y), a constitutively active form of a β-catenin plasmid, for a further 24 h. Then, cells were treated with different concentrations of mouse recombinant amelogenin (rM179) for 24 h. The cells were lysed and the luciferase activity was measured. Luciferase fluorescence was corrected for transfection efficiency by normalization with a Renilla construct (see the *Material and methods*). Each bar represents the mean ± standard deviation of triplicate samples. The results are representative of three separate experiments, with similar results obtained on each occasion (*$p < 0.05$).

Wnt3a. Bio is a chemical reagent with a strong and selective inhibitory effect for the enzyme GSK3. GSK3 is a kinase that phosphorylates β-catenin and prevents its activation. Thus, inhibition of GSK3 leads to accumulation of the active form of β-catenin and activation of the TOPGAL reporter. Wnt3a activates the canonical pathway for β-catenin, which is consistent with our observations in Fig. 3.

Amelogenin alters osteoblast gene expression

Using the osteoblast cell line, ROS17/2.8, we were able to show that treatment of these cells with amelogenin induced gene expression for a number of phenotypic markers related to osteoblast maturation. The rM179 protein characterized in Fig. 1, and used for cell treatments in Figs 4 and 5, was added to cultures of ROS17/2.8 cells for up to 7 d. rM179 was added at concentrations of 0.1, 1.0 and 5.0 μg/mL every 2–3 d. Total RNA was extracted and analyzed for type I collagen, alkaline phosphatase, osteocalcin, RANKL and osteoprotegerin. The data in Fig. 6 show that rM179 strongly upregulates osteocalcin mRNA levels, moderately upregulates type I collagen and RANKL mRNA levels, and has a minimal effect on the levels of alkaline phosphatase mRNA. rM179 had no effect on the regulation of osteoprotegerin mRNA levels. As osteocalcin is recognized to be a gene that is upregulated late in the osteoblast maturation pathway, it appears that amelogenin may be an important regulator of the final differentiation phases for bone formation.

Discussion

The salient points of this manuscript demonstrate that we have prepared a biologically active form of amelogenin that can be used to activate cells of the periodontal ligament and bone. We have also shown that the spatial expression of β-catenin signaling overlaps, to some extent, with the known and demonstrated localization of amelogenin, thus supporting a possible interactive role of these regulators. Moreover, our data support the possibility that amelogenin can directly upregulate the β-catenin pathway, resulting in the expression of genes necessary for alveolar bone formation. Because of its abundance *in vivo*, amelogenin could play a significant role in signaling, by modifying or supplementing canonical Wnt signaling either synergistically or additively.

Although we have shown that amelogenin leads to the activation of β-catenin, we are still uncertain as to the mechanism by which this might occur. It is possible that amelogenin may evoke β-catenin activation through its association with lysosomal membrane protein-1, but, without the knowledge of the signaling intermediates that are part of the lysosomal membrane protein-1 signaling pathway, no firm conclusion can be drawn.

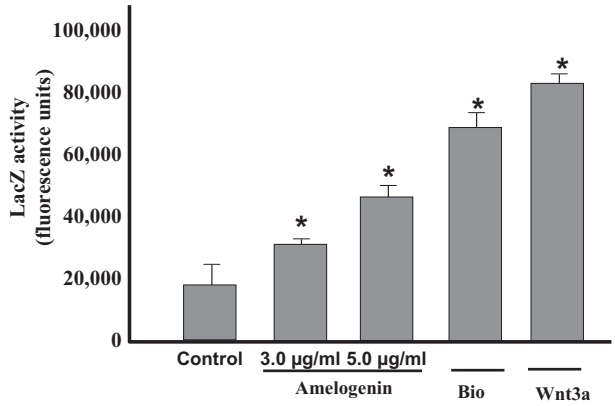

Fig. 5. Osteoblastic cells were isolated and cultured from the calvaria of 4-d-old TOPGAL mice. The cells were plated in 24-well plates (5.0×10^4 cells/well) for 24 h and then treated with mouse recombinant amelogenin (rM179), Bio and Wnt3a. (Bio is a chemical reagent with a strong and selective inhibitory effect for the enzyme GSK3. GSK3 is a kinase that phosphorylates β-catenin and prevents its activation. Thus, inhibition of GSK3 leads to accumulation of the active form of β-catenin and activation of the TOPGAL reporter. Wnt3a, a paracrine regulatory factor, activates the canonical pathway for β-catenin.) After 24 h of incubation, the cells were lysed with passive lysis buffer (200 μL/well) and the LacZ activity was determined using luciferase fluorescence (see the *Material and methods*). The activity was normalized relative to the total protein in the wells. Each bar represents the mean ± standard deviation of triplicate samples. The results are representative of three separate experiments, with similar results obtained on each occasion (*$p < 0.05$).

