Effects of enamel matrix proteins on adherence, proliferation and migration of epithelial cells: A real-time in vitro study

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Abstract. Enamel matrix derivative (EMD) can mimic odontogenic effects by inducing the proliferation and differentiation of connective tissue progenitor cells, stimulating bone growth and arresting epithelial cells migration. To the best of our knowledge, there is no data indicating that any active component of EMD reduces epithelial cell viability. The present study examines the impact of commercial lyophilized EMD, porcine recombinant amelogenin (prAMEL; 21.3 kDa) and tyrosine-rich amelogenin peptide (TRAP) on the adherence, proliferation and migration of human epithelial cells in real-time. The tongue carcinoma cell line SCC-25 was stimulated with EMD, porcine recombinant AMEL and TRAP, at concentrations of 12.5, 25 and 50 µg/ml. Cell adherence, migration and proliferation were monitored in real-time using the xCELLigence system. No significant effects of EMD on the morphology, adhesion, proliferation and migration of SCC-25 cells were observed. However, porcine recombinant AMEL had a dose-dependent inhibitory effect on SCC-25 cell proliferation and migration. Predominantly, no notable differences were found between control and TRAP-treated cells in terms of cell adhesion and migration, a decrease in proliferation was observed, but this was not statistically significant. EMD and its active components do not increase the tongue cancer cell viability.

Introduction

Regeneration of periodontal tissue requires the exclusion of the epithelium and, in some cases, the gingival connective tissue from the root surface. In addition, previous studies (1-3) strongly support the hypothesis that enamel matrix proteins (EMPs), known for their impact on the structural organization of tooth enamel, may serve an important role in periodontal tissue formation (1). It is believed that enamel matrix derivative (EMD), the active component of Emdogain (Straumann), has odontogenic effects through inducing the proliferation and differentiation of connective tissue progenitor cells, stimulating bone growth and arresting gingival epithelial cell migration (2,3).

EMD is comprised primarily of amelogenins (AMELs), a family of proline-rich peptides synthesized from the AMEL gene by alternative splicing and post-translational modifications. This family includes full-length AMEL (25 kDa), which is processed into a 20 kDa protein, and then into a tyrosine-rich AMEL peptide (TRAP) and a leucine-rich AMEL peptide (LRAP) (4,5). Only a small number of studies have directly described the effect of EMD and full-length recombinant AMEL in vitro (6-8). Furthermore, alternatively spliced products and degraded forms of AMEL have biochemical properties that are distinct from full-length AMEL that are critical for function (9,10), as well as between amelogenins with different molecular mass (11). Previous studies that have analyzed the influence of EMD on gingival epithelial cells are rare and the results ambiguous. A number of studies have demonstrated that EMD inhibits epithelial cell proliferation (12-15), while another indicated no effect (16) and another observed acceleration of epithelialization following EMD stimulation (17). Moreover, it is unclear which component of EMD is a direct inhibitor of epithelial cell growth. In previous studies, full-length recombinant AMEL was indicated to be the active component (18,19).

The aim of present study was to investigate the influence of commercial lyophilized EMD, porcine recombinant prAMEL and TRAP on the adherence, proliferation and migration of human epithelial cells. Real-time cell analysis (RTCA; xCELLigence) was used to facilitate label-free and
operator-independent investigation of cell behavior, through monitoring the cells in physiologically relevant conditions.

Materials and methods

Experimental proteins. Lyophilized EMD was provided by the Straumann AG Institute (Basel, Switzerland). Porcine recombinant AMEL (49 kDa) and TRAP (5.3 kDa) were synthesized, as described below. Cells were stimulated with protein extracts of 12.5, 25 and 50 µg/ml.

Porcine recombinant amelogenin synthesis

Construction of pGex4T-1-AMEL-GST. AMEL protein was provided by BLIRT S.A. (Gdańsk, Poland). The protein sequence of *Sus scrofa* AMEL was obtained from the UniProt database (accession no. Q861X0; uniprot.org/). This sequence, with an added glutathione S-transferase (GST) tag to increase protein solubility, is the following:

VLTPLKWKYQNMIRHPTYNGYEGPMGWFLHHQIPVVS QQTTPQSALHQPNIHPMVPAPQPGPPQPMPMLPGQH SMTPTQHHQPNLPLPAQFPQPQPQVQPQHQLPQPS MHPIQPMLPQPPLPMFSMQSLLLDPPLPEAWAPAT. The amelogenin construct contains prAMEL (21.3 kDa) and GST, yielding a molecular mass of ~49 kDa.

