Amelogenin Exons 8 and 9 Encoded Peptide Enhances Leucine Rich Amelogenin Peptide Mediated Dental Pulp Repair

Yulei Huang^{a, b} Michel Goldberg^c Thuan Le^b Ran Qiang^{b, d}
Douglas Warner^{b, e} Halina Ewa Witkowska^f Haichuan Liu^f Li Zhu^b
Pamela DenBesten^b Wu Li^b

^aDepartment of Oral Medicine, Guanghua School of Stomatology, Sun Yat-sen University, Guangzhou, PR China;

^bDepartment of Orofacial Sciences, University of California, San Francisco, Calif., USA; ^cInserm UMR-S 747, UFR Biomédicales des Saints-Pères, Université Paris Descartes, Paris, France; ^dSaierbio LLC, Tianjin, PR China;

^eUS Airforce, Misawa, Japan; ^fDepartment of Obstetrics, Gynecology and Reproductive Sciences, School of Medicine, University of California, San Francisco, Calif., USA

Key Words

Amelogenin · Alternative splicing · Amelogenin exons 8 and 9 · Dental pulp cells · Dental pulp capping

Abstract

Amelogenins containing exons 8 and 9 are alternatively spliced variants of amelogenin. Some amelogenin spliced variants have been found to promote pulp regeneration following pulp exposure. The function of the amelogenin spliced variants with the exons 8 and 9 remains unknown. In this study, we synthesized recombinant leucine rich amelogenin peptide (LRAP, A-4), LRAP plus exons 8 and 9 peptide (LRAP 8, 9) or exons 8 and 9 peptide (P89), to determine their effects on odontoblasts. In vivo analyses were completed following the insertion of agarose beads containing LRAP or LRAP 8, 9 into exposed cavity preparations of rat molars. After 8, 15 or 30 days' exposure, the pulp tissues were analyzed for changes in histomorphometry and cell proliferation by PCNA stainings. In vitro analyses included the effects of the addition of the recombinant proteins or peptide on cell proliferation, differentiation and adhesion of postnatal human

dental pulp cells (DPCs). These studies showed that in vivo LRAP 8, 9 enhanced the reparative dentin formation as compared to LRAP. In vitro LRAP 8, 9 promoted DPC proliferation and differentiation to a greater extent than LRAP. These data suggest that amelogenin exons 8 and 9 may be useful in amelogenin-mediated pulp repair.

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Abbreviations used in this paper

DPC	dental pulp cell
DSPP	dentin sialophosphoprotein
HPLC	high-performance liquid chromatography
LAMP-1	lysosome-associated membrane protein 1
P89	exons 8 and 9 peptide
LRAP (L-, A-4)	leucine rich amelogenin peptide
LRAP 8, 9 (L+)	LRAP plus exons 8 and 9 peptide
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Amelogenins are hydrophobic alternatively spliced proteins that are the major protein components of the developing enamel matrix [Fincham et al., 1999]. They have also been identified in smaller quantities in other cells and tissues, including odontoblasts [Veis et al., 2000; Dey et al., 2001; Oida et al., 2002; Veis, 2003]. A spliced form of amelogenins, containing exons 2, 3, 5, 6 (3' terminal) and 7 but lacking exons 4 and partial exon 6 (A-4), was identified by Veis et al. [2000] as having signaling properties mediated through lysosome-associated membrane protein 1 (LAMP-1) cell surface receptor. This protein was one of the alternatively spliced amelogenins previously isolated from the enamel matrix, known as leucine rich amelogenin peptide (LRAP) encoded by exons 2, 3, 5, 6d, and 7 [Veis, 2003]. Further in vivo studies by Goldberg et al. [2003] showed that A-4 (LRAP) could promote pulp repair in the rat molar model [Lacerda-Pinheiro et al., 2006; Jegat et al., 2007].