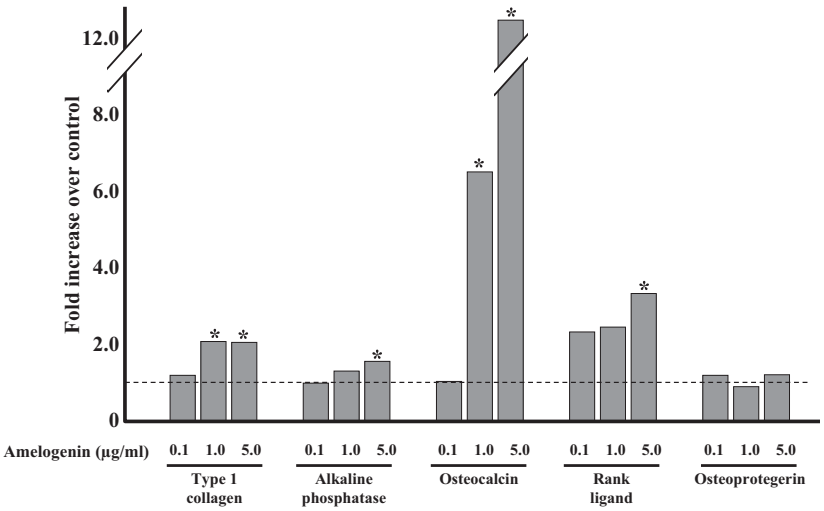

Fig. 6. Dose-dependent effect of mouse recombinant amelogenin (rM179) on gene expression by an osteoblast cell line (ROS17/2.8 cells). Cells were cultured in Dulbecco's modified Eagle's medium containing 7% fetal bovine seum and rM179 (0.1, 1.0 and 5.0 μg/mL) for 7 d. Total RNA was quantitatively analyzed for type 1 collagen, alkaline phosphatase, osteocalcin, receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin expression by real-time reverse transcription–polymerase chain reaction. Normalization was with β-actin. rM179 promoted the upregulation of all markers except osteoprotegerin. Each bar represents the mean ± standard deviation of triplicate samples. The results are representative of three separate experiments, with similar results obtained on each occasion (*$p < 0.05$).

Interestingly, as protein kinase A, protein kinase C and mitogen-activated protein kinase pathways have a ubiquitous role in many cell-differentiation mechanisms, and because crude extracts from enamel matrix are known to activate mitogen-activated protein kinase and Smad pathways (31), we considered the involvement of amelogenin interaction with G-protein-coupled mechanisms. However, as documented in the literature, there are few examples of interactions between β-catenin signaling and any of these G-protein-coupled pathways and thus it is less likely that amelogenin exerts its major effects through G-protein-coupled intermediates.

There are clinical applications that may relate to our studies. Periodontitis and orthodontic tooth movement are two processes in the oral cavity that depend a great deal on the integrated activity of bone resorption and bone formation. The development and maintenance of tissue structures surrounding teeth is a multifaceted process that is incompletely understood. The interplay of cells and factors that control the bone quality and structure of the enamel and bone matrix depend on the release of, and response to, factors by ameloblasts, epithelial cells, ligament cells and bone cells. In this report we propose that amelogenin may be another factor with both structural and regulatory properties. The signaling role of amelogenin, in addition to its structural role in development, adds another level of complexity to tooth formation and maintenance, while at the same time helping to unravel this process.

Acknowledgement

We thank Dr J. P. Simmer, University of Michigan (Ann Arbor, Michigan), for providing the rM179 expression vector.

References

1. Hammarstrom L, Heijl L, Gestrelius S. Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. *J Clin Periodontol* 1997;**24:**669–677.
2. Laurell L, Falk H, Fornell J, Johard G, Gottlow J. Clinical use of a bioresorbable matrix barrier in guided tissue regeneration therapy. Case series. *J Periodontol* 1994;**65:**967–975.
3. Peters H, Balling R. Teeth. Where and how to make them. *Trends Genet* 1999;**15:**59–65.