The DNA sequence encoding the AMEL-GST protein was synthesized using the GeneArt service (Thermo Fisher Scientific, Inc.; Waltham, MA, USA). The sequence obtained was cloned into the pGex4T-1 vector (Addgene, Inc., Cambridge, MA USA) with *NdeI* and *BamHI* enzymes and ligated into *E. coli* (Agilent Technologies, Inc., Santa Clara, CA, USA) using a chemical method. Plasmid DNA was added to 100 µl competent cells on ice. The whole mixture was incubated on ice for 30 min. The bacteria were shocked at 42°C and cooled on ice. Lysogeny broth (LB) medium was added and the culture was grown at 37°C for 45 min. The transformation mix was transferred on LB agar supplemented with ampicillin (100 µg/ml). The resulting colonies were sequenced using an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) to confirm that cloning had been performed correctly. The amelogenin construct included amelogenin (21.3 kDa) and GST, yielding a final molecular mass of 43 kDa.

Overexpression of AMEL-GST in *E. coli*. ArcticExpress (DE3) *E. coli* containing the pGex4T-1-AMEL-GST construct were cultured overnight in LB media, supplemented with ampicillin (100 µg/ml) and gentamicin (40 µg/ml). The cultures were then diluted to a 1:100 ratio in the same media and cultured at 30°C until they reached an optical density reading of 0.6 at a wavelength of 600 nm. The cultures were then cooled to 10°C and protein expression induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultivation was performed for ~40 h, prior to centrifugation at 3,500 x g for 30 min at 4°C. As expected, most protein remained in the soluble fraction. Following centrifugation the protein was bound via its GST tag to a 5 ml conditioned Glutathione Sepharose 4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 2 h at 4°C, rinsed twice with buffer A 4X column volume (CV) and eluted with buffer B (50 mM Tris, 50 mM NaCl, 20 mM GSH, pH 8) 3x1.5 CV. The fractions obtained were analyzed by 4-12% SDS-PAGE to confirm the presence of AMEL and the quantity from images of the gels analyzed using TotalLab Quant 1.2 software (Cleaver Scientific Ltd., Warwickshire, United Kingdom) to determine the purity. Protein concentration was determined by the Bradford assay (Thermo Fisher Scientific, Inc.) and a NanoDrop instrument (Thermo Fisher Scientific, Inc.).

Lyophilization of the AMEL-GST fusion protein. AMEL-GST and GST were lyophilized using the freeze-dry technique. Proteins (1 mg) in buffer A were aliquoted, frozen in liquid nitrogen and lyophilized overnight.

Reconstitution of the AMEL-GST fusion protein. Lyophilized AMEL-GST and GST were reconstituted in 1 ml of buffer A to concentration of 1 mg/ml. Reconstituted samples were centrifuged at 3,500 x g for 30 min at 4°C to remove any aggregated protein and separated by 4-12% SDS-PAGE to confirm reconstitution. Samples of AMEL-GST and GST were analyzed using the Bradford assay to confirm a concentration of 1 mg/ml and estimate the quantity of protein lost during reconstitution. Protein concentration prior to lyophilization and following reconstitution was 1 mg/ml, indicating that no protein was lost.

TRAP synthesis

Construction of pET-22b-TRAP. A TRAP gene construct was obtained by polymerase chain reaction (PCR) amplification of the clone containing human AMEL cDNA using the following modified primers: Forward, 5'-TTT CAT ATG CAT CAC CAT CAC CAT CAC GAT GAC GAT GAC AAG ATG CCT CTA CCA CCT CAT CC-3' and reverse, 5'-TTT AAG CTT CAC CAT CCA CCC ATG GGT CTC TCG TAC CCA TAG GAA GTG TAC GGA TGT CTT ATC ATG TTT GC-3'. Human AMEL cDNA clone was used as a template and modified primers converted the human TRAP coding sequence to the pig coding sequence of TRAP. These were capable of converting human TRAP coding sequence to pig coding sequence of TRAP, and contained a histidine tag and enterokinase recognition site. PCR was performed in a 25-µl total reaction volume containing 5 ng plasmid DNA (human AMEL cDNA clone), 1X KAPA2G Robust HotStart DNA Polymerase (KK5702; Robust HotStart ReadyMix PCR kit; Kapa Biosystems, Inc., Wilmington, MA, USA) and 0.125 µM of each primer. PCR thermal cycling conditions were as follows: 35 cycles of 30 sec at 94°C; 30 sec at 58°C, and 45 sec at 72°C. Subsequently, the PCR product was purified using a StrataPrep PCR Purification kit (400773; Agilent Technologies, Inc.) and sequenced using an automated 3130xl Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) to confirm correct cloning. The TRAP fragment was digested with *NdeI* and *Hind*III enzymes and ligated into...
the pET-22b(+) expression vector (Novagen; Merck & Co., Inc., Whitehouse Station, NJ, USA) digested with the same endonucleases.