Exons 8 and 9 were first identified in the rat enamel organ using rapid amplification of cDNA ends (3' RACE) [Li et al., 1995, 1998], and further analyses showed evidence of amelogenins transcribed from mRNA carrying exons 8 and 9 in ameloblasts, odontoblasts, enamel and dentin matrix in rodent teeth [Baba et al., 2002; Papagerakis et al., 2005]. In silico bioinformatic analyses have shown that the exon 9, but not exon 8 sequence, is present in the human genome, though mRNA expression of this sequence in amelogenin transcripts has not yet been identified [Sire, 2010].

Considering the conservation of these isoforms between species and their existence for millions of years, the exons should have their own unique functions in tooth development. Given the effects of amelogenins on mesenchymal proliferation and wound healing [Veis et al., 2000; Tompkins and Veis, 2002; Veis, 2003; Tompkins et al., 2005; Ye et al., 2006], in this study we determined the effect of the addition of the peptide sequence coded by exons 8 and 9 to LRAP on mesenchymal cells. Recombinant LRAP (L-), LRAP plus exons 8 and 9 peptide (LRAP 8, 9 or L+) and synthetic exons 8 and 9 peptide (P89) were used to compare their cellular functions on postnatal dental pulp cell (DPC) by analyzing the cell proliferation, differentiation and adhesion in vitro and in vivo in this study.

Materials and Methods

Expression, Purification and Characterization of Recombinant Human L-, L+ and P89

L- cDNA was amplified from a human ameloblast DNA library, cloned into pGEX-4T-1 (Amersham Biosciences, Piscat-

away, N.J., USA) vector and transformed into T7 Express *Escherichia coli* cells (New England Biolabs, Ipswich, Mass., USA). At an optical density of 0.4 at 600 nm, the cells were induced for protein expression using 0.4 mM isopropyl-1-thio- β -D-galactopyranoside (Invitrogen, Carlsbad, Calif., USA) at 37°C for an additional 2 h. The bacteria pellets were lysed in phosphate-buffered saline (PBS) at pH 7.4 containing 1% Triton X-100, 1% N-lauroylsarcosine sodium salt (Sigma, St. Louis, Mo., USA) and protease inhibitor cocktails (Roche, Mannheim, Germany). The soluble fraction of the GST-L- supernatant was purified on glutathione Sepharose 4B beads (GE Healthcare, Uppsala, Sweden), and the bound GST-L- was digested with thrombin (GE Healthcare) to remove the GST tag. The eluted L- was further purified by reverse-phase high-performance liquid chromatography (HPLC) on a C18 column (Varian, Lake Forest, Calif., USA).

An L+ expression vector was constructed from the L- vector by mutagenesis PCR using ExSite Mutagenesis Kit (Stratagene, La Jolla, Calif., USA) following the manufacturer's manual. Exon 8 was first inserted to replace exon 7 in the L- sequence in the pGEX vector using the primers LRAP_8 and LRAP_Down (table 1). A second round of mutagenesis was then done to add exon 9 to the 3' end of the product, using a new primer, LRAP_89_2 (table 1), along with the LRAP_Down primer. The final vector pGEX L+ was sequenced for confirmation before protein expression. Expression and purification of L+ were done using the same methods for L- as described above.

Purified L- and L+ were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue staining, Western blot and liquid chromatography mass spectrometry analyses. Western blots were done using rabbit antipeptide antibodies synthesized against L- peptide [Le et al., 2007] and against the peptide corresponding to the amino acids coded by the exons 8 and 9 cDNA sequence [Li et al., 1998]. P89 was synthesized and purified by CS Bio Co. (Menlo Park, Calif., USA). The mass of purified P89 was confirmed by MALDI-TOF mass spectrometry.

Dental Pulp Repair following Exposure to L- and L+ in vivo

Affigel agarose beads (70–150 μ m in diameter; Biorad, Hercules, Calif., USA) were equilibrated with PBS for use as controls. Additional beads were incubated with 50 μ g of L- or L+ in 75 ml of PBS at 37°C for 1 h, with an estimated 0.2 μ g of L- or L+ adsorbed onto each bead.

