The Epithelial-Mesenchymal Interaction Plays a Role in the Maintenance of the Stem Cell Niche of Mouse Incisors via Fgf10 and Fgf9 Signaling

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Abstract: The continuous eruption of mouse incisors throughout life is maintained by adult stem cells in the apical end. In these teeth, the continuous expression of Fgf10 in the mesenchyme plays a role in the maintenance of the epithelial stem compartment, referred to as the “apical bud.” However, little is known about the epithelial signaling that induces and maintains Fgf10 expression. Focusing on the epithelial-mesenchymal interaction during tooth development, we thoroughly investigated candidates expressed in the apical bud. In situ hybridization and immunostaining showed that Fgf9 mRNA and protein were detected in the basal epithelium, stellate reticulum, and inner enamel epithelium of the apical bud. Recombinant Fgf9 protein stimulated cell proliferation in cultures of apical end mesenchyme. Furthermore, Fgf9-releasing beads inhibited apoptosis in mesenchymal tissue cultures and maintained the expression of Fgf10. On the other hand, Fgf10-releasing beads induced Fgf9 expression in cultures of apical buds. Taken together, these results suggest that the stem cell niche in growing incisors is maintained by an epithelial mesenchymal interaction via Fgf9 and Fgf10 signaling.

INTRODUCTION

Mouse incisors are continuously growing teeth that are maintained by both cell proliferation at the apical end and attrition of the incisal edge. This type of tooth has a special epithelial bulge structure referred to as an “apical bud,” which maintains epithelial adult stem cells [1]. The apical bud consists of a large stellate reticulum (SR) which contains the stem cells, the basal epithelium (BE), the inner enamel epithelium (IEE), and the outer enamel epithelium (OEE). The stem cells divide slowly, giving rise to one daughter cell that remains in the SR and another cell that enters the zone of rapidly dividing IEE cells (the transit-amplifying cell population) to differentiate into ameloblasts that deposit the enamel matrix.

The processes of tooth development are regulated by the sequential and reciprocal interactions between the oral ectoderm and the neural crest-derived mesenchyme [2]. Several signaling pathways and transcription factors have been implicated in regulating molar crown development, but relatively little is known about the regulatory mechanisms of continuous incisor growth. We previously reported, using Fgf10-deficient mice, that Fgf10 maintains the epithelial stem cell compartments of mouse incisors [3]. However, the regulation of Fgf10 signaling has not been studied sufficiently in tooth development. Based on the epithelial-mesenchymal interactions during tooth development, the mesenchymal stem cells seem to rely upon epithelial cues for their survival and differentiation [4, 5]. Fibroblast growth factors (Fgfs) play a critical role in developing teeth [6, 7]. In this study, which was intended to elucidate the mechanism for the continuous growth of mouse incisors, we searched for growth factors maintaining Fgf10 expression by epithelial-mesenchymal interactions. We report a candidate molecule, Fgf9.

MATERIALS AND METHODS

RT-PCR Analysis

Mandibular incisors were carefully microdissected from neonatal, 1-day-old mice. Tissues were immediately suspended in RNA later RNA Stabilization Reagent (QIAGEN), and total RNA was extracted using an RNeasy Micro Kit (QIAGEN). After cDNA synthesis using oligo (dT) primers and reverse transcriptase, RT-PCR analysis was performed using the primer sets shown in Table 1. Amplification products were analyzed on 2.0% agarose gels and visualized by staining with ethidium bromide.

Immunohistochemistry

Mandibular incisors of neonatal 1-day-old mice were dissected, fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Serial 10-μm sections were taken in the sagittal plane. Antigens on the paraffin sections were activated in citric buffer at 121°C for 10 min. Rabbit anti-Fgf9 polyclonal antibody was used as the primary antibody (0.5 μg/ml) (CHEMICON, USA). Detection was carried out using a VECTASTAIN Elite ABC KIT (Vector, USA) and a DAB kit (Vector, USA) for color reactions according to the manufacturers’ instructions. The sections were counterstained by hematoxylin.
Table 1. Primer Sets for RT-PCR

