

## **Effects of human full-length amelogenin on the proliferation of human osteoblasts.**

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### **Abstract**

**Objective:** Amelogenins are known as a major constituent of the enamel matrix secreted by ameloblasts and play an important role in enamel formation. Amelogenin knockout mice exhibit enhanced osteoblast formation and resorption of tooth cementum. Recent studies have revealed that amelogenins also have cell signaling properties. However, the biological functions of amelogenin in osteoblasts remain unclear. In this study, we examined the effects of recombinant human full-length amelogenin (rh174) on the proliferation of cultured normal human osteoblasts (NHOst).

**Methods:** NHOst cells were cultured and treated with 100 ng/ml rh174. Cell proliferation was evaluated using MTS assay in a time-dependent manner. Expression of Lysosomal-associated membrane protein 1 (LAMP 1), a possible amelogenin receptor, in NHOst cells was analyzed. NHOst cells were cultured and treated with 100 ng/ml rh174 in the presence or absence of LAMP 1-blocking antibody. Cell proliferative activity was analyzed by BrdU assay. Phosphorylation of extracellular signal regulated kinases (ERK) 1/2 was measured by ELISA and western blotting analysis.

**Results:** Proliferation of NHOst cells was enhanced significantly ( $p < 0.01$ ) by treatment with rh174, and was inhibited significantly ( $p < 0.01$ ) by addition of anti-LAMP 1-blocking antibody. In addition, the ratio of phosphorylated ERK1/2 to total ERK1/2 was significantly larger ( $p < 0.01$ ) with rh174 treatment, and was reduced significantly by the addition of anti-LAMP 1-blocking antibody in NHOst cells.

**Conclusion:** These results confirmed that rh174 interacts with LAMP 1, and rh174/LAMP 1 interaction activates the ERK1/2 signaling pathway, enhancing the proliferation activity of NHOst cells.

**Keywords:** Osteoblasts, LAMP1, Osteoblast, Amelogenin.

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### **Introduction**

Several attempts have been made to restore periodontal tissue loss induced by periodontitis. Enamel matrix derivative (EMDOGAIN) (Straumann Institute, Basel, Switzerland), which is extracted from developing porcine tooth germs, has been widely used to regenerate periodontal tissue [1]. The clinical use of EMDOGAIN is based on the observation that Hertwig's epithelial root sheath deposits an enamel-like matrix on the dentin surface of the developing root [2]. Clinical studies have shown that EMDOGAIN successfully promotes cementum and alveolar bone formation in periodontal tissue [2,3]. In addition, other clinical studies of EMDOGAIN treatment for intrabony periodontal defects have found that it leads to regeneration of the periodontal ligament, and markedly enhances clinical attachment and alveolar bone growth [4-6]. However, the mechanism of its effects has not been fully clarified, possibly because the exact nature of the inducer among the numerous proteins included in EMDOGAIN remains unclear.

Among the protein components of the enamel extracellular matrix, amelogenins account for approximately 90% [7,8]. Amelogenins play a crucial role in the mineralization and structural organization of developing enamel [9]. In addition, recent studies have revealed tooth root resorption, as well as amelogenesis imperfecta, in amelogenin-deficient mice [10,11].

Lysosome-associated membrane protein 1 (LAMP 1) is a transmembrane protein that is expressed in late endosomes and lysosomes, and is often used as a marker for these two organelles [12]. LAMP 1 immunoreactivity is also observed at the plasma membrane and in early endocytic compartments [13]. The presence of LAMP 1 on the plasma membrane suggests that LAMP 1 acts as a cell surface receptor that can be shuttled to the lysosome through endocytosis. Thus, LAMP 1 may be involved in endocytosis, pinocytosis or phagocytosis [12]. A previous study demonstrated that amelogenins interact with LAMP 1 [14]. It is thus assumed that amelogenins affect the metabolism of osteoblasts through a LAMP 1-mediated mechanism.

The purpose of this study was to examine the effects of recombinant human full-length amelogenin (rh174) on proliferation and the contribution of amelogenin/LAMP 1 interaction in human osteoblasts.

## Materials and Methods

### *Synthesis of rh174*

A plasmid containing rh174 cDNA (GI: 6715562 in Genbank), including exons 2, 3, 5, 6 and 7 without the signal peptide and anti-human amelogenin antibody was provided by Drs. PK. DenBesten and W. Li of the Department of Orofacial Sciences, University of California at San Francisco. rh174 was synthesized and purified as described elsewhere[15,16].