General procedures of handling DNA were performed according to Sambrook and Russel (20). Plasmid DNA was isolated using the StrataPrep Plasmid Miniprep kit (400761; Agilent Technologies, Inc.). PCR reagents, restriction enzymes and T4 DNA ligase were purchased from Kapa Biosystems, Inc., (Wilmington, MA, USA), Thermo Fisher Scientific, Inc., and New England Biolabs, Inc., (Ipswich, MA, USA), respectively.

**TRAP overexpression in E. coli.** This construct was transformed into Rosetta 2 (DE3) pLysS E. coli (Novagen; Merck & Co., Inc.) using a chemical method. Plasmid DNA was added to 100 µl competent cells on ice. The whole mixture was incubated on ice for 30 min. The bacteria were shocked at 42°C and cooled on ice. LB medium was added and culture was grown at 37°C for 45 min. The transformation mix was transferred onto LB agar supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). E. coli was routinely grown overnight at 37°C, with standard antibiotic plate selection performed according to the manufacturer's instructions. Transformed E. coli were grown overnight in LB media supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). Cultures were then diluted in a 1:100 ratio in the same media and cultured at 37°C until they reached an optical density reading of between 0.6 and 0.8 at a wavelength of 600 nm. Then, protein expression was induced with 1 mmol/l IPTG and cultures were grown for 16 h at 37°C.

**Immobilized metal affinity chromatography of TRAP.** Pellets were collected by centrifugation at 3,500 x g for 30 min at 4°C (Heraeus Multifuge 3 S-R; Thermo Fisher Scientific, Inc.). Pellets were suspended in 2 ml of modified phosphate buffer solution (50 mmol/l phosphate buffer disodium hydrogen phosphate and potassium dihydrogen phosphate, pH 8.0, 300 mmol/l NaCl, 10% glycerol) and disrupted using a 10 ml tissue grinder for 15 min at 4°C. The resulting lysate was centrifuged at 14,000 x g for 20 min at 4°C. The supernatant from this was bound overnight at 4°C onto 2 ml of cobalt resin (TALON Metal Affinity Resin; Clontech; Takara Biotechnology Co., Ltd., Dalian, China) equilibrated with phosphate buffer. Then, the suspension was incubated on ice. LB medium was added at a ratio of 1:10, complete growth medium was added at a ratio of 1:10, and the cell suspension was transferred to new petri dishes.

**Cell adherence and monitoring.** Cell adherence and proliferation was monitored in real-time using the xCELLigence system and E-Plate 96 insert (ACEA Biosciences, Inc., San Diego, CA, USA). Instrument measures the electrical resistance of the sensor electrodes that is proportional to the number of cells attached to the sensors, which allows real time measurements by probing cell growth at different time intervals. The electrical impedance value of each well was automatically monitored by the xCELLigence system and expressed as a cell index (CI) value. Each experiment was performed five times. The external control plate contained cells that were not exposed to the experimental proteins.

For cell adherence measurements, after reaching 90% confluency, SCC-25 cells were passaged with 0.25% trypsin solution and seeded into wells of the E-plate 96 at 10,000 cells/well. Immediately, 96 wells were stimulated with the protein extracts (EMD, AMEL and TRAP at final concentrations of 12.5, 25 and 50 µg/ml, respectively), released by the metallic alloy material, and monitored every 15 min for 14 h.

For cell proliferation measurements, after reaching confluence, SCC-25 cells were passaged with 0.25% trypsin and seeded into wells of the E-plate 96 at 10,000 cells/well. Then, cells were left to obtain cell a CI value equal to ~1. Afterwards, cells were treated with EMD, prAMEL and TRAP (12.5, 25 and 50 µg/ml, respectively), released by the metallic alloy material, and monitored every 15 min for 48 h. Evaluation was performed 12, 24, 48, 60 and 77 h after stimulation.