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Sequence (5′→3′)</th>
<th>Size</th>
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| Fgf1 | Forward: ACCGAGAGTTCTCAACCTGCC  
Reverse: GCCATAGTTGTGGCAGAACCCGACC | 387 bp |
| Fgf2 | Forward: AGCGGCATACCTCTCCCTTCA  
Reverse: TGGAGAAACAGATGGCCTCCTGTCC | 438 bp |
| Fgf3 | Forward: GATGGACCTGATCTGCTTCTGCT  
Reverse: CACATCTCATGCTGCTTGGCACC | 573 bp |
| Fgf4 | Forward: ACCAGGAGGACGGCTGCGAAAA  
Reverse: CTTCACTACGAGGACACTCTGGA | 567 bp |
| Fgf5 | Forward: CATCTTCTGACGCACTGATCCA  
Reverse: AAGTCTCGGCTGTCGAGACTGCTT | 652 bp |
| Fgf6 | Forward: CACGTCGACCCCTGCTTTCCT  
Reverse: CAGTCATGATGGGACACTCTGTC | 542 bp |
| Fgf7 | Forward: CAATCTCACAATTCTACAGA  
Reverse: AGCCCCCTTTTTGATTAAAG | 622 bp |
| Fgf8 | Forward: CTGAGCTGCCTCGTGTGCTGACTTG  
Reverse: GTGATGGGAGAGTCCGATAG | 568 bp |
| Fgf9 | Forward: ATGGGTCCTTATTGGATGATTGG  
Reverse: GCCTCCGCTGAGAATCCCCTTTAAATG | 196 bp |
| Fgf10 | Forward: TCACCTGCAACCTTTGGT  
Reverse: CCGTTCTCTTACATGGTGA | 190 bp |
| Fgfr1b | Forward: GGCGCACTATGTTGAGTTCA  
Reverse: TTGGACAGCTCTGCTGAGACA | 278 bp |
| Fgfr1c | Forward: GGGCACAGTTGAGTTGACA  
Reverse: GTGATGGGAGAGTCCGATAG | 250 bp |
| Fgfr2b | Forward: GGAGGGGATGGGAGTTGTTG  
Reverse: ACTGGTTGTCGCTGCCTTATA | 244 bp |
| Fgfr2c | Forward: GAGGGGAGATGGGAGTTGTTG  
Reverse: CAGAACCTGTCACACCATGCA | 271 bp |
| Fgfr3b | Forward: TCAGTGGATGATGAGGACCA  
Reverse: TGCCAGCGTCATGCTTCC | 180 bp |
| Fgfr3c | Forward: TAAACCCAGCAAAGAGAAGGAG  
Reverse: TGCCAGCGTCATGCTTCC | 170 bp |
| Fgfr4 | Forward: CTGTTGCTGCACCTGCTGACCA  
Reverse: TGATGGAGGTTAAAGGATGC | 190 bp |
| CMHPRT | Forward: GCRCTGGCTATGCTATGATGTA  
Reverse: GTCRAGGGCTATCACAACAAC | 562 bp |

Cell Proliferation Assay

Primary dental mesenchymal cells were dissected from lower incisor germs. These cells were treated with trypsin and dispase. A total of 0.5x10^5 cells per well were inoculated onto 96-well plates containing 100 μl of medium (α-MEM, 10% FBS) per well, and recombinant human Fgf9 protein (CHEMICON, USA) was added to the medium at various concentrations (0 ng/mL, 1 ng/mL, 10 ng/mL, or 100 ng/mL). The cells were counted in 7 days. Points represent mean ± SEM of three replicates.

Recombinant Proteins and Treatment of Beads

To examine the effect of FGF proteins to each tissues, the experiment was designed. As FGFs bind to heparin, we used heparin acrylic beads (Sigma-Aldrich, USA) as biomaterials releasing FGFs. Heparin acrylic beads were incubated in FGF9 (CHEMICON, USA), FGF8 (R&D Systems, UK), or FGF10 (R&D) proteins (all 50 ng/μl). Control beads were incubated in bovine serum albumin (BSA, 1 μg/μl). About 100 beads were washed with PBS and soaked in 10 μl of growth factor for 45 min at 37°C and then stored at 4°C.