### *Cell cultures*

Normal human osteoblasts (NH0st) (CC-2538; Cambrex Bio Science Walkersville Inc., Walkersville, MD) were cultured in 100-mm dishes (Corning, New York, NY) with Clonetics Osteoblast basal medium (OBM) (CC-3208; Science Walkersville Inc) and osteoblast growth medium (OGM) (CC-4193; Science Walkersville Inc) containing ascorbic acid, fetal bovine serum (FBS), and gentamicin/amphotericin-B (CC-3207; Science Walkersville Inc) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. The culture medium was changed every other day until 80% confluence. Cultures were then detached with 0.025% Trypsin/0.01% EDTA Solution (CC-5034; Science Walkersville Inc), and were transferred to other dishes. NH0st in the 2<sup>nd</sup> to 4<sup>th</sup> passages were used for subsequent experiments.

### *Quantification of cell number by 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay*

NH0st were seeded at a density of  $4 \times 10^4$  cells/well in 6-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ), and cultured with OGM (Science Walkersville Inc) containing 10% FBS (Science Walkersville Inc) in the presence or absence of 100 ng/ml rh174 for 6 days. Medium was changed every other day. The degree of cell proliferation was examined by MTS assay using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, MI) according to the manufacturer's instructions. Culture medium was removed, and cells were incubated with 200  $\mu$ l MTS reagent in 1000  $\mu$ l PBS for 2 h at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Absorbance at 490 nm was recorded using a microplate reader (Model 550; Bio-Rad, Hercules, CA). Cell number was quantified based on a calibration curve.

### *Analysis of cell proliferation activity by BrdU immunoassay*

NH0st were seeded at a density of  $1 \times 10^3$  cells/well in a 96-well plate (FALCON, Franklin Lakes, NJ) and were cultured in OGM (Science Walkersville Inc) with 10% FBS until 60% confluence. These cells were treated with 100 ng/ml rh174 in

the presence or absence of 20 mg/ml rabbit anti-LAMP-1 antibody (ab24170; Abcam, Cambridge, MA) in serum-free OGM (Science Walkersville Inc). Cell proliferation activity was analyzed using a Cell Proliferation ELISA BrdU kit (Roche Diagnostics, Mannheim, Germany), in accordance with the manufacturer's instructions. Absorbance of samples was measured using a microplate reader (Model 550; Bio-Rad) at 450 nm.

### *Mitogen-activated protein kinases-extracellular signal regulated kinases (MAPK-ERK) activity*

NH0st were seeded at a density of  $2 \times 10^4$  cells/well in a 96-well plate (FALCON), and were cultured overnight in OGM (Science Walkersville Inc) containing 10% FBS. Cells were then treated with 100 ng/ml rh174 in the presence or absence of 20  $\mu$ g/ml rabbit anti-LAMP 1 polyclonal antibody (ab24170; Abcam) in serum-free OGM (Science Walkersville Inc). Rabbit polyclonal IgG antibody was used as the control. MAPK-ERK activity was measured at 30 min after treatment with rh174 using a Cellular Activation of Signaling ELISA (CASE) kit (SuperArray Bioscience, Frederick, MD). Expression of total ERK1/2 (tERK) and phosphorylated ERK1/2 (pERK) was examined in accordance with the manufacturer's protocol.

### *Western blot analysis*

NH0st were lysed in 5X sodium dodecyl sulfate (SDS) sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol]. Protein concentration was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL). After addition of 10% 2-mercaptoethanol and 0.01% bromophenol blue, samples were boiled at 100°C for 3 min. Ten micrograms of protein was resolved on 10% SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes using an iBlot gel transfer system (Invitrogen, Carlsbad, CA). Membranes were blocked with LI-COR blocking buffer (LI-COR, Cambridge, UK) at room temperature on a shaker for 1 h. Primary antibodies were incubated with the membranes at room temperature for 2 h. Anti-phospho ERK1/2 antibody (sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA), anti-total ERK1/2 antibody (p44/42 MAPK (Erk1/2), #4695; Cell Signaling Technology, Boston, MA) were used at 1:1000 dilution and anti- $\beta$ -actin antibody (AC-15; Sigma-Aldrich, St. Louis, MO) was used at 1:5000 dilution. After primary antibody incubation, membranes were washed three times for 5 min each with 15 ml of PBS and 0.1% Tween-20 before addition of secondary antibody conjugated to a fluorescent entity: IRDye 800CW-conjugated goat anti-mouse IgG in LI-COR blocking buffer (LI-COR). At the end of the incubation period, membranes were washed twice with 15 ml of PBS and 0.1% Tween-20 and once with 15 ml of PBS. Membranes were dried, visualized, and analyzed on the Odyssey® IR imaging system (LI-COR).