**Monitoring of cell migration.** The rate of cell migration was monitored in real-time with the xCELLigence system and the CIM-plate 16 insert (ACEA Biosciences, Inc.). Cells were passaged and placed in the upper chamber of CIM-plate 16 in FBS-free media. The lower chamber of the plate contained 160 µl of media with 10% of FBS as an attractant. Cell migration was measured by electrodes located between the lower and upper chambers. Immediately following seeding at 20,000 cells/well, cells were treated with EMD, prAMEL and TRAP (12.5, 25 and 50 µg/ml, respectively), and monitored every 15 min for 49 h. The control plate contained cells not exposed to the proteins.

**Statistical analysis.** Statistical analysis was performed using Statistica software (version 10; StatSoft, Inc., Tulsa, OK, USA). The Shapiro-Wilk test of normality was used on continuous variables. The results are described as the mean ± standard deviation.
deviation. One-way analysis of variance with the multiple comparisons Tukey's test was applied. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell morphology. None of the analyzed proteins affected SCC-25 cell morphology, regardless of the dose (Fig. 1). SCC-25 cells, characterized by a spindle-like shape. Only changes to a more circular shape with increasing cell density were observed. In addition, characteristic proliferation in clusters was observed (Fig. 1).

Cell adherence. The effect of experimental proteins on SCC-25 cell adhesion was monitored over 14 h in real-time. A representative graph comparing the rate of cell adherence, in terms of CI, when incubated with EMD, prAMEL or TRAP peptide is shown in Fig. 2. No significant difference in cell adherence was observed among all the groups, regardless of dose (Table I).

Cell proliferation. Cell proliferation was monitored using RTCA over a period of 77 h after EMD, prAMEL or TRAP stimulation. A representative graph comparing the rate of CI of SCC-25 cells is shown in Fig. 3. No significant difference
in the rate of proliferation was observed after 12-h incubation (Table II). RTCA analysis performed after 24-h incubation showed a significant decrease of CI in prAMEL (12.5 µg/ml) compared with cells stimulated with EMD 12.5 µg/ml (P=0.02) and 25 µg/ml-stimulated cells (P=0.02). Moreover, all doses of AMEL (12.5, 25 and 50 µg/ml) administered for 48-h caused a significant decrease in the proliferation rate in comparison with both control cells (P<0.001 for all doses) and all EMD doses (P<0.001 for all comparisons; Fig. 4) and EMD (50 µg/ml)-stimulated cells (P=0.005; Fig. 4).

Cell migration. Regardless of the type of ligand, dose and time following stimulation, no significant differences in SCC-25 cell migration were observed (Table III). A graph showing the rate of migration of SCC-25 cells when incubated with EMD, prAMEL or TRAP protein is shown in Fig. 5.

Discussion
Previous studies on the effects of EMD, conducted on a variety of research models, have been inconclusive (21,22). A number
of ambiguities have made it hard to compare the results between studies and have impeded the characterization of the functions of the different components of EMD. Firstly, previous in vitro studies have used various cell types (epithelial, tongue carcinoma, gingival fibroblast, periodontal ligament, bone marrow-mesenchymal stem cells) obtained from different species (such as, rat, pig and human) (18,19,21-24). Secondly, the studies used a number of different EMPs, such as commercially lyophilized EMD and different fractions isolated from it (<6 kDa, mainly TRAP; >6 kDa, LRAP, sheathing peptides and the full-length AMEL) (21), or numerous recombinants, such as full-length AMEL (22) and chemically synthesized 5.3 kDa TRAP (23). Furthermore, there are marked differences in the concentration of EMPs used; from between 10 ng/ml and 100 µg/ml. Finally, different techniques were applied in order to measure the biological effects of the EMPs. Conventional cell-based assays may be more prone to artifacts, due to considerable manipulation of the cell by labeling or over-expression of target or reporter proteins (25).

Numerous studies concerning the effects of EMD focus on periodontal tissue, including its stimulation (14,15,24). These have shown that the effects of EMD are different in mesenchymal and epithelial cells (14,18,19,24). Results concerning the influence of EMD on oral epithelial cells are particularly ambiguous; EMD was determined to have an anti-proliferative effect on epithelial cells (12,13), but numerous clinicians have observed accelerated epithelial soft-tissue healing upon intrasurgical application of EMD (26-29). It has been suggested that EMD may induce alterations in malignant mucosal tissue, which implies that patients with pre-malignant or malignant mucosal lesions should not be treated with EMD (27).