Forty-five male Sprague Dawley rats, 6–7 weeks old (approximately 150 g), were divided into 3 groups, each containing 15 animals. The rats were anesthetized, a cavity was drilled with a small tungsten burr and a small pulp exposure was created at the buccal cervical margin of the first molars as previously described [Lacerda-Pinheiro et al., 2006]. Agarose beads, containing L-, L+ or PBS-soaked controls, were placed on the exposed pulps, and covered with light-cured glass ionomer cement. All experiments were performed under an institutionally approved protocol for the use of animals in research.

Five animals from each group were sacrificed at 8, 15 and 30 days after implantations. The molars were demineralized, sectioned and stained with Masson's trichrome as previously described [Lacerda-Pinheiro et al., 2006]. Cell proliferation was characterized by immunohistochemical staining using an antibody against proliferating cell nuclear antigen (PCNA; NAO3 Oncogene Research Products, Cambridge, Mass., USA, dilution

Table 1. Sequences of the primers used in mutagenesis

Primer name	Sequences
LRAP_8	CGGATGCCTTGCCGTGCCCTGGTACCACTTCATAGGAGAAAATGCCACTTCTTCCCGCTTGGT
LRAP_Down	TAAGAATTCCCGGGTTCGACTC
LRAP_89_2	TCATTTTCTGTTGTGGTTTCCATGTTAAGCGGATGCCTTGCCGTGCCCTGGTACCACTTAG

1/75). In control sections, the primary antibody was omitted and the secondary antibody was used alone (polyclonal goat antibody 1/100, Dako, Glostrup, Denmark).

DPC Proliferation in vitro in the Presence of L-, L+ or P89

DPCs were obtained from adult human pulp tissues as previously described [Gronthos et al., 2000]. Passage 3 cells were plated at a density of 2×10^3 cells/well in clear-bottomed 96-well plates (Falcon, BD Bio Sciences, Bedford, Mass., USA) and grown in MEM containing 10% FBS (Invitrogen) and 1% penicillin/streptomycin (UCSF Cell Culture Facility, San Francisco, Calif., USA) until 60% confluence. The cells were then serum-starved for 24 h, and L-, L+ or P89 (30, 100 or 300 nM) was added to the cell culture media. Cells without peptide treatment were used as controls. They were maintained in this medium for 24 h before they reached complete confluence, and cell proliferation was then measured by BrdU incorporation using Cell Proliferation ELISA BrdU kit (Roche) according to the manufacturer's instructions.

Alterations in DPC Differentiation following Addition of L-, L+ or P89 in vitro

DPCs at passage 3 were cultured and grown to complete confluence in MEM with 10% serum. The medium was then supplemented with 10 mM β -glycerophosphate (Sigma), 50 μ g/ml ascorbic acid (Sigma), 10 nM dexamethasone (Sigma) and 150 nM L-, L+ or P89 was added to the culture dishes in triplicate. The cells were cultured for 21 days, with changes of media every 3 days. Cells without peptide treatment were used as controls.

Real-time PCR was used to compare dentin sialophosphoprotein (DSPP) and type I collagen gene expression, as markers of pulp cell differentiation [Bleicher et al., 1999; About et al., 2000; Domon et al., 2001; Liu et al., 2005; Zhang et al., 2005; Demarco et al., 2010]. Total RNA was isolated from the cultures using an RNeasy Mini Kit (Qiagen, Valencia, Calif., USA), and 2.5 μ g purified total RNA was reverse-transcribed into cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen). PCR amplification was done using FastStart TaqMan[®] Probe Master (Roche) and DSPP primers or type I collagen primers (Applied Biosystems Inc., Foster City, Calif., USA) in a 7500 Real Time PCR System (Applied Biosystems). The results were analyzed by Sequence Detection System software (Applied Biosystems). Conditions for PCR were 5 min at 94°C as an initial denaturing step, followed by 40 amplification cycles of 15 s at 94°C and 1 min at 60°C. The cycle threshold values from each primer pair were normalized with the cycle threshold values for an endogenous control 18S RNA.