### *Statistical analysis*

Experiments were repeated three times. Means and standard deviations were calculated from the data obtained, and

differences were examined using Student's t-test or one-way analysis of variance (ANOVA), followed by Scheffe's multiple comparison test at significance levels of  $p < 0.05$  and  $p < 0.01$ .

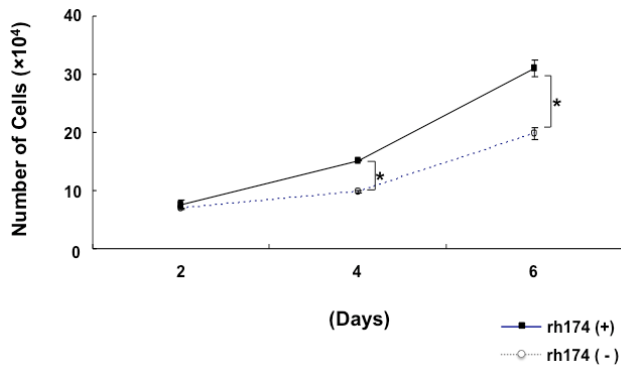
**Results**

**Effects of rh174 on proliferation of NHOst cells**

Proliferation of NHOst was enhanced significantly ( $p < 0.01$ ) by treatment with 100 ng/ml rh174, as compared to untreated controls, at 4 and 6 days after seeding (Figure 1).

**Effects of LAMP 1 blocking on enhancement of cell proliferation activity by rh174 in NHOst cells**

Proliferation activity of NHOst was enhanced significantly ( $p < 0.01$ ) by treatment with 100 ng/ml rh174, as compared to untreated controls (Figure 2). Meanwhile, anti-LAMP 1 antibody significantly ( $p < 0.01$ ) inhibited the effects of rh174 on the proliferation NHOst cells, with proliferation activity reduced to a level similar to that in controls.



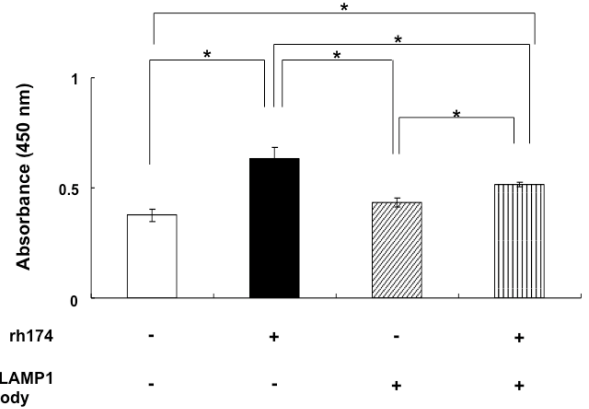
**Figure 1.** Effects of rh174 on proliferation of NHOst; NHOst were seeded at a density of  $4 \times 10^4$  cells/well in 6-well plates, and were cultured with OGM containing 10% FBS in the presence or absence of 100 ng/ml rh174. Cell proliferation was examined by MTS assay at 2, 4, and 6 days. \* $P < 0.01$ , vs. control,  $n = 3$ .

**Activation of ERK signaling pathway by interaction between rh174 and LAMP 1**

The ratio of pERK to tERK in NHOst became significantly larger ( $p < 0.01$ ) after treatment with 100 ng/ml rh174, as compared to untreated controls. In addition, the increase in pERK/tERK ratio by treatment with rh174 was reduced significantly ( $p < 0.01$ ) by addition of anti-LAMP 1 antibody (Figure 3).