| EMP added, µg/ml | 12-h incubation | 24-h incubation | 28-h incubation |
|------------------|----------------|----------------|----------------|
| Control          | 1.4±2.1        | 1.8±2.0        | 2.6±1.8        |
| EMD, 12.5        | 2.0±1.6        | 2.2±1.8        | 3.2±2.6        |
| EMD, 25          | 3.4±2.6        | 3.7±2.6        | 3.8±1.7        |
| EMD, 50          | 3.7±3.0        | 3.9±3.1        | 3.7±2.7        |
| prAMEL, 12.5     | 1.1±1.4        | 2.0±1.8        | 3.9±2.4        |
| prAMEL, 25       | 1.3±1.8        | 2.1±1.4        | 3.9±2.6        |
| prAMEL, 50       | 0.9±0.9        | 2.0±1.1        | 4.0±2.0        |
| TRAP, 12.5       | 1.7±2.1        | 2.5±2.3        | 3.7±2.0        |
| TRAP, 25         | 2.1±2.0        | 3.3±2.0        | 4.9±0.4        |
| TRAP, 50         | 2.1±2.5        | 2.9±2.1        | 4.0±2.2        |
| P-value >0.05    | >0.05          | >0.05          | >0.05          |

Cell index values were monitored using the xCELLigance system. Results are from three repeats. No significant differences were detected between the groups. EMP, enamel matrix protein; EMD, enamel matrix derivative; prAMEL, porcine recombinant amelogenin; TRAP, tyrosine-rich amelogenin peptide.
The aim of the present study was to determine the influence of EMD, AMEL and TRAP on human tongue carcinoma cells using a cell-based, label-free and real-time platform technology (xCELLigence). Label-free technologies have the advantage of being non-invasive. The real-time monitoring of cells provides important information regarding their biological status, such as cell growth, arrest and morphological changes. The qualities of this system made it possible to obtain physiologically relevant results.

The results of the present study indicate that EMD does not influence the morphology of SCC-25 significantly. Kawase et al (13) observed that SCC-25 cell cultures treated with 100 µg/ml EMD for 3 days became more flattened and had a slightly lower cell density. In the present study, cells were stimulated with EMD at concentrations of 12.5, 25 and 50 µg/l, which may explain the differences in the results obtained.

Real-time tests performed using the xCELLigence system indicate no significant effects of EMD on adhesion, proliferation and migration of SCC-25 cells. These results contradict the observations of Kawase et al (13), that EMD (in a dose-dependent manner) inhibited oral epithelial cell division and concomitantly arrested cell cycling at the G1 phase, although no apoptosis was observed. Kawase et al (13) concluded that EMD acts as a cytostatic, rather than cytotoxic, agent on
epithelial cells. In other studies the same group of researchers showed that EMD reduced DNA synthesis in a dose-dependent manner (12,14). Evidence from the literature suggests that the suppression of epithelial cell growth observed may be mediated by transforming growth factor β1 (TGF-β1) (14). Porcine TGF-β1 up-regulates p21 (WAF/CIP1) expression and inhibits epithelial proliferation (14). In addition, TGF-β1 phosphorylates the mitogen-activated protein kinase (MAPK) family, similar to EMD. Anti-TGF-β antibody completely blocks the up-regulation of p21 protein and anti-proliferative action by EMD or TGF-β1 in epithelial cells (14). In addition, anti-TGF-β antibody blocks other actions of EMD in epithelial cells, p38-MAPK and inhibition of DNA synthesis (14).

Anti-TGF-β antibody blocks TGF-β1- and EMD-induced SMAD family member 2 (SMAD2) translocation (14). Kawase et al (14) concluded that TGF-β1, as a principal bioactive factor in EMD, likely inhibits epithelial cell proliferation by a SMAD2-mediated, p21-dependent mechanism. Moreover, Kawase et al (12) showed that 50 µg/ml EMD promoted SCC-25 cell adherence and stimulated cytoketal actin polymerization. However, Laaksonen et al (30) did not confirm the inhibitory effects of EMD on tongue squamous cell carcinoma proliferation, no differences were found between the control and the EMD-treated (100 and 200 µg/ml) cells after 12, 24, 48, 72 and 96 h of incubation. Furthermore, Gestrelius et al (16) did not observe any statistically significant changes in rat tongue epithelial cell proliferation after exposure to 100 µg/ml EMD. In addition, Mirastschijski et al (17) revealed significant epithelialization after EMD treatment in vivo in rabbits. Moreover, a previous study indicated that EMD promotes re-epithelialization and neo-vascularization in full-thickness surgical wounds in rat oral mucosa (28). Maymon-Gil et al (29) observed that EMD had no effect on epithelial gap closure of an oral mucosa surgical wound in vivo in rats. The differences in the results of these studies may be associated with the method of EMD application; directly on the wound, or underneath the soft tissues.

