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Establishment and Characterization of Rat Dental Epithelial Derived Ameloblast-Lineage Clones

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Teeth are the hardest tissues covered with enamel produced by ameloblasts. The ameloblast differentiation is controlled by sequential epithelial-mesenchymal interactions during tooth morphogenesis. However, the molecular mechanism of ameloblast differentiation remains unclear. To address this question, we developed an *in vitro* assay system to evaluate the molecular mechanism of amelogenesis. First, we established dental epithelium-derived clones from 6-day-old rat incisors and established that cells of the clone SRE-G5 were the largest producers of amelogenin mRNA. Next, we analyzed the effects of several chemicals on the amelogenin expression in SRE-G5 cells. Only mitogen-activated protein kinase (MAPK) activators enhanced amelogenin mRNA expression. This finding corresponded to the immunohistochemical data showing the presence of phosphorylated forms of p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) during ameloblast differentiation. To examine the roles of MAPK signals, we compared the effects of anisomycin and sodium salicylate on the expression of tooth-related differentiation markers. Both anisomycin and sodium salicylate induced amelogenin, Abcg2, and Bmp4 mRNA and down-regulated p75NGFR mRNA. On the other hand, ALP, ectodin, Bmp2 and Fgf8 mRNA were up-regulated only by anisomycin. These results indicate that MAPK signaling functions, at least in part, as the inducer of ameloblast differentiation.

[**Key words:** ameloblast, amelogenin, differentiation, incisor, mitogen-activated protein kinase]

Stem cell therapy is a promising new medical treatment, not only for diseases but also for regeneration of lost tissues and organs. At present, the application of embryonic stem cells is still problematic both technically and ethically. Research data indicated the possible application of adult stem cells for regeneration therapy, including an achievement of bone marrow transplantation therapy for an aplastic anemia (1). In dentistry, the loss of teeth is a common problem in older people. According to the report of the Ministry of Health, Labour and Welfare in 2005 (http://www.mhlw.go.jp/houdou/2006/06/h0602-2.html), people aged 50–80 have lost more than 3 and 18 teeth, respectively. Loss of teeth causes several problems, such as the movement of the remaining teeth and the difficulty with chewing or swallowing. Hitherto, lost teeth have been replaced with dentures, bridges, and implants. However, many problems remain with these treatments, including discomfort of fitted dentures, secondary toothaches caused by bridges, and lack of tactile sensation from implants. Therefore, it would be beneficial to develop new techniques or tools to replace teeth.

During embryonic development, both sequential and reciprocal signals between dental epithelium and mesenchyme are required for normal tooth morphogenesis. Although several signaling pathways are reported to regulate tooth development (2), the molecular basis of tooth morphogenesis remains to be fully determined. Dental epithelial stem cells differentiate into four cell lineages: the inner enamel epithelium, the stratum intermedium, the stellate reticulum, and the outer enamel epithelium. The inner enamel epithelial cells subsequently differentiate into ameloblasts, which form the enamel. Amelogenin is the major enamel protein produced by ameloblasts and a tissue-specific ameloblast differentiation marker. However, little is known about the molecular mechanisms of ameloblast differentiation. In addition, no *in vitro* systems are currently available for manipulating dental epithelial cells.

We report here the development of an *in vitro* system to evaluate tooth development. Using this system, we demonstrated the involvement of the MAPK signaling pathway in expression of the amelogenin gene. This is the first report that relates MAPK signals to ameloblast differentiation. Here, we propose that this *in vitro* differentiation assay system, using a clone of ameloblast-lineage, is useful in analyzing the molecular basis of ameloblast differentiation, and in evaluating specific differentiation inducing activity in the

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screening of chemicals and drugs for using in cell therapy for tooth regeneration.

MATERIALS AND METHODS

Isolation of incisor-derived epithelial cells and cloning Dental epithelial cells were prepared from lower incisors of 6-day-old stroke-prone spontaneously hypertensive rats (SHRSP) (3) according Kukita's procedure (4). In brief, the lower incisors were dissected from the mandibles. The labial dental epithelial cell sheets were mechanically stripped from the area that contained cells of the ameloblast-lineage. The cell sheets were minced and digested sequentially with 0.25% collagenase, 0.125% trypsin, and 0.02% EDTA. They were then suspended in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Nissui, Tokyo) with 10% serum and the suspension was seeded onto the culture dishes. Single-cell cloning was performed using the limiting dilution method (0.3 cell/well). Rats were maintained and treated in accordance with the guidelines for Animal Experiments of the University of Tokushima. The experimental protocols were approved by the Committee of Ethics on Animal Experiments of the University of Tokushima.