The relative numbers of mineralized nodules formed by DPCs cultured for 21 days in the mineralization media containing L-, L+ or P89 were compared. The cells were grown as described

above, and after 21 days, the contents of each dish were fixed for 15 min in 10% neutral buffered formalin and then washed with PBS. After fixation, 5% silver nitrate was added to each dish, which was then exposed to light for 45 min, followed by incubation in 5% sodium thiosulfate solution for 5 min. The mineralized nodules were photographed using digital camera to allow comparisons of nodule formation.

Analyses of L-, L+ or P89 Mediated DPC Adhesion in vitro

Cell adhesion assays were done with a Vybrant Cell Adhesion Assay Kit (Invitrogen) according to the manufacturer's instructions. In brief, triplicate wells in a 96-well plate (Falcon, BD Bio Sciences) were coated with L-, L+ or P89, respectively, at concentrations of 30, 100 and 300 nM at 4°C overnight. Water-coated wells were used as controls. All wells were blocked for 30 min with 0.1% bovine serum albumin (Fisher Scientific, Pittsburgh, Pa., USA) which had been heat-inactivated at 80°C for 3 min. Equal amounts of calcein-labeled cell suspensions (2×10^5 DPCs at passage 3) were added to each treated microplate well, and the cells were incubated for 2 h at 37°C to allow adhesion. Following the incubation, nonadherent calcein-labeled cells were removed by careful washing, and the relative amounts of adhering cells were indirectly measured by quantitating total fluorescence per well at wavelength EX/EM = 494/517 nm on a SpectraMax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, Calif., USA).

Statistical Analysis

Experiments were performed in triplicate. Statistical analysis between groups was done by one-way ANOVA analysis, using Prism software (GraphPad Software Inc., San Diego, Calif., USA). $p < 0.05$ were considered as significant differences between groups.

Results

Recombinant L-, L+ and P89 Were Synthesized and Purified

The GST-tagged L- and L+ proteins were expressed in *E. coli* cells with apparent molecular weight of 33 kDa for L- and 35 kDa for L+. After purifying by glutathione affinity chromatography and removing the GST tag by thrombin, both L- and L+ showed single peaks in reversed-phase HPLC using a C18 column. L- was eluted at 57% acetonitrile while L+ showed a peak at 52% acetonitrile.

Fig. 1. Characterization of LRAP (L-) and LRAP 8, 9 (L+). **a** SDS-PAGE with Coomassie brilliant blue staining of L- and L+ after HPLC purification. **b** Western blot analysis of the purified L- and L+ using anti-L- antibody. **c** Western blot analysis of the purified L- and L+ using anti-exons 8 and 9 peptide antibody. M = SeeBlue Plus2 marker standard (Invitrogen).

Table 2. Mass spectrometry analyses of LRAP (L-), LRAP 8, 9 (L+) and P89

Proteins	Measured m/z, Da	Predicted m/z, Da
L-	6,838.8	6,838.5
L+	9,526.5	9,525.8
P89	2,824.3	2,825.2

Table 3. Evaluation of PCNA immunostaining

PCNA staining	Beads alone	LRAP (L-)	LRAP 8, 9 (L+)
Day 8	8.00 ± 1.42	9.15 ± 0.95	12.70 ± 1.92*
Day 15	7.43 ± 1.28	1.75 ± 0.04	3.14 ± 0.09*

Values are means ± SD given as density/5,000 μm².