Previous studies conducted on cervical cancer cells indicated that EMD has an inhibitory effect on epithelial cells (13,27). Lyngstadaas et al (15) showed that HeLa cells growing in the presence of EMD exhibited a highly increased intracellular level of cyclic adenosine monophosphate compared with controls. EMD primarily contains glycoproteins, and AMEL to non-AMEL proteins (such as, ameloblastin and enamelin) at a ratio of ~9:1 (AMEL:rest of proteins) (31,32). Full-length AMEL induces proliferation in periodontal cells, such as mesenchymal stem cells (33), cementoblasts (34), periodontal fibroblasts (18,34) and gingival fibroblasts (18).

The present study observed a dose-dependent inhibitory effect of porcine recombinant AMEL (21.3 kDa) on SCC-25 cell proliferation. These results are consistent with observations made by a previous study that indicated that recombinant AMEL inhibits the growth rate, adhesion and migration of gingival epithelial cells (18). Li et al (19) identified that recombinant 25 kDa porcine AMEL (5, 10 and 20 µg/ml) inhibited human gingival epithelial cell attachment, migration and growth rate in a time- and dose-dependent manner. In addition, a previous study demonstrated that recombinant AMEL inhibits epithelial cell proliferation in vitro. The results of Kuramitsu-Fujimoto et al (35) suggest that ameloblastin is the primary bioactive factor of EMD in regards to inhibition of epithelial cell proliferation. It has been suggested that EMPs, such as AMELs and ameloblastin, are required for enamel biomineralization and have synergistic cellular functions (36).

The present study examined the effects of recombinant 5.3 kDa TRAP on SCC-25 cells. No significant differences were found between the control and TRAP-treated (12.5, 25 and 50 µg/ml) cells in terms of adhesion, migration and proliferation. Villa et al (28) observed increased migration of epithelial cells following EMD treatment compared with recombinant TRAP stimulation in palatal wounds in rats. Numerous previous studies have analyzed the effect of the EMD protein fraction with a molecular weight of ~5 kDa, which is presumably composed by TRAP (4,5,37,38). These studies performed the following TRAP preparation methods: TRAP isolated from EMD; recombinant peptide TRAP; and synthetic TRAP, which resulted in different observations concerning their biological effects (4,5,37,38). This suggests that the method of TRAP preparation may be an important factor in influencing its biological activity. Jonke et al (37) demonstrated that TRAP isolated from EMD and synthetic TRAP (100 µg/ml) significantly decreased human umbilical vein endothelial cell proliferation and viability. No statistically significant decrease in proliferation of TRAP-treated cells was observed in the present study, although this was recombinant TRAP, 50 µg/ml was the highest concentration used and was on different cell line. EMPs are conserved as well as the TRAP cleavage site in humans and other mammals (1), however, because EMD contains porcine AMELs, porcine AMEL and porcine TRAP were used in the present study to minimize any differences. To the best of our knowledge, no previous studies used a similar research model of TRAP synthesis, which impeded the verification of results obtained.

In conclusion, the aim of the present study was to investigate effects of EMD, porcine recombinant AMEL and TRAP on SCC-25 cells using a real-time cell analysis platform (xCELLigence). The results demonstrated that EMD and its active components did not increase the tongue cancer cell viability, and that porcine recombinant AMEL inhibited epithelial cell proliferation and migration. To the best of our knowledge, no previous EMD studies concerning SCC-25 cells were conducted with the use of real-time monitoring. Thus, differences between the results of the present study and those obtained by previous studies are likely due to differences in the measurement technique used and the structure of applied ligands (AMEL and/or EMD). The amelogenin construct was coding a protein with a molecular mass of 49 kDa. The xCELLigence system enables real-time analysis of SCC-25 cells using a real-time cell analysis platform (xCELLigence). The results demonstrated that EMD and its active components did not increase the tongue cancer cell viability, and that porcine recombinant AMEL inhibited epithelial cell proliferation and migration. To the best of our knowledge, no previous EMD studies concerning SCC-25 cells were conducted with the use of real-time monitoring. Thus, differences between the results of the present study and those obtained by previous studies are likely due to differences in the measurement technique used and the structure of applied ligands (AMEL and/or EMD). The amelogenin construct was coding a protein with a mass of 21.3 kDa; however, a GST tag was added in order to increase the protein solubility. The final product used in the present research, comprising of amelogenin and GST, had a molecular mass of 49 kDa. The xCELLigence system enables better reproducibility than other instruments, which is an argument in favor of its use in real-time analysis of SCC-25 cells.