Cell culture Rat incisor-derived epithelial cells were maintained as monolayer cultures in DMEM/Ham's F12 with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA) at 37ºC with humidified air containing 5% CO_2. For experiments, after the cells were grown to 70% confluency, they were washed once with phosphate-buffered saline without calcium and magnesium [PBS(–)]. The cells were treated with bone morphogenetic protein 2 (provided by Yamanouchi Pharmaceutical, Tokyo), phorbol 12-myristate 13-acetate (Wako, Osaka), A23187 (Calbiochem, Darmstadt, Germany), anisomycin (Wako), forskolin (Alomone Labs, Jerusalem, Israel), okadaic acid (Wako) dissolved in dimethyl sulfoxide (Wako), and sodium salicylate (Wako) dissolved in DMEM/Ham's F12, and then added to DMEM/Ham's F12 with 2% FBS. Controls were treated with dimethyl sulfoxide (0.1% v/v).

Cell proliferation assay Cell growth was evaluated by counting the number of cells using the trypan blue exclusion method with triplicate samples at the indicated culture times.

RNA isolation, RT-PCR analysis, and real-time PCR Messenger RNA (mRNA) was isolated from cultured cells at the log phase using the method of acid guanidium thiocyanate-phenol/chloroform (5) with Tri Reagent (Molecular Research Center, Cincinnati, OH, USA). Reverse transcription (RT) was performed using Takara RNA PCR kit (AMV) ver. 3.0 (Takara, Shiga). The first strand of cDNA was then used as a template for PCR. Gene-specific primers and PCR conditions are shown in Table 1. Real-

TABLE 1 Primers for differerntiation markers

Gene	Primer	Anealing (°C)	Extension (s)	Cycle
Abcg2	5′-CAATGGGATCATGAAACCTG-3′ 5′-GAGGCTGATGAATGGAGAA-3′	58	30	38
CD133	5′-ATACTGGTGGCTGGGTGGC-3′ 5′-ACAAGGATCATCAATATCCAGC-3′	61	60	38
p63	5′-ACCTCTGAACAAAATGAACAGC-3′ 5′-CAGGGATCTTCAGACTTGCC-3′	58	30	30
E-cadherin	5′-CGTGATGAAGGTCTCAGCC-3′ 5′-ATGGGGGCTTCATTCACGTC-3′	58	60	30
p75 NGFR	5′-TGTGTGAAGAGTGCCCAGAG-3′ 5′-TCCACAGAGATGCCACTGTC-3′	58	60	40
ALP	5′-CTGGACCTCATCAGCATTTG-3′ 5′-GCTGTGAAGGGCTTCTTGTC-3′	58	60	40
Amelogenin	5′-CAAGAAATGGGGACCTGGATC-3′ 5′-GCTGCCTTATCATGCTCTGG-3′	60	30	40
Ameloblastin	5′-CATGTCTTATGGAGCAAACCA-3′ 5′-TCTTGCAGTGGAGAGCCTTCT-3′	60	60	40
Enamelin	5′-GTGAGGAAAAATACTCCATATTCTGG-3′ 5′-GTTGAAGCGATCCCTAAGCCTGAAGCAG-3′	60	60	40
Follistatin	5′-TTTTCTGTCCAGGCAGCTCCAC-3′ 5′-GCAAGATCCGGAGTGCTTCACT-3′	58	30	28
Ectodin	5′-GAGGCAGGCATTTCAGTAGC-3′ 5′-CATAGCCTCCTCCGATCCAG-3′	58	60	40
Bmp2	5′-TGAACACAGCTGGTCTCAGG-3′ 5′-GCTAAGCTCAGTGGGGACAC-3′	58	60	30
Bmp4	5′-CAGAGCCAACACTGTGAGG-3′ 5′-TCCACTCCCTTGAGGTAACG-3′	58	60	40
Fgf8	5′-TGTGGAGACCGATACTTTTGG-3′ 5′-CTCTGCTCTGTGGTGTGGTG-3′	60	60	40
Sp3	5′-ACTTTGACGCCTGTTCAGAC-3′ 5′-TGATGTTGTTGGTCCCCTTC-3′	58	30	35
C/EBPα	5′-TCTAACTCCCCCATGGAGTCGGCCGACTTC-3′ 5′-GCCTCAATGATGATGATGATGATGCGCGCAGTTGCCCATGGCCTTGACC-3′	63	90	40
C/EBPβ	5′-CGTTCATGCACCGCCTGCTGGCCTGGGA-3′ 5′-GCTAGCAGTGACCCGCCGAGGCCAGCAG-3′	63	90	30
C/EBPδ	5′-CTAGAGTTGTGCCACGACG-3′ 5′-CTTCTGCTGCATCTCCTGG-3′	54	60	38
GAPDH	5′-CATTGACCTCAACTACATGG-3′ 5′-CTCAGTGTAGCCCAGGATGC-3′	58	60	22