* p < 0.05 vs. L- group at the same time point.

trile, consistent with the presence of an extra hydrophilic domain at the C terminus in L+. L- and L+ appeared as single bands with molecular mass values of 6.8 and 9.5 kDa in SDS-PAGE with Coomassie brilliant blue staining, respectively (fig. 1a).

Western blots showed that the L- antibody could detect both L- and L+, while the antibody for exons 8 and 9 translated peptide identified L+ only (fig. 1b, c). Mass spectrometry showed that the mass values of L-, L+ and P89 matched the predicted mass values for these proteins and peptide (table 2).

L- and L+ Enhanced DPC Proliferation and Differentiation in vivo, with L+ Having Greater Effects

In vivo analyses were completed following the insertion of agarose beads containing L- or L+ into mechanically exposed cavity preparations of rat first molars. After 8, 15 or 30 days' exposure, the pulp tissue was analyzed for histological changes and cell proliferations by PCNA. PCNA immunostaining showed the presence of more mitotic cells at 8 days in pulps with L+ loaded agarose bead implants (fig. 2a; table 3), as compared with L- loaded beads (fig. 2b; table 3). Fewer PCNA-positive cells were present throughout the implanted pulps after 15 days in

the L+ group (fig. 2c; table 3), and very few mitotic cells were detected from the same period of time in the L- group (fig. 2d; table 3).

Masson's trichrome showed a stronger inflammatory reaction 8 days after implantation of the L+ implanted pulps as compared with the L- pulps (fig. 3a, b). The reaction was still intense 15 days after implantation with L- whereas with L+, a large part of the pulp was filled by reparative dentin formation (fig. 3c, d). In the 30 days' implanted pulps, some reparative dentin was formed after L- implantation (fig. 3e), but in a smaller amount than after L+ implantation (fig. 3f).

Effects of L-, L+ and P89 on Cell Proliferation of DPCs in vitro

In vitro, we used the BrdU incorporation method to measure the DPC proliferation mediated by L-, L+ or P89. DPC proliferation was significantly enhanced with the addition of 30, 100 and 300 nM L- or L+ in a dose-dependent manner as compared to the untreated control groups (p < 0.05). P89 significantly increased cell proliferation at concentrations of 100 and 300 nM. Cells in media containing L+ had significantly greater proliferation at concentrations of 100 and 300 nM proteins as compared to the same concentrations of L- (p < 0.05). Addi-

Fig. 2. Effects of LRAP 8, 9 (L+) and LRAP (L-) on pulp cell proliferation as measured by PCNA immunostaining. **a** Sections through molars implanted with agarose beads containing L+ after 8 days showed numerous mitotic cells (dark brown staining). **b** Implantation of L- resulted in relatively fewer positively stained cells. **c, d** These results, showing increased PCNA-immunostained cells in L+ as compared to L- implanted pulps, were more pronounced after 15 days. b = Bead. Scale bar: 150 μ m.

tion of 300 nM P89 peptides also significantly increased proliferation as compared to L- (fig. 4).

L+ Showed Greater Effects on DPC Differentiation in vitro as Compared to L- or P89

In vitro DPC differentiation mediated by L-, L+ or P89 was detected by the expressions of DSPP mRNA and type I collagen mRNA and mineralized nodule formation. The additions of L-, L+ or P89 at 150 nM significantly increased the expressions of DSPP mRNA and type I collagen mRNA ($p < 0.05$) as compared to the control groups. The L+ group showed significantly higher expressions of

DSPP and type I collagen than either the L- or P89 groups ($p < 0.05$) (fig. 5).

The enhancement of the differentiation by L-, L+ and P89 was also shown by in vitro mineralization assays. At day 21, Von Kossa staining showed more mineralized nodules formed in the proteins or peptide-treated groups as compared to controls. However, the L+ group had more calcified nodule formation, as compared to L- and P89. There were no obvious differences in mineral nodule formation when the L- and P89 groups were compared (fig. 6).