**Activation of ERK signaling pathway by interaction between rh174 and LAMP 1 (western blotting)**

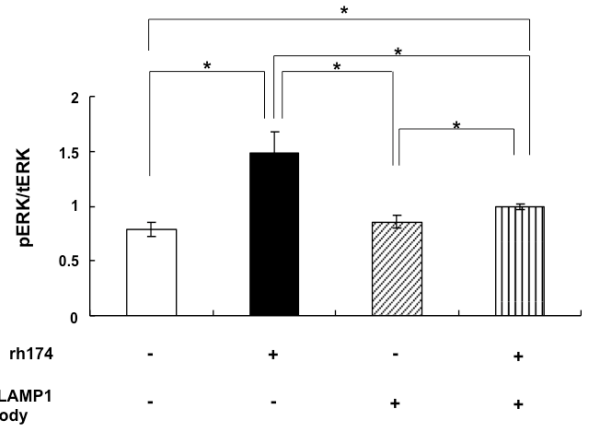
Western blot analysis revealed similar results of enhanced phosphorylated ERK1/2 signaling after addition of rh174. In addition, western blot analysis also revealed reduced phosphorylated ERK1/2 signaling after the blocking of LAMP 1 antibody. Lower bands show the abundance of total ERK and  $\beta$ -actin as a loading control (Figure 4).



**Figure 2.** Effects of LAMP 1 blocking on enhancement of cell proliferation activity by rh174 in NHOst. NHOst were seeded at a density of  $1 \times 10^3$  cells/well in a 96-well plate, and were cultured in OGM with 10% FBS until 60% confluence. Cells were treated with 100 ng/ml rh174 in the presence or absence of 20  $\mu$ g/ml rabbit anti-LAMP 1 antibody in serum-free OGM. Cell proliferation activity was analyzed using a Cell Proliferation ELISA BrdU kit. \* $P < 0.01$ ,  $n = 3$ .

**Discussion**

The phenotype of NHOst was characterized based on the expression levels of alkaline phosphatase (ALP), collagen type 1 collagen, osteocalcin (OC) and CD44, and the formation of mineralization nodules [17]. In the present study, the proliferation of NHOst cells was enhanced significantly by treatment with rh174, as compared to untreated controls.

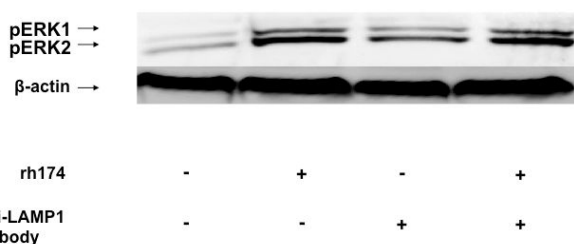


**Figure 3.** Activation of ERK signaling pathway by rh174/LAMP 1 interaction in NHOst (ELISA). NHOst were seeded at a density of  $2 \times 10^4$  cells/well in a 96-well plate, and were cultured in  $\alpha$ -MEM with 10% FBS until 60% confluence. Cells were treated with 100 ng/ml rh174 in the presence or absence of 20  $\mu$ g/ml rabbit anti-LAMP 1 antibody in serum-free OGM. ERK activity was measured at 30 min after treatment with rh174 using a CASE kit. The ratio of pERK to tERK was determined. \* $P < 0.01$ ,  $n = 3$ .

In both *in vitro/vivo* studies, the effectiveness of EMDOGAIN for periodontal regeneration, including the formation of new cementum, bone, and dentin has been reported [17-21]. It has also been demonstrated that EMDOGAIN enhances the

proliferation of various types of cells, such as immortalized murine cementoblasts (OCCM-30), pre-osteoblasts from mice OC-Tag (OCT-1) cells, and pre-osteoblasts from mouse calvaria cells (MC3T3-E1) [22]. In addition, it has been reported that EMDOGAIN stimulates proliferation of human gingival fibroblasts *via* the ERK signaling pathway [23]. However, it remains unclear whether the effects of EMDOGAIN are due to amelogenins, although approximately 90% of enamel matrix proteins are amelogenins, and the remaining 10% comprise non-amelogenin enamel matrix proteins and growth factors [24-26].

In addition, various isoforms and fragments of amelogenin in the enamel matrix may exert different functions; therefore, recombinant proteins have been used to examine the direct effects of amelogenins. For example, recent studies demonstrated that amelogenins enhance the proliferation of several cell types such as human periodontal ligament fibroblasts (PDLF) and gingival fibroblasts (GF). The proliferation activity of PDLF and GF was promoted by treatment with full-length porcine amelogenin. [27] Similarly, it has been reported that recombinant full-length porcine amelogenin (P172) induced a significant and dose-dependent increase in murine PDLF proliferation [28]. In addition, the proliferation of mouse PDL cells was enhanced by recombinant full-length murine amelogenin (rp(H)M180), [26] which is similar to the finding in this study that rh174 enhances the proliferation of NHOst cells.