Organ Culture

To separate the dental epithelium from the mesenchyme, postnatal day 1 incisors were dissected and incubated with 2% collagenase at 4°C for 3-4 h. Fgf9, Fgf8, and BSA beads were placed on the top of the isolated dental mesenchyme. Fgf10 beads and BSA beads were placed on the apical buds. All explants were cultured on 0.1 μm OMNIPOR filters (MILLIPORE, USA) at 37°C in a Trowell-type organ culture containing alpha minimum essential medium (α-MEM) supplemented with 10% fetal calf serum (PAALaboratories GmbH, Austria) and 1% penicillin-streptomycin (Gibco, UK). After culturing 24-36 h in vitro, tissues were treated for Annexin V staining, fixed in 4% PFA, and processed for whole-mount in situ hybridization analysis.

Annexin V Staining

Alexa 488-conjugated Annexin V (Molecular Probes, USA) was used to detect apoptotic cells in the tissue cultures. The tissues were incubated with HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2) containing 1 μg/ml Annexin V-Alexa 488 for 20 min. After rinsing in HEPES buffer, fluorescence was detected using a fluorescence microscope.

In Situ Hybridization and Whole-Mount In Situ Hybridization

Fgf10 cDNA was a kind gift from H. Ohuchi (University of Tokushima), and Fgf9 cDNA was from M. Abe (Osaka University). Digoxygenin-labeled antisense and sense riboprobes for Fgf10 and Fgf9 mRNAs were synthesized according to the manufacturer’s instructions (Roche Diagnostics GmbH, Switzerland). Lower jaws were fixed in 4% PFA, embedded in OTC and cryosectioned (10 μm intervals) were hybridized at 60°C (Fig. 1A). Processed dental tissues (Fig. 3) were hybridized at 55-57°C. The probes were detected by an alkaline phosphatase-coupled anti-digoxygenin antibody and NBT/BCIP (Roche) was used as the color substrates.

RESULTS AND DISCUSSION

Fgf9 mRNA and Protein Expression in the Epithelial Tissue in the Apical End of Mouse Incisor

To search for a candidate that induced or maintained Fgf10, we first examined the expression of mRNAs of several Fgfs and Fgfrs in the apical bud by RT-PCR. mRNAs of Fgf1, Fgf8, and Fgf9 were recognized clearly (Fig. 1). PCR products of Fgfr1b, Fgfr1c, Fgfr2b, and Fgfr2c were also detected (Fig. 1). In these detectable factors, the expression of only Fgf9 mRNA and protein was characteristically observed in the epithelial region adjacent to the mesenchyme.
expressing Fgf10 mRNA (Fig. 2). In situ hybridization analysis showed that Fgf9 mRNA was expressed in the apical bud, IEE (Fig. 2A, large arrow). Fgf9 protein was also strongly detected by immunohistochemistry in the BE and IEE (Fig. 2B, arrows) and weakly in the neighboring mesenchyme. It was not detected in the OEE or differentiated ameloblasts (Fig. 2C-E).

Murine incisors are continuously growing teeth, and all stages of odontogenesis, including amelogenesis and dentinogenesis, can be surveyed if serial sections of the teeth are prepared from the apical end to the incisal edge. The morphological transition of the epithelial-mesenchymal compartment by serial transverse sections of the apical end toward the incisal direction likely reflects the development of the tooth germ in the prenatal stage [8]. In murine molar development, Fgf9 mRNA is expressed in the dental epithelium from E11 to E18 [9]. In particular, it is expressed in the enamel knot as a signaling center that regulates tooth shape at the cap stage and in the IEE at the early bell stage, and is no longer seen in the differentiated ameloblasts of the late bell stage. Fgf9 immunohistochemistry of serial transverse sections showed that in the incisors, Fgf9 expression patterns were very similar to those of the Fgf9 and Fgf10 mRNA of the developing molar germ (Fig. 2C-F). The expression pattern of Fgf10 mRNA during molar tooth development is detected faintly around the tooth bud (E13), and continued to express strongly in the dental papilla from cap stage (E14) to bell stage (E16). Though after birth, its expression is slowly decreased [6], and finally disappeared [10].

These results suggested that, as seen in molar development, the expression of Fgf10 mRNA was associated with the localization of Fgf9 protein. It is well known that the early developmental process of organogenesis is regulated by several Fgf families of intercellular signaling molecules that are utilized repeatedly at successive stages of development.