Fig. 3. Masson's trichrome staining of pulps implanted with LRAP (L-) (a) showed minimal inflammation after 8 days. b Implantation of LRAP 8, 9 (L+) for 8 days showed the presence of more small round inflammatory cells. After 15 days, inflammatory cells remained in L- treated pulps (c) as well as in L+ treated pulps (d). However, with exposure to L+ a large part of the pulp was filled with reparative dentin. The reparative dentin (green parts) locates between two calciotraumatic lines (including the residual pulp). The original dentin outside the calciotraumatic lines is grey/pink. After 30 days' exposure to L- (e) some reparative dentin was formed, while the amount of reparative dentin formed in the presence of L+ was further increased (f). D = Dentin; C = cementum; P = pulp. Scale bar: 1,000 μ m.

L- and L+ Similarly Enhanced Adhesion of DPCs in vitro

We utilized Vybrant Cell Adhesion Assay to compare the effects of L-, L+ and P89 on cell adhesion of DPCs. L-, L+ and P89 increased the adhesion of DPCs to the culture dishes in a dose-dependent pattern, though there

was no statistically significant difference for L- at the lowest concentration (30 nM) as compared to the control. At 100 nM and 300 nM, both L- and L+ showed significantly higher effects on cell adhesion than P89 alone, while no significant difference was found between the L- and L+ groups (fig. 7).

Fig. 4. DPC proliferation as measured by BrdU incorporation. LRAP (L-) and LRAP 8, 9 (L+) significantly enhanced DPC proliferation relative to controls at each concentration, while P89 enhanced the proliferation at the concentrations of 100 and 300 nM. The effect of L+ was greater than that of L- at the concentrations of 100 or 300 nM. P89 had a greater effect than L- at the concentration of 300 nM. Results represent the means $\pm$ SE. * $p < 0.05$ ($n = 4$). rlu/s = Relative light units per second.

Fig. 5. Relative expressions of DSPP mRNA (a) and type I collagen mRNA (b) by DPC. LRAP (L-), LRAP 8, 9 (L+) and P89 significantly enhanced both DSPP and type I collagen expressions. L+ had a significantly greater effect on DSPP and type I collagen expressions than either L- or P89. Results represent the means $\pm$ SE. * $p < 0.05$ ($n = 3$).

Discussion

Odontoblasts are known to express amelogenins, including full-length and alternatively spliced short isoforms, and these amelogenins have been implicated as having a functional role in odontoblast differentiation [Veis et al., 2000; Tompkins and Veis, 2002; Veis, 2003; Tompkins et al., 2005; Ye et al., 2006]. We found that similar to previous studies [Goldberg et al., 2003; Lacerda-Pinheiro et al., 2006; Jegat et al., 2007], L- (LRAP, A-4) enhanced pulp healing, resulting in a narrower pulp chamber as compared to controls. In the current study we found these same effects were further amplified by the addition of exons 8 and 9.

In vivo studies of pulp healing using agarose bead implants showed that the replacement of the exon 7 by exons 8 and 9 in LRAP resulted in increased cell proliferation (detected after 8 days) followed by an increased reparative dentin formation. It is interesting that previously conducted similar studies showed that A+4 (L- plus exon 4) had less effect on pulp cell proliferation and differentiation as compared to A-4 (L-) [Veis et al., 2000; Jegat et al., 2007]. It is interesting to note that although exon 4 adds 11 residues in the middle of L-, reducing activity, the replacement of exon 7 (only 1 amino acid) by a 24-residue peptide coded by the exons 8 and 9 sequence is at the end of LRAP, resulting in increased proliferation and differentiation. As compared to exon 4, exons 8 and 9 contain more charged and polar amino acids, which adds an extra

Fig. 6. Mineralization of postnatal human DPCs visualized by Von Kossa staining. More mineralization nodules formed in LRAP (L-), LRAP 8, 9 (L+) and P89 groups as compared to control group. L+ showed the greatest effect on DPC mineral formation. No obvious differences were observed between L- and P89 mediated mineral formation.