References

1. Fincham AG, Belcourt AB, Termine JD, Butler WT and Cothran WC: Amelogenins. Sequence homologies in enamel-matrix proteins from three mammalian species. Biochem J 211: 149-154, 1983.
2. Miron RJ, Caluseru OM, Guillemette V, Zhang Y, Gemperli AC, Chandad F and Sculean A: Influence of enamel matrix derivative on cells at different maturation stages of differentiation. PLoS One 8: e71008, 2013.
3. Miron RJ, Wei L, Yang S, Caluseriu OM, Sculean A and Zhang Y: Effect of enamel matrix derivative on periodontal wound healing and regeneration in an osteoporotic model. J Periodontol 85: 1603-1611, 2014.

4. Fincham AG, Belcourt AB, Termine JD, Butler WT and Cothran WC: Dental enamel matrix: Sequences of two amelogenin polypeptides. Biosci Rep 1: 771-778, 1981.

5. Fincham AG, Hu YY, Pavlova Z, Slavkin HC and Snejad ML: Human amelogenins: Sequences of TRAP molecules. Calcif Tissue Int 45: 243-250, 1989.

6. Grayson RE, Yamakoshi Y, Wood EJ and Ageren MS: The effect of the amelogenin fraction of enamel matrix proteins on fibroblast-mediated collagen matrix reorganization. Biomaterials 27: 2926-2933, 2006.

7. Hoang AM, Klebe RJ, Steffensen B, Ryu OH, Simmer JP and Cochran DL: Amelogenin is a cell adhesion protein. J Dent Res 81: 497-500, 2002.

8. Matsuzawa M, Sheu TJ, Lee YJ, Chen M, Li TF, Huang CT, Holz JD and Puzas JE: Putative signaling action of amelogenin utilizes the Wnt/beta-catenin pathway. J Periodontal Res 44: 289-296, 2009.

9. Sun Z, Fan D, Fan Y, Du C and Moradian-Oldak J: Enamel proteases reduce amelogenin-apatite binding. J Dent Res 87: 1133-1137, 2008.

10. Tan J, Lough W, Moradian-Oldak J, Zeichner-David M and Fincham AG: Quantitative analysis of amelogenin solubility. J Dent Res 77: 1388-1396, 1998.

11. Yamakoshi Y: Porcine Amelogenin: Alternative splicing, proteolytic processing, protein-protein interactions, and possible functions. J Oral Biosci 53: 275-283, 2011.

12. Kawase T, Okuda K, Momose M, Kato Y, Yoshih H and 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 Periodontal Res 36: 367-376, 2001.

13. Kawase T, Okuda K, Yoshih H and Burns DM: Cytostatic action of enamel matrix derivative (EMDOGAIN) on human oral squamous cell carcinoma-derived SCC25 epithelial cells. J Periodontal Res 35: 291-300, 2000.

14. Kawase T, Okuda K, Yoshih H and Burns DM: Anti-TGF-beta antibody blocks enamel matrix derivative-induced upregulation of p21WA F1/cip1 and prevents its inhibition of human oral epithelial cell proliferation. J Periodontal Res 37: 255-262, 2002.

15. Lyngstadaa SP, Lundberg E, Ekdahl H, Andersson C and Gestrelius S: Autocrine growth factors in human periodontal ligament cells cultured on enamel matrix derivative. J Clin Periodontol 28: 181-188, 2001.

16. Gestrelius S, Andersson C, Lidström D, Hammarström L and Somerman M: In vitro studies on periodontal ligament cells and enamel matrix derivative. J Clin Periodontol 24: 685-692, 1997.

17. Mirastichyjii U, Konrad D, Lundberg E, Lyngstadaa SP, Jorgensen LN and Agren MS: Effects of a topical enamel matrix derivative on skin wound healing. Wound Repair Regen 12: 100-108, 2004.

18. Li X, Shu R, Liu D and Jiang S: Different effects of 25-kDa amelogenin on the proliferation, attachment and migration of various periodontal cells. Biochem Biophys Res Commun 394: 581-586, 2010.

19. Li XT, Shu R, Song ZC and Zhou YB: The effects of recombinant porcine amelogenin on human gingival epithelial cells. Shanghai Kou Qiang Yi Xue 21: 257-261, 2012 (In Chinese).

20. Sambrook J and Russel DW: Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory Press, New Youk, Cold Spring Harbor Laboratory Press, 2001.

21. Amin HD, Olsen I, Knowles JC and Donos N: Differential effect of amelogenin peptides on osteogenic differentiation in vitro: Identification of possible new drugs for bone repair and regeneration. Tissue Eng Part A 18: 1193-1202, 2012.