**Role of Fgf9 in Maintenance of Stem Cell Niche in a Growing Mouse Incisor**

To examine the effects of Fgf9 on the mesenchymal tissues of the apical end, we carried out cell proliferation assays using primary mesenchymal cultures. We found that Fgf9 stimulated proliferation in a dose-dependent manner (Fig. 3A).

Next, we examined the appearance of apoptotic cells in the presence and/or absence of Fgf9 by Annexin V staining. In the organ cultures with Fgf9-releasing beads, most mesenchymal cells escaped from apoptosis around the Fgf9-releasing beads after 24 h (Fig. 3D). Apoptotic cells were also rarely seen after 48 h of culture (Fig. 3H). In the cultures with BSA beads as a control, most mesenchymal cells reacted with Annexin V after 48 h (Fig. 3I, arrows).

The survival of mesenchymal cells is reportedly associated with epithelial cells [2]. These results suggested that Fgf9 was expressed in the apical buds, and was a mesenchymal survival/mitogenesis factor.

Furthermore, we examined whether Fgf9 maintains Fgf10 expression using an organ culture system of the incisal mesenchyme. The isolated mesenchymal tissues were cul-
tured in the presence of Fgf9-releasing beads, and the expression of Fgf10 was analyzed after 24 h by whole-mount in situ hybridization. Fgf10 mRNA was expressed in explants with Fgf9-releasing beads (Fig. 4A, arrows). No expression was detected in the presence of Fgf8- or BSA-releasing beads (Fig. 4B and 4C).

These results suggested that Fgf9 is a signaling factor that aids the ability of the apical end mesenchyme to maintain Fgf10 expression. Finally, to examine the effect of Fgf10 on Fgf9 expression in the apical bud, we carried out organ cultures of the buds in the presence and/or absence of Fgf10.

Fig. (3). Effect of Fgf9 on the proliferation and apoptosis of dental mesenchymal cells.

(A) Mesenchymal cells cultured in a medium containing recombinant Fgf9 increased the cell number in a concentration-dependent manner. Data are presented as means ± SD (n=3). (D, E, H, I) Apoptotic cells were stained with Alexa 488-conjugated Annexin V. (B, C, F, G) These pictures were phase contrast images to D, E, H, I respectively. The tissues were cultured with Fgf9-releasing beads for 24 h (B, D) or 48 h (F, H) or with BSA-releasing beads for 24 h (C, E) or 48 h (G, I). The presence of Fgf9 clearly maintained the cells, and apoptotic cells bound to Annexin V were sparse (D, H). In the explants cultured with BSA, apoptotic cells began to appear after 24 h (E, arrows). Those cultured for 48 h exhibited a larger number of apoptotic cells (I, arrows). Bar, 200 μm.

Fig. (4). Effect of Fgf9 on the induction of Fgf10 mRNA in the dental mesenchyme, and effect of Fgf10 on the induction of Fgf9 mRNA expression in the epithelium.

Stimulation of Fgf10 mRNA expression was seen around the Fgf9 beads in the organ culture mesenchyme (A, arrows), but not around the Fgf8 or BSA beads (B, C). Stimulation of Fgf9 mRNA expression is seen around Fgf10 beads in an apical bud (D, arrows). No specific expression is observed around BSA-soaked beads in the apical bud (E). Bar, 200 μm.

Fgf10-releasing beads maintained Fgf9 expression in the apical buds (Fig. 4D, arrows). But in the presence of BSA-releasing beads, the apical buds were destroyed (Fig. 4E). Fgf10 maintained the expression of Fgf9 in the apical buds of growing incisors.

The epithelial-mesenchymal interaction is a well-known developmental mechanism. Here, our results suggested that Fgf9 and Fgf10 act as direct regulatory factors for reciprocal interactions and contribute to the maintenance of the stem cell compartment of mouse incisors (Fig. 5). We suggest that the epithelial-mesenchymal interaction is also essential for supporting continuous growth. However, we reported previously that cessation of Fgf10 signaling induces the transition from crown morphogenesis to root formation [11]. At the transitional stage, Fgf9 expression also disappears from the dental epithelium [9]. Upstream factors regulating the cessation of the expression of these factors have not yet been elucidated. We suggest that the epithelial-mesenchymal interaction is also essential for supporting continuous growth. Future studies, which should attempt to better understand the...
molecular mechanisms, will contribute to the development of bioengineered teeth created using human dental epithelial and mesenchymal stem cells.

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