**Figure 4.** Activation of ERK signaling pathway by rh174/LAMP 1 interaction in NHOst (Western blot analysis). Western blot analysis revealed similar results for enhanced phosphorylated ERK1/2 signaling after addition of rh174. In addition, western blot analysis showed reduced phosphorylated ERK1/2 signaling after blocking of LAMP 1 antibody. Lower bands show the abundance of total ERK and  $\beta$ -actin as a loading control ( $n=3$ ).

LAMP 1 was originally identified as a major protein component of the lysosomal membrane [12]. It was identified as a cell surface receptor for a small isoform of amelogenin, [A-4]/M59 (leucine-rich amelogenin peptide; LRAP) in mesenchymal-derived mouse fetal myoblasts [14]. In addition, it was reported that several fragments derived from rp(H)M180 bind to LAMP 1 [29]. A recent study revealed that LAMP 1 serves as a cell surface binding site for amelogenins on mouse dental follicle cells and mouse cementoblasts [30].

It was recently reported that rh174 increased the proliferation of human mesenchymal stem cells (MSCs) by interaction with

LAMP 1 through the MAPK-ERK signaling pathway, indicating the possibility of MSC application to tissue regeneration in the orofacial region [31]. Similarly, in the present study, proliferation of NHOst was enhanced significantly by treatment with rh174, as compared to untreated controls, but was inhibited significantly by the addition of anti-LAMP 1 blocking antibody. Furthermore, pERK1/2 expression in NHOst was significantly up-regulated by treatment with rh174, and was inhibited in the presence of anti-LAMP 1 blocking antibody. These results suggest that rh174 interacts with LAMP 1, and that rh174/LAMP 1 interaction activates the ERK1/2 signaling pathway, leading to the enhancement of cell proliferation activity in NHOst cells.

In a previous study, it has been reported that rh174 significantly enhances the proliferation of cultured human cementoblast lineage cells through cluster of differentiation (CD) 63 receptor-mediated activation of the ERK1/2 signaling pathway [32-36]. In this study, the proliferation activity of NHOst was enhanced significantly by treatment with rh174 and anti-LAMP1 antibody as compared to the non-treated control group. Alternatively, the ratio of pERK to tERK in NHOst became significantly larger by treatment with rh174 and anti-LAMP1 antibody, as compared to untreated controls. In addition, western blot analysis revealed similar results for enhanced phosphorylated ERK1/2 signaling after addition of rh174 and anti-LAMP1 antibody. It is assumed that amelogenins affect the metabolism of osteoblasts through both CD63 and LAMP1-mediated mechanisms. In addition, recent study has been demonstrated that amelogenin promotes the differentiation of odontoblast-like cells *via* the ERK1/2 and p 38 MARK pathways [37]. Our previous study showed that rh174 and C-terminal amelogenin increased proliferation of human cementoblasts through MAPK-ERK signaling pathway [38]. A recent *in vitro* study suggested that bone marrow MSC proliferation was increased in the presence of C-terminal of amelogenin and inhibited by anti-LAMP1 antibody or U0126. Increased phosphorylated ERK1/2 was observed in the presence of C-terminal of amelogenin, and decreased phosphorylated ERK1/2 was seen in the presence of anti-LAMP1 antibody or U0126 [39]. Recently it has been demonstrated that human recombinant amelogenin remarkably enhanced LAMP-1 staining in mouse cementoblasts [40]. In addition, it has been revealed that human recombinant amelogenin enhances the mineralization accompanied by the upregulation of bone markers (BSP and OCN) in mouse cementoblasts [40]. From these studies may be valuable for future periodontal or bone regenerative medicine. Furthermore, comprehensive knowledge of these pathways is important for optimization of future amelogenin tissue engineering strategies.

In conclusion, it was shown that rh174 significantly enhances the proliferation of cultured NHOst *via* LAMP 1-mediated activation of the ERK1/2 signaling pathway, suggesting a possible application of amelogenin in cell proliferation and periodontal tissue engineering.

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