22. Frasher I, Ern C, Diegritz C, Hickel R, Hristov M and Folwaczny M: Full-length amelogenin influences the differentiation of human dental pulp stem cells. Stem Cell Res Ther 7: 10, 2016.

23. Amel HD, Olsen I, Knowles JC, Dard M and Donos N: A tyrosine-rich amelogenin peptide promotes neovascularization in vitro and ex vivo. Acta Biomater 10: 1930-1939, 2014.

24. Grandin HM, Gumperli AC and Dard M: Enamel matrix derivative: A review of cellular effects in vitro and a model of molecular arrangement and functioning. Tissue Eng Part B Rev 18: 181-202, 2012.

25. Xi B, Yu N, Wang X, Xu X and Abassi YA: The application of cell-based label-free technology in drug discovery. Biotechnol J 3: 484-495, 2008.

26. Sazn M, Tonetti MS, Zahabugi I, Sicilia A, Blanco J, Rebelo H, Rasperini G, Merli M, Cortellini P and Suvan JE: Treatment of intrabony defects with enamel matrix proteins or barrier membranes; Results from a multicenter practice-based clinical trial. J Periodontol 75: 726-733, 2004.

27. Laaksonen M, Sorsa T and Salo T: Emdogain in carcinogenesis: A systematic review of in vitro studies. J Oral Sci 52: 1-11, 2010.

28. Villa O, Wohlfarth JC, Mda I, Peltzold C, Reseland JE, Snead ML and Lyngstadaa SP: Proline-rich peptide mimics effects of enamel matrix derivative on rat oral mucosa incisional wound healing. J Periodontol 86: 1386-1395, 2015.

29. Maymnon-Gil T, Weinberg E, Nemcovsky C and Weinreb M: Enamel matrix derivative promotes healing of a surgical wound in the rat oral mucosa. J Periodontol 87: 601-609, 2016.

30. Laaksonen M, Suojanen J, Nurmenniemi S, Lääri E, Sorsa T and Salo T: The enamel matrix derivative (Emdogain) enhances human tongue carcinoma cells gelatinase production, migration and metastasis formation. Oral Oncol 44: 733-742, 2008.

31. Schwartz Z, Carnes DL Jr, Pulliam R, Lohmann CH, Sylvia VL, Liu Y, Dean DD, Cochran DL and Boyan BD: Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2T9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOST cells. J Periodontol 71: 1287-1296, 2000.

32. Shimizu-Ishura M, Tanaka S, Lee WS, Debari K and Sasaki T: Effects of enamel matrix derivative to titanium implantation in rat femurs. J Biomed Mater Res 60: 269-276, 2002.

33. Huang YC, Tanimoto K, Tanne Y, Kaimiya T, Kunimatsu R, Michida M, Yoshioke M, Yoshini Y, Kato Y and Tanne K: Effects of human full-length amelogenin on the proliferation of human mesenchymal stem cells derived from bone marrow. Cell Tissue Res 342: 205-212, 2010.

34. Hatakeyama J, Philip D, Hatakeyama Y, Haruyama N, Shum L, Aragon M, Yuan Z, Gibson CW, Sreenath T, Kleinman HK and Kulkarni AB: Amelogenin-mediated regulation of osteoclastogenesis, and periodontal cell proliferation and migration. J Dent Res 85: 144-149, 2006.

35. Kuramitsu-Fujimoto S, Ariojishi W, Saito N, Okinaga T, Kamo M, Ishisaki A, Takata T, Yamaguchi K and Nishihara T: Novel biological activity of ameloblastin in enamel matrix derivative. J Appl Oral Sci 23: 49-55, 2015.

36. Hatakeyama J, Fukumoto S, Nakamura T, Haruyama N, Suzuki S, Hatakeyama Y, Shum L, Gibson CW, Yamada Y and Kulkarni AB: Synergistic roles of amelogenin and ameloblastin. J Dent Res 88: 318-322, 2009.

37. Jonke E, Gumperli AC, Zhang T, Ozmemin B, Dard M, Rausch-Fan X and Andrukhov O: Effect of tyrosine-rich amelogenin peptide on behavior and differentiation of endothelial cells. Clin Oral Investig Feb 12, 2016 (Epub ahead of print).

38. Ravindranath RM, Tam WY, Nguyen P and Fincham AG: The enamel protein amelogenin binds to the N-acetyl-D-glucosami ne-mimicking peptide motif of cytokeratins. J Biol Chem 275: 39654-39661, 2000.