relatively hydrophilic terminal to amelogenin and its alternatively spliced proteins. In this 24-residue short peptide encoded by exons 8 and 9, there are 6 charged amino acids including 2 lysines, 2 aspartic acids, 1 arginine and 1 histidine. These residues change the hydrophobicity of the alternatively spliced amelogenin proteins. The different amino acid compositions and location may further alter the folding of these peptides to change its structure-related functions. In addition, the DPC adhesion assay showed that both L- and L+ enhanced cell adhesion, but no significant difference was found in these two groups. This indicates that the enhanced effects of these 2 exons on cell proliferation and differentiation may not be mediated by cell adhesions.

Cell differentiation in tissue culture may result from cell-cell interactions (cell contact effects) when the cells confluence [Ben-Ze'ev et al., 1988; Nakajima and Kaneko, 2008]. The proteins and peptide used in this study showed

Fig. 7. Effects of LRAP (L-), LRAP 8, 9 (L+) and P89 on postnatal human DPC adhesion. DPCs showed significantly greater adhesion to peptide-coated dishes as compared to uncoated dishes. Adhesion was highest in L- and L+-coated dishes with no significant difference between them. Results represent the means $\pm$ SE. * $p < 0.05$ ($n = 3$). RFU = Relative fluorescence units.

that they had different strengths in cell proliferation; therefore, the cultured cells may reach confluence at different times, and this may lead to difficulties in comparing and observing their effects on cell differentiation. To avoid this problem and determine the in vitro effects of these proteins and peptide on DPC proliferation and differentiation separately, we used different strategies for the assays of cell proliferation and differentiation. For the proliferation assay, we grew the cells to 60% confluence and assayed them before they reach complete confluence. While for differentiation experiments, we first grew these cells to complete confluence and then treat them to compare the enhancement of these proteins and peptide on cell differentiation. These studies showed that L- separately increased DPC proliferation and differentiation (indicated by increased DSPP and type I collagen expressions and nodule formation) and the effects were further enhanced by the L+, carrying exons 8 and 9 coded domains.

In previous studies, upregulation of LAMP-1 was associated with increased cell proliferation and differentiation [Cella et al., 1996; Kunzli et al., 2002; Journet and Ferro, 2004; Sarafian et al., 2006; Le et al., 2007]. The N-terminal region from Tyr¹² and Ser²⁷ in L- was considered to be involved in interactions with membrane recep-

tor LAMP-1 [Tompkins et al., 2006]. In our studies, however, we found that the P89 alone was also functional, indicating that the C terminal domain may have direct interactions with membrane receptors. In current studies, we have not tested whether the exons 8 and 9 also bind to LAMP-1. The identification of receptors and the studies on the mechanism of the cellular functions of exons 8 and 9 coded peptide region remain to be determined in our future studies.

Our in silico sequence analyses [Sire, 2010] have identified the presence of putative amelogenin exon 9 in the human X and Y chromosomes. The existence of these 2 exons in various mammals for millions of years suggests that this peptide must have its own unique functional role in tooth formation. Unfortunately, our attempts to find transcripts including exon 9 in human tooth germs were unsuccessful (unpubl. data). It is possible that such transcriptions could be stage specific as our cDNAs were prepared from human fetuses younger than 23 weeks when the tooth enamel was still in presecretory and earlier stag-

es. In these early stages, the expression of exon 9 may not be at a detectable level. Our functional studies on human DPC still indicate its possible role in human dentin formation and repair.

In conclusion, exons 8 and 9 peptide enhanced the reparative dentin formation by promoting cell proliferation and differentiation. The L+ containing exons 8 and 9 peptide may be useful in pulp repair. Future studies to delete portions of this peptide to determine the functional domains will be useful in generating unique dental materials aimed at enhancing pulp repair.

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