4. Fincham AG, Moradian-Oldak J. Recent advances in amelogenin biochemistry. *Connect Tissue Res* 1995;**32:**119–124.
5. Simmer JP, Fincham AG. Molecular mechanisms of dental enamel formation. *Crit Rev Oral Biol Med* 1995;**6:**84–108.
6. Moradian-Oldak J. Amelogenins: assembly, processing and control of crystal morphology. *Matrix Biol* 2001;**20:** 293–305.
7. Fincham AG, Moradian-Oldak J, Diekwisch TG *et al*. Evidence for amelogenin ''nanospheres'' as functional components of secretory-stage enamel matrix. *J Struct Biol* 1995;**115:**50–59.
8. Zeichner-David M, Diekwisch T, Fincham A *et al*. Control of ameloblast differentiation. *Int J Dev Biol* 1995;**39:** 69–92.
9. Smith CE. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med* 1998;**9:**128–161.
10. Gibson CW, Yuan ZA, Hall B *et al*. Amelogenin-deficient mice display an amelogenesis imperfecta phenotype. *J Biol Chem* 2001;**276:**31871–31875.
11. Lagerstrom M, Dahl N, Nakahori Y *et al*. A deletion in the amelogenin gene (AMG) causes X-linked amelogenesis imperfecta (AIH1). *Genomics* 1991;**10:**971–975.
12. Hatakeyama J, Sreenath T, Hatakeyama Y *et al*. The receptor activator of nuclear factor-kappa B ligand-mediated osteoclastogenic pathway is elevated in amelogenin-null mice. *J Biol Chem* 2003; **278:**35743–35748.
13. Hatakeyama J, Philp D, Hatakeyama Y *et al*. Amelogenin-mediated regulation of osteoclastogenesis, and periodontal cell proliferation and migration. *J Dent Res* 2006;**85:**144–149.
14. Veis A, Tompkins K, Alvares K *et al*. Specific amelogenin gene splice products have signaling effects on cells in culture and in implants in vivo. *J Biol Chem* 2000;**275:**41263–41272.
15. Boabaid F, Gibson CW, Kuehl MA *et al*. Leucine-rich amelogenin peptide: a candidate signaling molecule during cementogenesis. *J Periodontol* 2004;**75:** 1126–1136.
16. Viswanathan HL, Berry JE, Foster BL *et al*. Amelogenin: a potential regulator of cementum-associated genes. *J Periodontol* 2003;**74:**1423–1431.
17. Wang W. Ectopic bone induction by human fetal enamel proteins. *Zhonghua Kou Qiang Yi Xue Za Zhi* 1993;**28:** 362–364.
18. Tompkins K, George A, Veis A. Characterization of a mouse amelogenin [A-4]/M59 cell surface receptor. *Bone* 2006; **38:**172–180.
19. Lacerda-Pinheiro S, Septier D, Tompkins K, Veis A, Goldberg M, Chardin H. Amelogenin gene splice products A+4 and A−4 implanted in soft tissue determine the reorientation of CD45-positive cells to an osteo-chondrogenic lineage. *J Biomed Mater Res A* 2006;**79:**1015–1022.
20. Zeichner-David M, Chen LS, Hsu Z, Reyna J, Caton J, Bringas P. Amelogenin and ameloblastin show growth-factor like activity in periodontal ligament cells. *Eur J Oral Sci* 2006;**256:**381–382.
21. Yamamoto H, Kishida S, Kishida M, Ikeda S, Takada S, Kikuchi A. Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. *J Biol Chem* 1999;**274:**10681–10684.
22. Simmer JP, Lau EC, Hu CC, Aoba T *et al*. Isolation and characterization of a mouse amelogenin expressed in Escherichia coli. *Calcif Tissue Int* 1994;**54:**312–319.
23. Kohn MJ, Leung SW, Criniti V, Agromayor M, Yamasaki L. Dp1 is largely dispensable for embryonic development. *Mol Cell Biol* 2004;**24:**7197–7205.
24. Chen M, Lichtler AC, Sheu TJ *et al*. Generation of a transgenic mouse model with chondrocyte-specific and tamoxifen-inducible expression of Cre recombinase. *Genesis* 2007;**45:**44–50.
25. Welbury R, Walton AG. Continued apexogenesis of immature permanent incisors following trauma. *Br Dent J* 1999;**187:**643–644.
26. Bykov VL. Epithelial cell rests of Malassez: tissue, cell, and molecular biology. *Morfologiia* 2003;**124:**95–103.
27. Slavkin HC. Towards a cellular and molecular understanding of periodontics. Cementogenesis revisited. *J Periodontol* 1976;**47:**249–255.
28. Schonfeld SE, Slavkin HC. Demonstration of enamel matrix proteins on root-analogue surfaces of rabbit permanent incisor teeth. *Calcif Tissue Res* 1977; **24:**223–229.
29. Hens JR, Wilson KM, Dann P, Chen X, Horowitz MC, Wysolmerski JJ. TOPGAL mice show that the canonical Wnt signaling pathway is active during bone development and growth and is activated by mechanical loading in vitro. *J Bone Miner Res* 2005;**20:**1103–1113.
30. DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* 1999; **126:**4557–4568.
31. Kawase T, Okuda K, Momose M, Kato Y, Yoshie H, Burns DM. Enamel matrix derivative (EMDOGAIN) rapidly stimulates phosphorylation of the MAP kinase family and nuclear accumulation of smad2 in both oral epithelial and fibroblastic human cells. *J Periodont Res* 2001;**36:** 367–376.